GENETIC AND ENVIRONMENTAL INFLUENCES ON THE CONTROL OF
EXPRESSION OF FIBRINOGEN AND FACTOR VII GENES

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ABSTRACT

Raised levels of plasma fibrinogen and factor VII coagulant activity (FVIIc), both involved in blood coagulation, have been shown in a number of prospective epidemiological studies to be independent risk factors for coronary heart disease (CHD). Lipid-loaded macrophages, or 'foam cells', form a large proportion of the advanced atherosclerotic plaque. Cytokine production by lipid-loaded human peripheral macrophages was investigated in vitro to determine a possible effect on plasma fibrinogen levels. Conditioned medium from these cells after 3 days in culture produced a slight stimulation of fibrinogen synthesis in HepG2 cells, but contained little apoE protein. This stimulation was blocked by an anti-IL-6 antibody. Conditioned medium from 17-day-old-cultured monocyte-macrophages treated with acetylated low density lipoprotein (AcLDL) caused no stimulation of fibrinogen synthesis in HepG2 cells, contained no IL-1, TNFα or IL-6, but did contain increased levels of apoE protein. These results suggest that it is unlikely that cytokine production by foam cells in the atherosclerotic lesion is a major contributing factor in determining the raised fibrinogen levels associated with ischaemic heart disease (IHD). IL-6 induced expression of β-fibrinogen mRNA in HepG2 cells was inhibited in a dose-dependent fashion by TGF-β. A C to T polymorphism at position -148 in the promoter region of the β-fibrinogen gene, near to an IL-6 responsive element and previously shown to be associated with plasma fibrinogen levels, was found to be within a TGF-β inhibitory consensus sequence. The single base change appeared to alter the strength of binding of a number of hepatoma cell nuclear proteins in 'bandshift' assays both under basal and cytokine-stimulated conditions. It did not alter promoter strength of the β-fibrinogen gene in transient expression assays.
The association between a common polymorphism in the coding region of the FVII gene which alters an amino acid from an arginine to a glutamine at position 353 (Arg/Gln\textsubscript{353}) was investigated in a number of different population samples. These were population samples from white European men and women from north-west London, Gujarati Indian men and women, Afro-Caribbean men and women, and from our different centres in Europe (Belfast, Lille, Strasbourg and Toulouse). There was a statistically significant association in all the population samples studied between plasma FVIIc and genotype, and in one study between FVIIag and genotype, with the Gln\textsubscript{353} allele associated with a 20% reduction in FVIIc and FVIIag levels. There was also evidence for an interaction between genotype and plasma triglyceride level in determining FVIIc level. Carriers of the Gln\textsubscript{353} allele showed no correlation between FVIIc and triglyceride level while in Arg\textsubscript{353} homozygotes there was a positive correlation. These results suggest that the Arg/Gln\textsubscript{353} polymorphism may be affecting the interaction of FVII with triglycerides and also the production and/or secretion of FVII rather than being a marker for another functional polymorphism elsewhere in the FVII gene.

In conclusion, this study provides evidence that genetic variation at the fibrinogen and FVII gene loci may determine plasma levels of the respective gene products and their response to 'environmental' factors such as acute-phase stimuli and plasma triglyceride level.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AcLDL</td>
<td>Acetylated LDL</td>
</tr>
<tr>
<td>AMP</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CsCl</td>
<td>Caesium chloride</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosinetriphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytosinetriphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanosinetriphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymidinetriphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FVIIa</td>
<td>Activated factor VII</td>
</tr>
<tr>
<td>FVIIag</td>
<td>Factor VII antigen</td>
</tr>
<tr>
<td>FVIIc</td>
<td>Factor VII coagulant activity</td>
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</table>
Gln Glutamine
GT Guanidinium isothiocyanate
HBSS Hanks balanced salts solution
IHD Ischaemic heart disease
IL-6 Interleukin-6
Kb Kilobase(s)
LDL Low density lipoprotein
LPS Lipopolysaccharide
MCM Monocyte/macrophage conditioned medium
MI Myocardial infarction
mRNA Messenger RNA
NaOAc Sodium acetate
NP40 Nonidet-P 40
OD Optical density
PBS Phosphate buffered saline
PCR Polymerase chain reaction
PCV Packed cell volume
PDGF Platelet-derived growth factor
RFLP Restriction fragment length polymorphism
rhIL-6 Recombinant human IL-6
RNA Ribonucleic acid
SDS Sodium dodecyl sulphate
SE Standard error of the mean
SSC Standard saline citrate solution
Taq Taq polymerase thermostable DNA polymerase from Thermus aquaticus
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRIG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>(v/v)</td>
<td>Volume by volume</td>
</tr>
<tr>
<td>WHIP</td>
<td>Waist/hip ratio</td>
</tr>
<tr>
<td>(w/v)</td>
<td>Weight by volume</td>
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1. Introduction

1.1 Atherosclerosis and the structure of the atherosclerotic plaque

Cardiovascular disease is accepted as the most common cause of death in western countries (Tunstall-Pedoe et al 1986). Atherosclerosis and thrombosis together cause the majority of these deaths.

In the mid 19th century there were two main hypotheses as to the aetiology of atherosclerosis: the first described it as an "encrustation" of the intima of the artery wall, resulting from fibrin deposition with lipid accumulation being secondary, and the second as a chronic irritation of the vessel wall with subsequent accumulation of lipid which formed complexes with acid mucopolysaccharides (reviewed by Fuster et al 1992). Much more recently Ross and Harker (1976) developed the "response to injury" hypothesis based on the fact that upon removal of the arterial endothelium by balloon catheterisation a lesion or lesions resembling that of the atherosclerotic plaque develop. This hypothesis accommodated both the previous theories. He since modified this hypothesis and re-defined the term "injury" as any minor alteration in the artery wall (Ross 1986). Other groups have put forward two different hypotheses for which there are varying amounts of evidence. Gerrity (1981a, 1981b) has carried out investigations on animals fed high cholesterol diets which suggests that lipid insudation and accumulation in the artery wall, with accompanying adherence and infiltration of monocytes, is the initiating step in the development of the atherosclerotic plaque. The work of Joris et al (1983) and Faggiotto et al (1984a, 1984b) also supported this hypothesis. Benditt and Benditt (1973) proposed the "monoclonal theory of
atherogenesis" from their findings that cells in a plaque appear to be the result of the proliferation of a single cell. This theory is supported by the observation that cells transfected with DNA from atherosclerotic plaques become tumorigenic (Penn et al 1986) but Thomas and Kim (1983) found that cells in lesions are of mixed origin.

The arterial lesions that characterise atherosclerosis appear to develop from the fatty streak, which can commonly be found in childhood (Stary 1983). The fatty streak is a narrow, slightly raised, yellow area consisting of foam cells, whose cytoplasm is filled with lipid droplets, mostly cholesteryl esters and free cholesterol. The majority of these cells have been found to be macrophages and the rest are smooth muscle cells (McGill 1968, Aqel et al 1985, Vedeler et al 1984). The fibrous plaque is found in advanced atherosclerosis and typically consists of three main areas (see fig). Firstly, a fibrous cap of smooth muscle cells, some leukocytes and connective tissue containing elastin, collagen fibrils, proteoglycans and basement membrane (Joris et al 1983, Murata et al 1986 and Yla-Herttuala et al 1986). Secondly, an area beneath the cap consisting of macrophages, smooth muscle cells and T lymphocytes (Jonasson et al 1986). Thirdly, a necrotic core which contains cellular debris, extracellular lipid droplets, cholesterol crystals and calcium deposits. Around the periphery of the lesion, proliferating blood vessels are sometimes detectable, indicating neovascularization from the direction of the adventitia (Munro and Cotran 1988).
Fig 1 Diagram of a cross section through an advanced atherosclerotic plaque showing the position of the fibrous cap and the lipid core or necrotic centre. 

1.1 Fibrous Cap
1.2 Necrotic/Lipid Core
2.0 Smooth Muscle Cells, Leukocytes, Elastin, Collagen Fibrils and Proteoglycans
3.0 Macrophages, Smooth Muscle Cells and T-lymphocytes

1.2 Immune mechanisms and atherosclerosis

Saphir and Gore (1950) and later Joris and Majno (1977) described the similarities between atherosclerosis and the inflammatory response. Many of the features described in the last section are also common features of inflammation. The main function of macrophages which, as already described, make up a large part of the atherosclerotic plaque, is to participate in the immune response by phagocytosing foreign antigens. The electron microscopy studies of Gerrity (1981a and 1981b) and Faggiotto et al (1984a, 1984b) in swine and non-human primates led to the hypothesis that monocytes act as
an important defence mechanism in cholesterol-induced atherosclerosis by mobilizing cholesterol from the lipid-laden intima and the work of Hansson et al showed that monocytes bind to injured endothelium (Hansson et al 1981). Monoclonal antibody technology identified monocytes in the human atherosclerotic plaque (Vedeler et al 1984, Aqel et al 1985). Subsequent studies have shown that monocyte-derived macrophages are present in the early fatty lesion and in the later plaque (Jonasson et al 1986, Klurfeld 1985, Hansson et al 1988).

Lymphocytes have been shown to be present at all stages of atherosclerosis (Gown et al 1986, Jonasson et al 1986, Hansson et al 1988, Munro et al 1987, Emeson and Robertson 1988). Jonasson et al found (1986) that two types of T lymphocyte were present in the plaque, those recognised by the CD-4 antibody and those recognised by the CD-8 antibody. CD-4 T-lymphocytes induce antibody production and regulate cell-mediated immune responses and were found to be present in the later, complicated plaque. CD-8 T lymphocytes, which have cytotoxic activity, were more frequent in the early plaque and fatty streak and were concentrated around the intimal thickening that surrounds the late plaque. Hansson suggests that the CD8 cells may be involved in the initiation of lesion, with the CD-4 cell type becoming more predominant during progression of the plaque (Hansson et al 1989). Smooth muscle cells are involved in lesion enlargement through migration from the media into the intima with accompanying proliferation and deposition of connective tissue within the intima (Ross et al 1990). Smooth muscle cells also form a large proportion of foam cells in the plaque (Aqel et al 1985, Vedeler et al 1984).

Monocytes, T lymphocytes and smooth muscle cells produce a wide range of cytokines
and growth factors under different conditions. Several of these cytokines have been shown to affect growth and gene expression in vascular cells. However the actions and interactions of cytokines are too wide to be covered in detail here and only selected ones will be discussed, namely, interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β) which are all produced by monocytes (Lomedico et al 1984, Auron et al 1984, March et al 1985, Old 1985, Pennica et al 1984, Wang et al 1985, Marmenout et al 1985, Beutler et al 1985b, Derynck et al 1985, Gauldie et al 1987).

Activated T-lymphocytes produce interferon-γ (IFN-γ) (Vilcek et al 1985 for review), and IL-6 (Ray et al 1989). Stimulated smooth muscle cells produce TNFα (Warner and Libby 1989), PDGF (Libby et al 1988) and large amounts of IL-1 (Libby et al 1986).

Platelets appear also to be involved in the development of the plaque when endothelial retraction occurs and they adhere to exposed underlying foam cells. Degranulation of these platelets releases a number of growth factors including platelet derived growth factor (PDGF), epidermal growth factor (EGF), TGF-β and IL-1 (Hawrylowicz 1989). IL-1 exists in two forms, IL-1α and IL-1β, both having similar effects (Le and Vilcek 1987 for review). IL-1 induces a number of changes in cultured endothelial cells including stimulation of proliferation (Ooi 1983), induction of procoagulant activity (Bevilaqua 1984) and induction of expression of adhesion proteins (Bevilaqua 1984) and it has been suggested that the overall effect of IL-1 on endothelial cells may be to produce a pro-inflammatory phenotype (Hansson et al 1989). IL-1 also stimulates its own production from endothelial cells and smooth muscle cells in a positive-feedback loop (Warner 1987a, 1987b) and has been shown to induce secretion of platelet-derived growth factor (PDGF) by smooth muscle cells (Raines et al 1989). IL-1 could therefore promote the development of the atherosclerotic plaque by stimulating adhesion of
monocytes and lymphocytes through its effects on endothelial cells and by increasing the migration and proliferation of smooth muscle cells in the atherosclerotic plaque indirectly by its induction of PDGF (Ross et al 1990). IL-1 can also stimulate IL-6 production by monocytes and macrophages and may therefore indirectly contribute to levels of circulating IL-6 (Bauer et al 1988). However IL-1 also stimulates production of prostaglandins which inhibit growth of vascular smooth muscle cells in vitro (Libby et al 1988), which makes its involvement in plaque formation unclear.

TNF-α is produced by smooth muscle cells as well as macrophages and induces vascular and inflammatory responses similar to those exhibited by IL-1. It can also inhibit lipoprotein lipase (LPL) secretion in adipocytes (Cerami and Beutler 1988). LPL, of which macrophage-derived foam cells are the major source in the plaque, may be involved in local lipolysis in the atherosclerotic plaque causing modification of triglyceride-rich lipoproteins at the endothelial surface into atherogenic remnants (O'Brien 1992). In the atherosclerotic and normal artery, the smooth muscle cell is also a source of LPL (Jonasson et al 1987), but where these have been activated (by either interferon-γ or TNF-α) no LPL can be detected, suggesting that TNF-α may inhibit LPL production in the plaque (Hansson et al 1989).

TGF-β is a 25kD homodimeric protein, expressed by mammalian cells such as platelets, activated macrophages and lymphocytes and released by cells around sites of tissue repair (Sporn et al 1987, Derynck et al 1985, Assoian et al 1987). TGF-β1 is the most common form and acts as a regulatory protein that modulates a variety of biological functions relating to developmental processes (Roberts et al 1988). There is evidence that TGF-β acts in opposition to the actions of TNF-α (Ranges et al 1987, Espevik et
TGF-β is also chemotactic for monocytes and fibroblasts (Wahl et al 1987, Postlethwaite et al 1987) and induces the synthesis of extracellular matrix proteins by fibroblasts (Ignotz and Massague 1986, Raghow et al 1987, Penttinen et al 1988, Chen et al 1987). Vascular endothelial and smooth muscle cells produce TGF-β (Antonelli-Orlidge et al 1989, Sato and Rifkin 1989) which has been shown to inhibit smooth muscle cell proliferation and T and B cell activation. Wahl et al (1989) suggested that one of the functions of TGF-β might be to act in a negative feedback pathway to inhibit inflammation, while allowing monocyte-derived IL-1 and other cytokines to act in tissue repair, in effect to protect from prolonged inflammatory events.

ApoE secretion has been shown to be increased in lipid-loaded macrophages (Werb and Chin 1983, Takemura and Werb 1984). ApoE is an integral component of the surface of several mammalian lipoproteins and a significant proportion of apoE appears to be synthesised peripherally, as well as in the liver. Extrahepatic apoE is thought to play a significant role in cholesterol metabolism by acting as a recognition signal for the hepatic removal of peripheral cholesteryl ester-rich HDL-subfractions through the hepatic apoE receptor (Mazzone et al 1987). ApoE synthesized by cultured cholesteryl ester-loaded macrophages associates with co-incubated HDL and may therefore potentiate the movement of cholesterol from cells to HDL (Gordon et al 1983) and its subsequent removal. TGF-β has been shown to upregulate macrophage apoE secretion (Zuckerman 1992) and so may increase transport of cholesterol out of the cell. Other effects of TGF-β also suggest a role in plaque regression. THP-1 cells, which are a monocyte-derived cell line, have decreased scavenger receptor activity on treatment with TGF-β. Generation of superoxide anions which are thought to modify LDL and enhance its uptake by macrophages via the scavenger receptor, is inhibited by TGF-β.
in IFNγ-stimulated macrophages (Tsunawaki et al 1988). TGF-β down regulates IL-1 receptors and may therefore down regulate cytokine receptors on smooth muscle cells, also involved in plaque formation. TGF-β has been detected in atherosclerotic lesions from cholesterol-fed rabbits however it is not clear whether its role is in the development or the regression of atheroma (Ross et al 1990).

IL-6 acts as a hepatocyte-stimulating factor and induces a number of acute phase proteins in liver cells, notably fibrinogen (Gauldie et al 1987). It can stimulate terminal maturation of activated B-cells (Hirano 1985) and, in synergy with IL-3, supports proliferation of haematopoietic cells (Ikebuchi 1987). However IL-6 can also inhibit the proliferation of other cell types and is induced by TNFα, PDGF and IL-1 (Kohase et al 1986). IL-6 also has a number of interactions with IL-1, TNFα, IFN-β, and PDGF (Gauldie et al 1987, Darlington et al 1986, Kohase et al 1987). Human vascular endothelial and smooth muscle cells have been shown to express the IL-6 gene in vitro (Jirik et al 1991, Loppnow and Libby 1989, Loppnow and Libby 1990, Sironi et al 1989). IL-6 has been shown to be one of the major regulators of the acute phase response and this is described further in section 1.5.

Since a large proportion of cells in the atherosclerotic plaque consist of foam cells derived from macrophages, activation by lipoproteins may alter their secretion of certain cytokines. This may effect the evolution of the plaque itself and may also influence circulating levels of cytokines. Fig 2 shows a schematic representation of some of the immune mechanisms described above.
Fig 2 Schematic representation of postulated interactions and effects of cytokines in the atherosclerotic plaque. PDGF may be involved in SMC migration and proliferation and may be released from adherent degranulated platelets and from SMC through the action of IL-1. IL-6 released from activated macrophages, SMC and endothelial cells may contribute to circulating levels. TNF-α is produced by both SMC and macrophages and induces vascular and inflammatory responses. TGF-β produced by activated macrophages may contribute to circulating levels. TGF-β may be involved in regression of plaque formation but some of its actions seem to be opposed to these effects (see text). Broken arrows represent possible inhibitory effects.
1.3 The role of factor VII and fibrinogen in the coagulation cascade

Higher animals have developed a complex series of mechanisms, known as haemostasis, to limit blood loss after vascular damage. There are three main stages in this process, firstly transient vasoconstriction, secondly adhesion and aggregation of circulating platelets and lastly coagulation. Coagulation of the blood is the culmination of a series of enzymatic reactions known as the coagulation cascade. One of the features of this process is the series of zymogen activations where the activated form of one factor catalyses the activation of the next factor. The coagulation cascade has been divided into the intrinsic and extrinsic pathways both of which culminate in the activation of factor X (FX) to activated FX (FXa). The intrinsic pathway is initiated when blood comes into contact with an anionic surface, activating both prekallikrein and factor XII. FXI is converted to FXIa by FXIIa and high molecular weight kininogen as cofactor. FXIa converts FIX to FIXa which binds to FVIIIa and in the presence of calcium ions and phospholipid converts FX to FXa. Prothrombinase, which consists of a complex of FXa, FVa, calcium ions and phospholipid then converts prothrombin to thrombin which cleaves fibrinogen to form the fibrin clot. The extrinsic pathway requires the exposure of tissue factor (TF) to plasma, where FVII is converted to FVIIa. The complex of TF and FVIIa then cleaves FX to FXa (see fig 3). However these pathways are not entirely separate but are becoming increasingly viewed as interconnecting networks.

Factor VII is a vitamin K dependent coagulation factor secreted by the liver as a single chain glycoprotein (Mr 50,000). It is secreted into the blood as a zymogen. It is cleaved
Anionic surface
Kininogen
Prekallikrein

Factor XII → Factor XIIa

Factor XI → Factor XIa

Factor IX → Factor IXa

Factor VII → Factor VIIa

Factor X (INTRINSIC PATHWAY)

Factor IXa + Ca++ + Phospholipid → Factor VIIIa

Factor Xa + Ca++ + Phospholipid

Factor Va + Factor VIIIa + Tissue Factor

Prothrombin → Thrombin

Fibrinogen → Fibrin

Fibrin → Fibrin degradation products

Fibrinolysis (plasmin)
in the blood by factor Xa and a number of other coagulation proteases to the activated form factor VIIa (Broze and Majerus 1980). Factor VIIa is composed of two polypeptide chains held together by a disulphide bond and in the presence of tissue factor, phospholipid and Ca$^{2+}$, converts factor X to factor Xa (Rao and Rapaport 1988) (Fig 3 clotting cascade) and as described above can convert IX to IXa. Factor VII differs from the other coagulation cascade zymogens in that it has some protease activity without prior activation (Zur et al 1982), although this point remains controversial.

The amino-terminal region of FVII contains 10 $\gamma$-carboxyglutamic acid residues which require vitamin-K for their $\gamma$-carboxylation and are in analogous positions to the $\gamma$-carboxyglutamic acid residues in the other vitamin-K dependent proteins. This region is known as the $\gamma$-carboxyglutamic acid (Gla) domain of FVII, contains a Ca$^{2+}$-binding domain (Hagen 1986) and has been shown to be essential for the interaction of FVII with tissue factor (TF) (Sakai et al 1990). The Gla domain of two other coagulation factors, factor IX and factor X, have been shown to interact with phospholipid (Wildgoose et al 1988) and the Gla domain of FVII was also thought to interact with phospholipid by binding Ca$^{2+}$ ions which acted as "bridges" interacting between lipid membranes and the protein (Nemerson 1988). However the factor VII Gla-domain has been shown not to be involved in phospholipid interaction or to interact with the extracellular domain of tissue factor apoprotein but has been postulated to induce calcium-dependent conformational changes in FVIIa which then expresses one or more neoepitopes which specifically interact with TF (Wildgoose et al 1992). Two potential epidermal growth factor domains are adjacent to this region and are also present in FX, FIX and protein C (Hagen 1986). Studies on these growth factor domains implicate them in the high affinity tissue factor recognition determinant of factor VIIa (Toomey
et al 1991). A recent study of an Arg-Gln substitution at amino acid 304 which is spatially distant from the active site, showed that this caused reduced factor VIIc activity and reduced affinity for tissue factor (O'Brien et al 1991) suggesting that this region is also involved in tissue factor interaction. Activation of FVII to FVIIa results from the cleavage of a peptide bond between arginine-152 and isoleucine-153 which gives a light chain of 152 amino acids and a heavy chain of 254 amino acids. The heavy chain contains the three principal residues involved in the catalytic activity of the enzyme while the light chain contains the growth factor domains and the Gla-domain (see fig 4).
Fig 4 Amino acid sequence and tentative structure for prepro-factor VII. The prepro-leader sequence of 60 amino acids (numbered -60 to -1) or 38 amino acids is removed by a signal peptidase and a processing protease that cleaves the arginine-alanine (R-A) bond between -1 and 1. FXa cleaves FVII at amino acid 152 to give the two chain FVIIa. The Gla domain and potential growth factor domains are located within residues 1-152 and constitute the light chain of factor VIIa. The serine protease or catalytic domain of factor VIIa contains 254 amino acids from the point of cleavage by factor Xa to the carboxy terminal. The site of the Arg355/Gln353 polymorphism (see section 1.9) is at amino acid 353 (bracketed and marked with a *) (adapted from Hagen 1986).

Regulation of the catalytic activity of the FVIIa/TF complex is through the tissue factor
pathway inhibitor (TFPI), previously known as extrinsic pathway inhibitor (Rao and Rapaport 1987) or lipoprotein-associated coagulation inhibitor (Broze et al 1988), which is present in the plasma at a concentration of 2.5nM (Novotny et al 1991), a quarter of that of FVII. A large intravascular pool of heparin-releasable TFPI is present on the luminal surface of vascular endothelium (Sandset and Abildgaard 1991) and a small amount of TFPI is present in the platelets (Novotny et al 1988). TFPI inhibits FVIIa/TF in a two-step stoichiometric reaction by firstly binding to the active site of FXa to form a FXa/TFPI complex and secondly by this complex binding to FVIIa when it is complexed to TF (Girard et al 1989). The initial binding of TFPI to FXa is slow (Broze et al 1990) and this appears to explain why disseminated intravascular coagulation (DIC) continues in patients with normal or elevated levels of TFPI, since the formation of FVIIa/TF complexes would be too rapid to allow inhibition by TFPI (Warr et al 1990, Rapaport and Rao 1991). The role of TFPI in humans has been suggested as that of a natural anticoagulant, protecting against thrombotic complications which may otherwise arise from events where circulating blood is exposed to low levels of TF such as child birth or mild diseases where cytokine production is stimulated (Rapaport and Rao 1991). However, this point remains controversial.

Fibrinogen

Fibrinogen is a protein of 340kD, consisting of two identical, disulphide-linked subunits each containing one Aα, one Bβ and one γ chain held together by a number of disulphide bonds (reviewed by Henschen and Mc Donagh 1986). It is synthesised in the liver and is found circulating in plasma and also in platelets. There has been some controversy as to whether the platelet fibrinogen is metabolically, structurally or
functionally different from plasma fibrinogen but recent studies indicate that the origin of platelet fibrinogen is from the plasma and not megakaryocyte precursors (Handagama et al 1990, Louache et al 1991). Fibrinogen is converted to fibrin by the proteolytic action of thrombin. This results in the release of A and B peptides (A peptide from the Aα chain and B peptide from the Bβ chain) and the fibrin monomer which then associates with other fibrin monomers and is cross-linked by FXIII to form the insoluble fibrin clot (Doolittle 1973) (Fig 3). Deficiency of fibrinogen or afibrinogenenaemia, and dysfibrinogenenaemia in which the fibrinogen molecule is functionally altered by amino-acid substitution, result in bleeding disorders which can be severe (Flute 1977, Uzan et al 1984).

The amino acid sequence of the human Aα chain is 15 amino acids shorter at the carboxy-terminal end than that predicted from the nucleotide sequence (Rixon et al 1983, Kant et al 1983c). This is probably due to proteolysis of this part of the chain either before it leaves the cell or shortly after secretion in the circulation (Hantgan 1986). There are two forms of the fibrinogen γ-chain: γ-chains and the longer, γ'-chains. The γ'-chain contains a different and longer carboxy-terminal section than the 'normal' γ chain. The corresponding mRNAs for the γ- and γ'-chain variants are also of differing length. In humans about 10% of cytoplasmic γ-chain mRNA is 531 nucleotides longer than that for the major γ-chain and in the rat this figure is nearer 30%. In the rat this is due to concurrent alternative splice patterns of transcripts from a single gene, with the longer γ'-chain resulting from the failure to remove the final intron (Crabtree and Kant 1982). In the human the longer γ'-chain is produced by a different mechanism where an alternative processing and polyadenylation reaction occurs within the final exon to produce an mRNA which encodes the longer γ'-chain
(Fornace et al 1984). The function of the \( \gamma' \)-chain of fibrinogen is uncertain. There is also heterogeneity in the length of the \( \beta \)-chain mRNA because of different lengths of 3' untranslated region (Chung et al 1983).

The fibrinogen molecule is assembled in the rough endoplasmic reticulum and studies in the human hepatoma cell line HepG2 have shown that newly synthesized B\( \beta \) chains are utilized more rapidly than A\( \alpha \) or \( \gamma \) chains and that a large intracellular pool of free \( \gamma \)-chains exists. This suggests that there are also intracellular pools of A\( \alpha \) chains and that synthesis of the B\( \beta \) chain is rate limiting in the assembly and secretion of the mature fibrinogen molecule since individual chains are not secreted (Yu et al 1983, 1986). In the chicken however, there is evidence that production of the \( \beta \)-chain may not be the rate limiting step, although this was found in a chick embryo hepatocytes grown under abnormal conditions (Plant 1986).

1.4 Epidemiology of fibrinogen and factor VII as risk factors for atherosclerosis and MI

Much of the interest in the relationship between thrombosis and atherosclerosis has stemmed from the finding that the plasma levels of factor VII and fibrinogen were stronger predictors of risk of IHD than plasma cholesterol levels (Wilhelmsen et al 1984, Stone and Thorp 1985, Meade et al 1986, Kannel et al 1987).

The Northwick Park Heart Study showed that there was an association between the mean levels of FVII coagulant activity (FVIIc) and fibrinogen and an individual’s risk
of ischaemic heart disease (Meade et al 1986). This was a five year prospective study of 1511 middle-aged white men from the UK in which haemostatic variables were measured, as well as other variables that had been previously associated with risk. The principal results of the Northwick Park Heart Study showed that an increase of one standard deviation in factor VII and fibrinogen raised the risk of IHD death within 5 years of recruitment by 55% and 67% respectively, and by 62% and 84% for all IHD events compared to 43% for cholesterol (Meade et al 1986). Cholesterol was not found to exert an independent effect on IHD deaths within 5 years of recruitment (Meade et al 1986). Preliminary data from the Prospective Cardiovascular Munster Study (PROCAM) confirmed these observations (Balleisen et al 1986). Three other prospective studies have shown that plasma fibrinogen level is a risk factor for IHD (Wilhelmsen et al 1984, Stone and Thorp 1985, Kannel et al 1987). Plasma fibrinogen levels have been found to be higher in patients with a greater degree of coronary artery disease (Lowe et al 1980, Broadhurst et al 1990) and have also been found to have a synergistic effect on stroke together with blood pressure (Wilhelmsen et al 1984).

In both sexes, increasing age and body mass index are positively correlated with FVII level (Meade and North 1977, Balleisen et al 1985). In women, use of oral contraceptives and menopausal status is associated with increased factor VII level (Ballaiiesen et al 1985, Scarabin et al 1990), and it has been suggested that in menopause this increase may be due to the rise in VLDL (Folsom et al 1991). Carbohydrate metabolism has also been implicated in the control of FVII levels, which were found to be correlated with fasting glucose level and also to be positively correlated with induced hyperglycaemia in one study (Hultin 1991). Dietary studies have also shown a positive relationship between hypertriglyceridaemia and some of the
vitamin-K dependent coagulation factors including FVII and this will be described in more detail in 1.7. Decreased FVIIc levels were found to be associated with ethanol intake in one study (Folsom et al 1991).

Increased plasma fibrinogen levels are associated with age, obesity, use of oral contraceptives and menopause (Balleisen et al 1985a, Meade and North 1977, Meade et al 1979, Balleisen et al 1985b, Meade et al 1976). Fibrinogen levels can also be increased through smoking (Wilhelmsen et al 1984, Stone et al 1985, Balleisen et al 1985 and Meade et al 1987). Decreased fibrinogen levels have been found to be associated with ethanol intake (Meade et al 1979, Yarnell et al 1983, Lee et al 1990 and Folsom et al 1991) and with physical activity in one study (Møller and Kristensen 1991).

There are also ethnic differences in risk of CHD. Within the UK, individuals of different ethnic origin are known to differ in their risk of CHD, with the incidence being low in individuals of Afro-Caribbean origin (Marmot et al 1984) and high in individuals from the Indian sub-continent (Balarajan et al 1984, McKeigue and Marmot 1988). These different ethnic groups are also known to exhibit differences in factor VIIc and triglyceride levels (Meade et al 1978, Miller et al 1988), with Afro-Caribbeans having a lower mean factor VIIc and triglyceride level than Europeans (Meade et al 1987). The differences in plasma FVIIc levels have been suggested to be a consequence of differences in dietary fat intake because of the correlation between plasma triglyceride level and FVIIc level (Miller et al 1989, see also 1.8).

Although thrombolytic therapy has been established in the treatment of acute myocardial
infarction (ISIS-2 1988, GISSI-2 1990, The International Study Group 1990) the relative importance of acute thrombosis in the presence of atherosclerosis is not fully understood. The presence of fibrinogen and fibrin in atherosclerotic plaques has been known for some time (Duguid 1948, Woolf 1961, Woolf and Carstairs 1967, Kao et al 1965, Haust et al 1965), and there is evidence for an association between fibrin present in the atherosclerotic plaque and the binding of LDL (Smith et al 1979), which would contribute to the evolution of the plaque. Fibrinogen is chemotactic for vascular smooth muscle cells and so may contribute to intimal thickening (Naito et al 1989).

Plasma fibrinogen level is also related to the extent of coronary atherosclerosis in CHD patients (Lowe et al 1980, Hamsten et al 1986, Handa et al 1989). Plasma fibrinogen has been directly implicated in promoting the thrombotic occlusion of arteries already narrowed by atherosclerotic lesions and six prospective epidemiological studies have shown a positive association between fibrinogen and subsequent cardiovascular events (Wilhelmsen et al 1984, Stone and Thorp 1985, Meade et al 1986, Kannel et al 1987, Balleisen et al 1987, Yarnell et al 1991). Recent studies have shown a relationship between CHD, raised plasma fibrinogen concentration, raised LDL and low in vitro fibrin gel porosity formed, suggesting that these factors cause intravascular deposition of fibrin networks which may be more thrombogenic and more resistant to mechanical forces of blood flow (Fatah et al 1992).

Formation of the FVII/FVIIa and TF complex is regarded as a major initiating stimulus of coagulation, and increases in plasma FVII may contribute to the development of CHD by increasing blood coagulability (Meade et al 1987). In experimental models it has been shown that tissue factor interacts with FVIIa in the generation of fibrin at exposed subendothelial surfaces and that when levels of tissue factor are limiting, tissue
factor dependent coagulation proceeds largely through activation of factor IX by FVIIa (Nemerson 1988). Macrophages can also express tissue factor and, as already discussed (1.1), there is good evidence that macrophages are involved in development of the atherosclerotic plaque. These observations have led to the hypothesis that FVII and tissue factor may be involved in the development of the plaque as well as in thrombogenesis (Hultin 1991). Therefore factors which affect the circulating levels of FVII and its activation are important in atherosclerosis where an increased predisposition to coagulation can lead to a thrombotic event.

FVII is the vitamin-K dependent coagulation factor most rapidly and extensively modified by warfarin, a drug congeneric with dicumarol, which acts as a vitamin-K antagonist and inhibits the $\gamma$-carboxylation, for which vitamin-K acts as a cofactor, of the glutamic acid residues in these coagulation factors (McTigue and Sutie 1983). Studies on the prevention of venous thrombosis using lower than conventional doses of warfarin have shown this to be effective and a pilot study of the effects of low doses of warfarin (Thrombosis Prevention Trial-TPT) on the prevention of IHD in men at high risk has shown that FVIIc levels can be reduced to 70% of control levels using these lower doses without significant increases in bleeding side effects (Meade et al 1988). The final results of the full TPT are not yet available.

1.5 Fibrinogen and the acute phase response

The acute phase response is a set of highly complex systemic reactions accompanying acute inflammation. It is characterized by significant changes in plasma levels of a number of proteins known as acute phase reactants. These include positive acute phase
proteins, C-reactive protein, serum amyloid A, \(\alpha_1\)-antichymotrypsin, \(\alpha_1\)-acid glycoprotein (orosomucoid), haptoglobin, haemopexin, ceruloplasmin, complement C3, \(\alpha_1\)-antitrypsin and fibrinogen, the levels of which may increase from several-fold up to 1000-fold relative to the normal levels of these proteins. There is also a set of negative acute phase proteins, including albumin, transferrin, HS\(_2\)-glycoprotein and apolipoprotein A-I, the levels of which are reduced during an acute phase response (Reviewed in Fey and Fuller 1987).

Fibrinogen concentration rises 2-20 fold during an acute phase response with a comparable rise in mRNA levels (Fuller et al 1985, Birch and Schreiber 1986, Otto et al 1987). The increase in mRNA levels can be inhibited by actinomycin D and has been shown to be correlated with increased transcription from all three fibrinogen genes (Fuller et al 1985, Birch and Schreiber 1986, Otto et al 1987) suggesting that the increased fibrinogen levels seen in the acute phase are due to increased transcription and not changes in mRNA stability or protein synthesis.

There are a number of cellular and molecular factors thought to be involved in the regulation of the hepatic acute phase response. It has been shown that, on incubation with plasmin-derived fragments of fibrin or fibrinogen, leucocytes secrete a factor which can increase the production of fibrinogen 4-6 fold in rat hepatocyte monolayers (Ritchie et al 1982). More specifically, monocytes are thought to play key role in the regulation of the acute phase. They are known to migrate from peripheral blood to sites of tissue damage and produce many factors which mediate inflammation in response to various stimuli. Monocyte conditioned medium produces an acute phase-like response when incubated with hepatocytes, with an increase in fibrinogen protein and

HSF purified from monocyte-conditioned medium has been shown to produce an acute phase-like response in cultured hepatocytes and hepatoma cell lines; recombinant human IL-6 has been shown to produce a similar response, and in both cases there is an increase in fibrinogen protein and mRNA levels along with other positive acute phase reactants (Fuller et al 1985, Gauldie et al 1987, Castell et al 1988, Jambou et al 1988, Morrone et al 1988, Castell et al 1989). This HSF and IL-6 appear to be the same molecule, which has also been independently identified as interferonβ-2 (IFNβ-2), B cell stimulating factor-2 (BSF-2), and hybridoma growth factor (Gauldie et al 1987, Yasukawa et al 1987), illustrating its pleiotropic action.

IL-1 and glucocorticoids were found to be involved in achieving an optimal acute phase response in vitro using the human hepatoma cell line HepG2 (Baumann et al 1987). These studies however, emphasized the fact that hepatoma cell lines may not reflect the in vivo situation in normal liver cells. Some mouse and rat hepatoma cell lines do not respond to IL-6 or IL-1 (Baumann 1983) and two human hepatoma cell lines HepG2 and Hep3B respond differently to IL-1 and TNF when compared with human hepatocytes stimulated in the same way. In Hep3B cells, IL-1 and TNF only stimulated production of complement C3 (Darlington et al 1986). In HepG2 cells IL-1 reduced basal levels of fibrinogen but TNF had no effect (Baumann et al 1987). In human
primary hepatocytes IL-1 and TNF both reduced fibrinogen, albumin and transferrin production (Castell et al 1989). Even though some of this evidence is conflicting, it would seem that IL-1 may have a direct effect on fibrinogen synthesis. IL-1 and TNF both induce expression of IL-6 in various cell types and so may provide a co-ordinated regulation of the acute phase response in the liver (Ray et al 1988, Zhang et al 1988).

TGF-β, as previously discussed, is a multifunctional polypeptide which appears be important as a modulator of inflammation and tissue repair (Wahl et al 1989). Cells that are involved in the development of the atherosclerotic lesion, such as monocyte-macrophages, lymphocytes and platelets release significant amounts of TGF-β when activated (Assoian et al 1987). TGF-β appears to be involved in the control of hepatocyte growth (Braun et al 1988) and has been proven to regulate the expression of several genes, such as the inhibition of growth factor and oncogene induced expression of stromelysin (Kerr et al 1990). In vitro studies have shown TGF-β to be a possible counterpart to IL-6 in acute phase regulation, by its post-transcriptional inhibition of Apo-AI and albumin, which are both negative acute phase reactants (Morrone et al 1989). Interaction between IL-6 and TGF-β may therefore be of importance in the regulation of the acute phase response and in the expression of acute phase proteins.

Most of the investigation into the action of glucocorticoids has centred around the synthetic glucocorticoid, dexamethasone. Fibrinogen protein levels, but not mRNA levels, were found to be increased in primary rat hepatocytes and the FAZA rat hepatoma cell line by dexamethasone. Further investigation suggested that when IL-6 and dexamethasone were added together they acted synergistically, producing an effect
similar to but greater than that elicited by IL-6 alone, since both protein and mRNA levels were increased under this stimulus (Otto et al 1987). Dexamethasone has been found to enhance the action of IL-6 in rat and human hepatocyte and hepatoma cell cultures (Baumann et al 1984, 1986, 1987). Woloski et al have proposed a plausible negative feedback pathway based on the interactions of IL-1, IL-6, adrenocorticotropic hormone (ACTH) and glucocorticoids (Woloski et al 1985). Briefly, monocyte production of IL-1 and IL-6 in response to tissue injury stimulates the release of ACTH from the pituitary gland. ACTH stimulates glucocorticoid production and these act synergistically to produce an acute phase response from the liver. The circulating glucocorticoids then act to inhibit further monocyte release of IL-1 and IL-6 and at the same time block ACTH release from the pituitary which in turn inhibits further glucocorticoid production.

Evans et al, while investigating which factors were involved in the induction of fibrinogen during the acute phase response, found that the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), and the synthetic diacylglycerol, 1-oleoyl-2-acetylglycerol (OAG), both induced fibrinogen mRNA. Since both these substances induce protein kinase C at the same doses found to induce the production of fibrinogen, and both mimic the effects of IL-6, it was proposed that IL-6, in its capacity as HSF, acts through the protein kinase C mechanism (Evans et al 1987).

1.6 Fibrinogen gene; structure and expression

The fibrinogen gene locus has been mapped to the distal third of the long arm of human chromosome 4 (Olaisen et al 1982, Humphries et al 1984, Kant et al 1985).
described in the preceding section, the fibrinogen molecule consists of three polypeptide chains \( \alpha \), \( \beta \), and \( \gamma \). This six chain \( \alpha_2\beta_2\gamma_2 \) structure has been found to be conserved in even the most primitive vertebrates, where 50% homology exists between the amino acid sequences of the \( \beta \) and \( \gamma \) chains and those in the human genes (Strong et al 1985, Bohonus et al 1986). Each polypeptide chain is encoded by a separate mRNA transcribed from three single-copy genes (Imam et al 1983, Kant et al 1983b, Kant et al 1985). Cloning of the cDNAs for each polypeptide chain and comparison of the cDNAs and the amino acid sequences showed a strong homology between these genes, suggesting they are evolutionarily related (Henschen and McDonagh 1986). Further investigation showed that these three genes lie in close proximity to each other over a region of 50 kilobases (Kant et al 1985) and that the \( \beta \) gene is in the opposite orientation to the \( \alpha \) and \( \gamma \) genes (Kant et al 1983b, Kant et al 1985). Kant suggested that gene duplication and inversion of a single ancestral gene resulted in the present organization of the fibrinogen genes, which have the same arrangements of introns and exons (Kant et al 1985). The entire sequences of the fibrinogen \( \alpha^- \), \( \beta^- \), and \( \gamma^- \)-chain genes are known and are deposited in the Genbank databank (Rixon et al 1983, Chung et al 1983, Rixon et al 1985).

The process of transcription of a DNA sequence into RNA is central to gene expression and is therefore one of the major points of control. Tissue-specific or signal-specific protein production has been shown to be dependent largely on transcriptional control (Darnell 1982). In eukaryotic genes, regions upstream of the sequence which encode the final protein have been found to contain two types of elements: those found in many genes, which appear to be involved in the basic transcription process, and those found in genes whose transcription is tissue or signal specific. These sequences are
known as *cis*-acting elements, and the proteins which bind to such sequences are known as *trans*-acting factors.

The TATA box is an AT-rich region (consensus TATAA/TAA/T) which is found about 30 base pairs upstream of the transcriptional start site in many genes. The TATA box is involved in binding RNA polymerase II, the enzyme responsible for transcribing protein coding genes, and is involved in the accurate positioning of the start site of transcription (Goodwin et al 1990). The Sp1 box is a GC-rich sequence, one or more copies of which are found upstream of the promoter in many genes, that dramatically enhances the low activity of the promoter itself (Maniatis et al 1987). The CCAAT box is also found typically upstream of the TATA box and is present in many but not all genes. The importance of these elements is shown by the fact that the elimination of any of these sites by mutation or deletion results in the abolition of transcription (Mc Knight et al 1984).

Sequences involved in regulated transcription are less frequent and are found interspersed with the elements described above. There are a variety of elements found in signal-specific genes: the heat shock consensus element is involved in the regulation of transcription in response to heat and is found in, among others, the human heat-shock protein (hsp 70) gene. Other signal-specific elements are the glucocorticoid response element which can bind the glucocorticoid hormone-receptor complex, the metal response elements (MRE) which allow activation of the gene in response to treatment with heavy metals such as zinc and cadmium and elements which bind a transcription factor AP2 which allows activation of the gene in response to cyclic AMP and phorbol esters. Other similar elements are found in the promoters of tissue-specific
genes, but these bind transcription factors which are active only in tissue where the gene is expressed. These include the octamer motif in the enhancers of the immunoglobulin heavy- and light-chain genes which mediates B cell-specific expression by binding the transcription factor Oct-2 and a sequence in the promoters of the prolactin and growth hormone genes which mediates expression only in pituitary cells (Reviewed by Latchman1991).

The fibrinogen genes each contain elements involved in the basic transcriptional process and elements involved in tissue- and signal-specific transcription. As previously described, formation of the β-fibrinogen chain is the rate limiting step in the biosynthesis of fibrinogen and therefore the control of the expression of the β-fibrinogen gene may be important in determining plasma levels of fibrinogen ( Yu et al 1983, 1986).

All the human fibrinogen genes have a TATA box and a CAAT box (Courtois et al 1987, Morgan et al 1988). In the β gene there is a TATA box at -21 to -29 and a CAAT box at -58 to -63 (Huber et al 1987). The TATA box of the γ-gene was found to be different and closer to the transcription start point although the CAAT boxes of both genes are identical and at approximately the same distance from the transcription start site (Rixon et al 1985). There are also homologous sequences present in the α-fibrinogen gene (Rixon et al 1985, Huber et al 1987). The β fibrinogen gene has both distal and proximal elements which regulate its expression in response to cytokines and hormones.

Studies on the β fibrinogen gene promoter showed that it was functional in hepatoma cells but not in non-hepatic cell lines, and this correlated with the presence or absence
of a liver-specific nuclear factor, hepatic nuclear factor (HNF-1) (Courtois et al 1987). This factor was found to interact with a short sequence between -89 and -77 upstream of the \( \beta \) fibrinogen gene (Courtois et al 1987). The HNF-1 binding site was also found to be present in the \( \alpha \)-fibrinogen promoter and in the promoters of other liver-specific genes (Courtois et al 1988, Kugler et al 1988, Costa et al 1989, Baumhueter et al 1990).

The control of the \( \gamma \)-fibrinogen gene appears to be different from that of the \( \alpha \)- and \( \beta \)-genes and the \textit{cis}- and \textit{trans}-acting factors which have been found in the promoter of this gene are not hepatic-specific. Binding sites for a CAAT-binding protein, an Sp1 transcription factor and the adenovirus major late transcription factor were found (Chodosh et al 1987, Morgan et al 1988). Since none of these factors are liver-specific, there may still be undiscovered elements which are involved in the tissue specific expression of this gene, or it has been suggested that perhaps the HNF1 element of the \( \alpha \)-fibrinogen gene may act as an enhancer to control the expression of the \( \gamma \)-fibrinogen gene as well as the \( \alpha \)-fibrinogen gene (Green and Humphries 1989).

The recent studies by Huber et al also identified upstream sequences involved in the dexamethasone and IL-6 stimulation of the \( \beta \)-fibrinogen gene (Huber et al 1987). A sequence determining dexamethasone response was far from the promoter, between -2900 and -1503 from the transcription start point. The studies found a discrepancy between expression of the bacterial reporter gene chloramphenicol acetyl transferase (CAT) when driven by part of the \( \beta \)-fibrinogen gene in response to dexamethasone and \( \beta \)-fibrinogen mRNA levels from HepG2 cells under dexamethasone stimulation. This suggests that there are either additional dexamethasone responsive elements in the \( \beta \)-
fibrinogen gene or increased messenger RNA stability under the action of dexamethasone (Huber et al 1990). The IL-6 responsive elements were found to be between -150 and -82 in the 5'-flanking sequence of the gene and, since there were no differences between β-fibrinogen mRNA analysis and CAT assays, this region was taken to contain all the IL-6 responsive elements, although this may be a reflection of the limited sensitivity of the in vitro assay used. As described above, the HNF-1 binding-site is in the same region so there may be some relationship between these two regulatory elements (Huber et al 1990). In the rat α₂-macroglobulin gene a short sequence -CTGGGA- has been shown to be important in the IL-6 mediated induction of this gene (Ito et al 1989, M Hattori et al 1990, Hocke et al 1992) and this has been suggested to be a possible binding site for an acute phase transcription factor. This sequence is found in a number of acute phase reactant genes (Majello et al 1990, Oliviero and Cortese 1989, Poli and Cortese 1989, Wilson et al 1990, Won and Baumann 1990) and is present in all three fibrinogen genes and in the case of the β-fibrinogen gene, this sequence is present within the sequence found to be required for IL-6 stimulation in the studies of Huber et al described above.

1.7 Genetic variation in the β-fibrinogen gene

The extent to which genetic factors are involved in determining plasma fibrinogen level is of interest since individual and environmental factors influencing fibrinogen level only accounts for around 20%-28% of the population variance in fibrinogen (Meade et al 1979, Thompson et al 1987). Genetic heritability of plasma fibrinogen levels has been estimated at 51% in one family study and 29% in another study in monozygotic twins (Hamsten et al 1987, Berg and Kierulf 1989). No evidence is available at present
for the existence of one major gene determining fibrinogen levels and variation at number of unlinked loci is likely to be involved in determining between-individual differences in plasma fibrinogen levels. However it seems reasonable that the fibrinogen genes themselves are candidate genes that may be involved in determining between-individual differences in plasma fibrinogen level. Studies on the association between fibrinogen levels and two-allele restriction fragment length polymorphisms (RFLPs) of the fibrinogen cluster have shown that variation at the fibrinogen gene locus does determine some of the between-individual differences in fibrinogen levels (Humphries et al 1987, Snowden et al 1992). Two other studies however did not confirm this association (Berg and Kierulf 1989, Connor et al 1992) but this may be due to differences in the samples studied or the intra-individual variability in plasma fibrinogen levels which can account for as much as 28% of the sample variance (Thompson et al 1987). All of the RFLPs described in these studies lie in non-coding regions of the gene (Thomas, Green, Humphries unpublished data) and there is a possibility that they may therefore be markers in linkage disequilibrium with other sites in the fibrinogen gene which are functionally significant.

More recently, a G/A^455 polymorphic site has been described in the 5' flanking region of the β-fibrinogen gene and found to be associated with differences in plasma fibrinogen level and in linkage disequilibrium with the BclI RFLP (Thomas 1991). The sequence change underlying this polymorphism has been found to affect the binding of a hepatic nuclear protein (Dr F Green personal communication). Since the formation of β-fibrinogen is rate-limiting in the production of mature fibrinogen protein, any factors which affect transcription of this gene could have a direct effect on plasma fibrinogen levels. The G/A^455 polymorphism was found to account for 3.1% of the
variance in fibrinogen levels (Thomas et al 1991) and further studies on a C/T\textsuperscript{148}
polymorphism which is close to the G/A\textsuperscript{455} site, near to an IL-6 responsive element
(Fig 5), have found it to account for a similar percentage of variance in fibrinogen
levels and to be in almost complete linkage disequilibrium with the G/A\textsuperscript{455} site (Dr A
Thomas, personal communication). The G/A\textsuperscript{455} polymorphism has also been shown to
be in linkage disequilibrium with a polymorphism in the \(\beta\)-fibrinogen gene at +448
which results in the substitution of an arginine for a lysine and is also associated with
plasma fibrinogen levels (Henschen and Mc Donagh 1991), and so it remains a
possibility that this amino acid change may be functional and that the other \(\beta\)-fibrinogen
polymorphisms are acting as markers for it.
FIBRINOGEN LOCUS

POLYMORPHIC SITES
AND
TRANSCRIPTIONAL CONTROL SEQUENCES

Fig 5 Fibrinogen gene locus showing proposed binding sites for transcription factors. MLTF, adenovirus major late transcription factor; CAAT, CAAT binding factor; Sp1, transcription factor Sp1; HNF1, hepatic nuclear factor 1; IL-6, interleukin-6-responsive element. Horizontal arrows indicate direction of transcription of genes. The positions of the variable site for the enzymes HindIII, HaeIII, BclI and Taq I are shown.
The Northwick Park Heart Study found that FVII coagulant activity (FVIIc), serum cholesterol level and plasma triglyceride level were all positively associated with risk of CHD. Both lipid levels were positively correlated with FVIIc, and once the FVIIc level had been accounted for, neither made a statistically significant contribution to risk (Meade et al 1986). Hypertriglyceridaemia is associated with increased coagulability (Miller et al 1985, Simpson et al 1983) and there is good evidence to suggest that triglycerides have an effect on factor VII levels and factor VII activation (Miller et al 1985, Miller et al 1986). The large, negatively charged, triglyceride rich lipoprotein particles, very low density lipoproteins (VLDLs) and chylomicrons are thought to activate the intrinsic coagulation pathway via contact surface activation and therefore ultimately to catalyse factor VII activation (Mitropoulos et al 1987a, Mitropoulos et al 1987b, Miller et al 1986, Mitropoulos et al 1989, Miller et al 1989a). The fractional catabolic rate of factor VII is also reduced by its binding to lipoprotein particles which would result in raised plasma levels of factor VIIc, enhancing the effect on activation (Carvalho de Sousa et al 1989). It has also been suggested that in prolonged hyperlipidaemia, where sustained factor VII activation causes an increase in procoagulant activity, hepatic production of factor VII and other vitamin-K dependent clotting factors is stimulated by the resulting increase in the levels of prothrombin fragment F1.2 (Mitropoulos and Esnouf 1990). 'Autoactivation' of purified FXII has been observed in vitro where micellar long-chain fatty acids provide a contact surface (Mitropoulos and Esnouf 1991) and activation of FXII and FVII observed when long-chain fatty acids are added to plasma (Mitropoulos and Esnouf 1990), suggesting that fatty acid generation may account for the high FVIIc found to accompany
hyperlipidaemia (Mitropoulos et al 1992). Castelli (1986) and Malekzadeh (1991) et al showed that hypertriglyceridaemia was only associated with CHD in the presence of lipoprotein lipase (LPL), the enzyme which releases unesterified fatty acids from the triglyceride core of chylomicrons and VLDL. Further studies by Mitropoulos et al (1992) provided evidence for the dependence of FVII activity on lipolytic activity via FXII activity, by their findings that the addition of LPL to plasma from LPL-deficient patients resulted in rapid activation of FXII. Cold activation of FVII with a contact surface of glass was similar in the LPL-deficient patients and the control samples studied. Incubation of plasma at 4°C inactivates C-I inhibitor, the major plasma inhibitor of the contact pathway, and so allows contact activation to proceed unhindered. This suggested that the high concentration of triglyceride-particles in the patients' plasma was a relatively ineffective contact surface without the action of LPL (Mitropoulos et al 1992).

1.9 Factor VII gene; structure and expression

The gene for Factor VII is adjacent to the gene for Factor X, on the long arm of chromosome 13 (Pfeiffer et al 1985, Miao 1992). There is some evidence that there is a regulatory mechanism controlling expression of FVII associated with region p23.1→p23.2 of chromosome 8 although the mechanism of this regulation has not yet been deduced (Fagan et al 1988). The gene for FVII contains nine exons and eight introns, and is approximately 12.8kb long (O’Hara et al 1987). There are five regions of imperfect tandem repeats, one of which, in intron 7, has been found to be polymorphic (O’Hara and Grant 1988). Polymorphisms in minisatellite tandem repeats is a common phenomena (Jeffreys et al 1985). There appears to be an optional first
exon, exon 1b, and mRNAs with or without this exon can be found in normal liver, although mRNA lacking exon 1b is far more abundant (Berkner 1986). Exons 1a and 1b and part of exon 2 encode a prepro leader sequence removed during processing. The mature circulating protein of 406 amino acids is encoded by the rest of exon 2 and exons 3-8.

The FVII gene also contains sequences known to be associated with control of transcription and translation. There is a TCATCATGG sequence which shares 7 of the 9 nucleotides of the eukaryotic consensus sequence for the translation initiation site of CCACCATGG (Kozak 1984). There is homology between the upstream regions of factor IX, protein C and FVII, all of which contain classical eukaryotic promoter elements. There are three polyadenylation sites at 15, 19 and 41 nucleotides downstream from the poly(A) signal. Downstream of the poly(A) signal FVII also has multiple copies of elements resembling sequences thought to play a role in mRNA stability (O'Hara et al 1987). The FVII gene shows similarities with other vitamin-K dependent coagulation factors in exon sequence and the nonoptional introns being similar in position to those in human protein C, factor IX, FX and the first three introns of prothrombin. This has led to the suggestion that the preservation of intron position and phase (which preserves the reading frame), and the encoding of discrete domains of these coagulation factors, such as the γ-carboxylase region and the growth factor domain, by separate (O'Hara et al 1987) exons has resulted from exon shuffling during evolution of the vitamin-K dependent family of coagulation factors.
1.10 Genetic variation in the factor VII gene

Although there is considerable within-individual variation in factor VIIc levels (Thompson et al 1989), 47% of the total variance in factor VIIc levels is explained by the variability between individuals (Meade and North 1977). At present there are no data on the heritability of factor VIIc levels but because of the increased risk of IHD associated with increased factor VIIc levels (1.4) the effect of genetic variation on factor VIIc levels is of interest.

A polymorphism was identified in exon 8 of the FVII gene which was found to be a G to A substitution, which alters an amino acid at position 353 from an arginine to a glutamine (Green et al 1991). This base change altered the cutting site for the enzyme MspI, making genotype determination simple by PCR and MspI digestion (Fig 6).

In a sample of 284 men from the UK the frequency of the allele coding for the factor VII Gln\textsubscript{353} was 0.1 and carriers for the Gln\textsubscript{353} allele had levels of Factor VIIc 22% lower than the sample mean. The 3 individuals homozygous for the Gln\textsubscript{353} allele had both low factor VIIc and low factor VII antigen levels suggesting that the amino acid substitution may alter the conformation of the protein, leading to reduced secretion of the protein from the liver or increased catabolism (Green et al 1991). Although the Arg-Gln\textsubscript{353} substitution is close to the active site serine (aminoacid 344) in the primary sequence, a three dimensional model of factor VII suggests that aminoacid 353 is on the opposite surface of the molecule to the active site (personal communication, Dr EGD Tuddenham) (Fig 7a and Fig 7b). The peripheral location of aminoacid 353 suggests that the Arg-Gln\textsubscript{353} substitution may not directly affect enzyme activity, however it
Fig 6 Position of the PCR amplified region for the detection of the Arg/Gln\textsubscript{333} polymorphism in the factor VII gene, showing sizes of MspI digestion products for the two alleles.
could influence interactions of factor VII with lipid surfaces or cofactors, thereby affecting factor VII activation or activity indirectly. However, since levels of both factor VIIc and antigen are low in homozygotes for the Gln$^{333}$ allele (Green et al 1991), it seems likely that the aminoacid substitution may affect either secretion or catabolism of the factor VII protein in addition to any indirect effects.
Fig 7a and 7b: overleaf, view of factor VIIa showing the Arg₃₅₃ and Gln₃₅₃ variants with the core region modelled on trypsin and putative surface loops. The core structure is realistic but the structure of the surface loops and their side chains is based on supposition. However the position of the Arg/Gln₃₅₃ on the opposite face of the molecule to its active site cleft is clear (Model by kind permission of D.Liney and S.Pemberton unpublished data). Large turquoise spheres represent amino acids in the active site triad.
Approaches

The studies presented in this thesis cover two main topics, FVII and fibrinogen. Elevated levels of both are known to be associated with an increased risk of IHD (Wilhelmsen 1984, Stone 1985, Meade 1986, Kannel 1987) and both are known to be influenced by a number of environmental and genetic factors. Because atherosclerosis and thrombosis are multifactorial polygenic disorders, genetic variation in one gene alone, or even in a number of genes, is not likely to be the sole cause of IHD in an individual. Rather it is more likely that development of atherosclerosis resulting in MI is due to interaction between a number of genes and the environment.

Two different approaches have been used in the studies presented in this thesis. Firstly, as in the fibrinogen gene expression studies, to investigate which environmental or other factors influence expression of the gene product under study. The gene is then examined to identify any mutations which are common in the population and which may influence these effects, and to elucidate the molecular mechanism of this interaction.

Secondly, as in the studies on the FVII genetic polymorphism, to look for mutations in functional regions of molecules, i.e. promoter regions, coding regions for enzyme catalytic domains, and examine the association in populations with levels of the gene product. Gene-environment interaction, i.e. with measured environmental factors such as plasma triglyceride level can then also be investigated, and ultimately the molecular mechanism of this interaction elucidated.
Aims

The overall aim of the project is to determine the contribution of common genetic variation at factor VII and fibrinogen loci to the differences in levels seen in individuals in the healthy population and to the elevated levels associated with atherosclerosis and thrombosis and to investigate molecular mechanisms for these effects.

Fibrinogen

Experiments were designed to:

(1) determine contribution of cytokine production from lipid-laden macrophages, or foam cells, in the atherosclerotic plaque to the raised plasma fibrinogen levels associated with IHD using peripheral human monocyte/macrophages in vitro

(2) investigate cytokine effect on fibrinogen mRNA in vitro using a human hepatoma cell line, HepG2 and recombinant human IL-6 and purified TGF-β

(3) investigate effect of -148 C→T base change in the 5' flanking region of the β-fibrinogen gene on the binding of hepatoma cell nuclear proteins in the presence of cytokines

(4) determine effect of -148 C→T base change in the 5' flanking region of the β-fibrinogen gene on transcriptional regulation using the bacterial CAT reporter gene
Factor VII

Experiments were designed to:

(1) determine the extent of association of the factor VII Arg/Gln$_{353}$ polymorphism with plasma factor VII coagulant activity in larger population samples from different ethnic backgrounds and in MI patients.

(2) Investigate genotype-specific interaction with environmental factors known to be associated with factor VIIc levels, most particularly triglycerides and warfarin.

(3) examine the association between Arg/Gln$_{353}$ genotype with risk of MI by studying allele frequency in patient samples and in controls from different European localities at differing risk of MI.
2 Materials and methods

2.1 Materials

Chemicals were obtained from BDH, Gibco-BRL or Sigma.

Enzymes and biochemicals were obtained from Pharmacia, Sigma, BRL, Promega or Anglian Biotechnology unless otherwise stated.

Radiochemicals, Hybond-N and X-ray film were obtained from Amersham.

Tissue culture media was obtained from Gibco-BRL and plasticware from Nunc or Falcon.

2.2 Methods

2.2.1 Cell culture

a Monocyte-macrophages

Monocytes were isolated from 25mls of fresh anticoagulated blood from healthy volunteer donors using Ficoll/Hyphaque centrifugation (Boyum et al 1968). The cells were seeded in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) in 24-well plates at a density of 1.5x10^6/well. After incubation for 2 hours at 37°C in 5%CO₂ humidified incubator, non-adherent cells (mainly lymphocytes) were removed by washing with Hanks Balanced Salts Solution (HBSS) and fresh medium was added. Twenty four hours after seeding, the cells were washed again. Experiments were performed 3 or 17 days after seeding, in the latter case when the remaining adherent
cells had assumed features characteristic of macrophages and were capable of taking up modified low density lipoprotein (LDL).

**b HepG2 cells**

HepG2 human hepatoma cells were obtained from the American Type Culture Collection and maintained in MEM (Gibco) supplemented with 10% FCS, 2mM sodium pyruvate, 1 x non-essential amino acids, 100 U/ml penicillin, 100μg/ml streptomycin at 37°C, in a humidified incubator equilibrated with 5% CO₂ and 95% air. Cells were seeded at a density of approximately 6.6 x 10⁶ cells/10cm² well in 6-well tissue culture plates.

**c Human Detroit foreskin fibroblasts**

Human Detroit foreskin fibroblasts were obtained from Flow Laboratories and maintained in DMEM supplemented with 10% FCS, 100U/ml penicillin and 100μg/ml streptomycin.

**2.2.2 Cell treatments**

**a Lipid loading of monocyte-macrophages**

Monocyte-macrophages to be treated with acetylated LDL (AcLDL) were cultured for 17 days and washed with phosphate buffered saline (PBS). These cells were then incubated with LDL (gift from Dr. Arvin Jaddah, Hammersmith Hospital) previously
treated with acetic anhydride (Basu et al. 1976) at 50μg/ml for 48 hours. Monocyte-macrophages to be treated with lipopolysaccharide (LPS) (from Salmonella typhimurium, Sigma chemical company Ltd. Poole, England) were cultured for either 3 or 17 days, washed with PBS and incubated for 48 hours with LPS at a concentration of 100μg/ml. Cells which were left untreated had medium replaced at 17 days with normal tissue culture medium. The conditioned medium was removed and stored in aliquots at -20°C until used to treat hepatoma cells.

b Treatment of HepG2 cells with recombinant human IL-6 (rhIL-6) and anti-IL-6 antibody

At the time of these experiments there were no reports of treatment of HepG2 cells with rh IL-6. The dose of IL-6 was calculated to give a maximal response since the reported Kd of the IL-6 receptor is 0.1-1nm depending on the cell type and a dose of 1000U/ml IL-6 is approximately 400 pmoles and therefore within the range of the receptor Kd (Taga et al 1987). When almost confluent, HepG2 cells were incubated in 6-well plates for 24 hours with 1ml of medium containing rhIL-6 at 1000U/ml (Gift from D. Novick, Weizmann Institute, Israel). Cells to be treated with anti IL-6 antibody were incubated for 24 hours with medium containing 80μl of 1:10 dilution of polyclonal anti-IL-6 antibody (Gift from L. Aarden, Leiden, Netherlands). Where both rhIL-6 and anti-IL-6 were used, appropriate amounts were preincubated together in 100μl of medium for 1 hour at room temperature before diluting into 1ml of medium and incubating with the cells. Control cells were treated in the same way as test cells, omitting additives.
c Treatment of HepG2 cells with conditioned medium

HepG2 cells were treated with a 1 in 10 dilution in fresh medium of conditioned medium from AcLDL-treated and untreated monocyte-macrophages. The dilution of conditioned medium used was determined in a dose response experiment. After 48 hours the medium was removed and RNA extracted from the cells using NP-40 as described in 2.2.3a.

2.2.3 RNA preparation from cell cultures

a Nonidet P-40 extraction

RNA was extracted from monocyte-macrophages by the Nonidet P-40 (NP-40) lysis method which allows RNA extraction from small numbers of cells (White et al 1982). Briefly, medium was aspirated and the cells were washed once with PBS. Two-hundred microlitres of TE buffer followed by 40μl 0.5% NP-40 solution were added to each well of a 6-well plate, mixed well and incubated for 3-4 minutes to allow cell lysis. The resulting mixture was transferred into 1.5 ml sterile microfuge tubes on ice. The mixture was centrifuged for 15 minutes and the supernatant transferred to fresh 1.5 ml tubes. The RNA was extracted from the supernatant with equal volumes of TE-saturated phenol, phenol:chloroform and chloroform and then precipitated overnight at -20°C after adding 1/10th volume 3M sodium acetate and 2 volumes absolute ethanol. Finally the RNA pellet was vacuum dried, resuspended in 30μl sterile water and stored at -20°C until RNA analysis.
b Guanidinium Isothiocyanate (GT) extraction

Total cellular RNA from HepG2 and fibroblast cells was isolated by the guanidinium isothiocyanate-caesium chloride method (Chirgwin et al 1979), which requires approximately $1 \times 10^6$ cells. Briefly, cells were trypsinized, resuspended in PBS and centrifuged for 5 minutes at 1000 rpm. Five millilitres of GT solution were added to the pellet, mixed well and incubated at 4°C for 15 minutes. The GT mixture was carefully pipetted onto 3mls CsCl solution in a 12ml Sorvall polyallomer tube and centrifuged at 33,000rpm at 20°C for 24 hours in a Sorvall OTD-55B ultracentrifuge using a TH641 swing-out rotor. The GT/CsCl solutions were then poured off and the final RNA pellet was vacuum dried and dissolved in 200μl sterile water. Total RNA was quantitated by measuring its absorbance at 260nm, and stored at a concentration of 1mg/ml at -20°C until RNA analysis.

2.2.4 DNA preparation

a DNA preparation from whole blood

DNA was extracted from whole blood by a rapid small scale method (Talmud et al 1991). 100μl whole blood was mixed with 400μl 0.17M NH₄Cl and incubated at room temperature for 20 minutes. After spinning in a microfuge, the pellet was washed 2-3 times with cold 0.9% NaCl, resuspended in 200 μl 0.05M NaOH, boiled for 10 minutes and neutralised with 25 μl 1M Tris (pH8.0). The DNA preparation was then stored at -20°C.
b DNA preparation from bacterial cultures

i Bacterial strains

Competent cells for transformation with recombinant plasmids were from the *E. Coli* strain XL1-blue and were prepared by Dr F Green.

ii Miniprep

DNA was extracted in small quantities using the alkali lysis method (miniprep) from 1.5 ml fresh overnight culture. The cells were pelleted and lysed in 50mM glucose, 10mM EDTA, 25mM Tris-HCl (pH 8.0). The bacterial cell membranes, chromosomes and proteins were removed by precipitation with 0.2M NaOH, 1% SDS followed by 3M potassium/5M acetate solution and centrifugation. The DNA was ethanol precipitated and the DNA pellet was resuspended in TE and treated with 10 μl of 10mg/ml DNase-free pancreatic RNase at 37°C for 30 minutes. The DNA was stored at -20°C.

iii Midiprep

Plasmid DNA for use as cDNA probes was prepared from 500ml overnight cultures. The cell pellet was thoroughly resuspended in buffer I and incubated on ice for 10 minutes. Freshly prepared buffer II was added, mixed very gently to lyse the cells and incubated on ice for exactly 10 minutes. The mixture was neutralised by the addition of buffer III and incubated on ice for 20 minutes. The mixture was then spun at
10,000rpm in a Sorvall RC5 centrifuge using an SA-600 rotor, at 4°C for 30 minutes. The supernatant was transferred to 2 fresh centrifuge tubes and after RNase A treatment, the supernatant was phenol/chloroform/isoamylalcohol (25:24:1 by volume) extracted twice to remove protein, and the DNA was ethanol precipitated overnight at -20°C. The DNA pellets were resuspended in 1.6ml sterile water and mixed with 0.4ml 4M NaCl. Two millilitres of 13% polyethylene glycol (PEG) 8000 solution were added and the mixture was incubated on ice for 1 h. The solution was centrifuged as above for 20 minutes and the DNA pellet was washed once with 70% ethanol, dried, resuspended in TE and stored at -20°C.

Buffer I: 25mM Tris-HCl pH8.0
10mM EDTA
15% sucrose
2mg/ml lysozyme (added immediately prior to use)

Buffer II: 0.2M NaOH
1% SDS
prepared immediately before use

Buffer III: 60ml 5MKoAc
11.5ml glacial acetic acid
28.5ml water
iv Maxiprep

Large scale preparation of plasmid DNA was performed for plasmids required for transfection experiments. One colony from a plate grown overnight was inoculated into 10mls of Circlegrow medium (BIO101 INC., California) containing 50μg/ml ampicillin (AMP) and incubated overnight at 37°C. The pRSVCAT plasmid required amplification and 500mls Circlegrow + AMP were inoculated with 1ml of overnight culture and incubated until the A_{600} was 0.4. Eighty-five milligrammes chloramphenicol were added and the culture incubated overnight. The plasmid was extracted using a Circleprep kit (BIO 101 INC., California) according to manufacturer's instructions. The resulting DNA was quantified spectrophotometrically and stored at -20°C. The other plasmids used were pUC based i.e. high copy number, and so chloramphenicol amplification was not necessary.

c Purification of DNA using Geneclean

This procedure was used in the purification of PCR products prior to ligation. The Geneclean kit (BIO 101 INC., California) was used according to manufacturer's instructions.

d Probe preparation

Human apo E cDNA and human β-fibrinogen cDNA probes were used to detect their respective mRNAs on Northern and slot blots. The apo E cDNA probe is a 930bp Xho II fragment in a pKT218 vector and contains most of the coding region (Wallis et al
The β-fibrinogen cDNA probe is a 700 bp PstI fragment from pFB5 (Humphries et al 1984). A mouse α-actin cDNA probe was used as a control (Humphries et al 1981). Plasmids were grown up and DNA was extracted using the midiprep method and the cDNA was cut out of the vector using the appropriate restriction enzyme. After digestion, the insert was separated from the vector on a 2% low-melting point agarose gel containing EtBr. The bands were visualised under U.V. light and the appropriate band cut out from the agarose gel. This was weighed and 3x the weight of the agarose was added in water. The mixture was then boiled for 5 minutes to melt the agarose, aliquotted and stored at -20°C. The probe was then labelled using the random oligonucleotide primer extension technique (see section 2.2.8a).

2.2.5 Electrophoresis

a Agarose gel electrophoresis

Agarose gels were made with 1-2% agarose in 1xTAE containing 1μg/ml EtBr. The solution was boiled and allowed to cool before being poured into a gel former with a comb to form wells for loading DNA samples. The gel was left to set, the comb was removed and the gel placed in a horizontal electrophoresis tank containing 1xTAE. The samples to be loaded were first mixed with one tenth volume 10x loading buffer. After sample loading, the gel was run at a suitable voltage to separate the DNA fragments and the resulting bands were then visualised using U.V. transillumination.

10x TAE buffer: 0.4M Tris
0.01M EDTA
pH 8.0 with glacial acetic acid

10x loading buffer: 0.25% bromophenol blue (w/v)
25% Ficoll 400 (w/v)
0.1M EDTA

b Denaturing polyacrylamide gel electrophoresis for DNA sequencing

0.4mm spacers were placed between two clean plates, one of which had been siliconised using Repelcote (BDH) and these were then taped together to prevent leakage of the acrylamide solution. 75mls of 6% acrylamide solution was prepared with 19:1 acrylamide:bisacrylamide solution, 42% urea and 1xTBE. The solution was warmed at 37°C to dissolve the urea. Polymerization was achieved by the addition of 75μl of a crosslinking agent, TEMED (NN N’N’-tetramethylethylenediamine) and 75μl of freshly made 25% ammonium persulphate solution. The solution was then poured carefully between the glass plates, avoiding the formation of bubbles. Two 0.4mm sharks tooth combs were inserted upside down and clipped in place to form a horizontal surface at the top of the gel. Once the gel had set (approx. 1 hour) it was untaped, placed in a vertical electrophoresis apparatus and the combs were replaced with the teeth forming small wells at the surface of the gel. The gel was pre-run in 1xTBE until warm (approx. 20 minutes) and the wells were flushed out to remove any urea which may have leached out of the gel. Samples to be loaded were mixed with a 0.5 volume of formamide dye, boiled for 3 minutes, immediately placed on ice and quickly loaded. The gel was run at a constant power of 60W (maximum current 55mA, maximum voltage 2000V) until the required separation was achieved. After the appropriate time
the gel was transferred to 3MM filter paper (Whatmann) and dried under vacuum at 80°C for at least 1 hour. Since the radioisotope used in sequencing was 35S, autoradiography was then carried out overnight at room temperature.

10x TBE: 89mM Tris-borate
2mM EDTA
pH 8.3

Formamide dye: 100ml deionized formamide
0.1% xylene cyanol
0.1% bromophenol blue
20mM EDTA

c Non-denaturing polyacrylamide gel electrophoresis for bandshift assays

Seventy-five millilitres of acrylamide solution was prepared containing 7% 30:1 acrylamide:bisacrylamide and 0.5x TBE. This was polymerized with 75µl TEMED and 75µl of freshly prepared 25% ammonium persulphate. The gel was poured between two clean glass plates (20x20cm) separated by 1mm spacers and a wide toothed comb was inserted and clipped in place. Once the gel had set it was untaped and pre-run for 30 minutes (and for not longer than 1 hour) at 4°C. The samples were then loaded and the gel run for a further 3 to 4 hours at 150V (maximum current 20mA) at 4°C, dried on to DE81 paper (Whatmann) and, since the isotope used was 32P, autoradiography was carried out at -70°C overnight.
2.2.6 PCR

Standard conditions were used for the polymerase chain reaction (PCR) (Saiki et al 1988; Mullis and Faloona 1987). 200-500ng of genomic DNA were used as template with 200ng of each primer in a 40μl volume. The PCR contained 200μM each of dTTP, dGTP, dCTP and dATP, 10mM Tris-HCl (pH8.3), 1.5mM MgCl₂, 50mM KCl, 10% dimethyl sulphoxide and 0.8 units BRL Taq polymerase. Samples were overlaid with paraffin oil and typically underwent one cycle of 5 minutes at 95°C, 1 minute at 55°C and 2 minutes at 72°C followed by 30 cycles of 1 minute at 95°C, 1 minute at 55°C and 2 minutes at 72°C on a Cambio Intelligent Heating Block. Primers were obtained from The Advanced Biotechnology Centre, Charing Cross and Westminster Medical School or Severn Biotech Ltd, Kidderminster.
TABLE 1 Polymerase chain reaction conditions and primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR purpose</th>
<th>Primer sequence (5’-3’)</th>
<th>Reaction Conditions *</th>
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</thead>
<tbody>
<tr>
<td>Factor VII</td>
<td>Detection of Arg/Gln353 polymorphism</td>
<td>GGGAGACTCCCCCA AATATCAC</td>
<td>Denaturing 95°C (1min)</td>
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<tr>
<td></td>
<td></td>
<td>ACGCAGGCTTGGCT TTTCTCTC</td>
<td>Annealing 55°C (1min)</td>
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<td></td>
<td>Extension 72°C (2min)</td>
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<td>Production of inserts for transient expression constructs</td>
<td>AAATAAGCTTTGC TGG</td>
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<tr>
<td></td>
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<td>CCAGCAAAACTTA TTTA</td>
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<td>Extension 72°C (2min)</td>
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<td></td>
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<td>Fragment Size (bp) 240</td>
</tr>
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</table>

* Reaction mixtures were heated initially to 95°C for 5 minutes to denature DNA
2.7 Restriction enzyme digestion of DNA

Restriction enzyme digests were set up in 20μl volumes for PCR products and 50μl for plasmid DNA. 10μl of PCR product were mixed with 2μl of 10x digestion buffer and 15 units of the appropriate restriction enzyme and incubated at 37°C for at least 4 hours. Plasmid digests were set up in the same way but were only incubated for 1-2 hours. For genotyping samples from PCR the whole volume was electrophoresed on a 2% agarose gel in 1xTAE buffer containing Et Br. For plasmid digests one tenth of the volume was run on an agarose gel to check whether digestion was complete. In the event of incomplete digestion, more enzyme was added and the incubation was continued. The volume of enzyme added did not exceed one tenth of the total sample volume.

2.2.8 Radiolabelling

Random oligonucleotide primer extension

The α-actin, β-fibrinogen and apoE cDNA probes were labelled by the method of Feinberg and Vogelstein (1983) for hybridization of Northern and slot blots. Agarose containing the cDNA probe to be labelled was boiled for 3 minutes to separate the DNA strands and placed on ice to prevent reannealing. Thirty-two microlitres of probe (approximately 50 ng) were added to a labelling mixture of 10μl oligolabelling buffer and 2μl bovine serum albumin(10mg/ml). Five microlitres of 32P α-dCTP (800Ci/mmol) and 1μl of Klenow enzyme (1U/μl) were added and left at room temperature overnight. Separation of labelled DNA from nucleotides was acheived with a Sephadex G50
column equilibrated with 3xSSC. Generally $10^6$ cpm were used per ml of hybridization solution.

Oligolabelling buffer: Solutions A, B and C were mixed in the ratio 1:2.5:5

Solution A: 1ml solution X
18μl beta-mercaptoethanol
5μl 0.1M dATP
5μl 0.1M dTTP
5μl 0.1M dGTP

Solution B: 2M Hepes pH 6.6 with 4M NaOH

Solution C: Hexadeoxyribonucleotides, 90 OD U/ml

Solution X: 1.25M Tris pH 8.0
0.125M MgCl₂

2.2.9 RNA analysis

Northern blotting and hybridisation

RNA samples were mixed with 5μl 10x MOPS buffer, 8.75μl 37% formaldehyde and 25μl deionized formamide. The samples were incubated for 15 minutes at 55°C, mixed with 10μl formaldehyde loading buffer and loaded onto a denaturing formaldehyde
agarose gel. The gel was then run for 3 hours at 80V in 1xMOPS running buffer and rinsed several times in ddH₂O. The RNA was transferred from the gel to the membrane (Hybond N Amersham) by sandwiching a piece of Hybond N between the gel, which was placed on an inverted perspex gel former covered with two layers of 3MM dipping into 20xSSC, and two layers of 3MM soaked in 3xSSC, taking care to avoid air bubbles between the layers. A stack of paper towels was placed on top with an approximate 500g weight and left overnight. The positions of the wells were marked on the filter with an indelible marker. The filter was then removed, rinsed in 3xSSC, dried for 30 minutes in an 80°C oven and fixed by U.V. on a transilluminator for exactly 2 minutes. The remaining gel was viewed by U.V. light to ensure that the RNA had successfully transferred.

10x MOPS running buffer: 0.2M MOPS pH7.0 with NaOH/acetic acid
50mM sodium acetate
5mM EDTA

Formaldehyde loading buffer: 1mM EDTA
0.25% bromophenol blue
0.25% xylene cyanol
50% glycerol

20xSSC: 3M NaCl
0.3M sodium citrate

The filter was cut to size, rolled up and placed in a 50ml falcon tube with 4mls of
hybridisation buffer. The filter was prehybridised for at least one hour at 42°C in a rotary oven, after which time the hybridisation buffer was replaced, 1x10^6 cpm/ml of boiled labelled probe was added and the filter was hybridised overnight. After hybridisation the filter was removed and washed for 30 minutes in 3xSSC/0.2%SDS at 65°C, 15 minutes in 1xSSC/0.2% SDS at 65°C and finally 0.1xSSC/0.2%SDS at 65°C if required. The filter was monitored with a Geiger counter at each stage to prevent overwashing. The filter was then wrapped in cling film and autoradiographed at -70°C overnight with intensifying screens.

Hybridisation buffer:  
15% deionized formamide  
0.2M phosphate buffer  
1mM EDTA  
1% bovine serum albumin  
7% SDS  

2.2.10 Sequencing  

The constructs used in the transient expression assays (2.2.13) were sequenced to ensure that they differed by only the C/T. Three microgrammes of the double-stranded plasmid DNA was denatured in 0.2M NaOH, 0.2mM EDTA for 30 minutes at 37°C. The mixture was then neutralized by adding 0.1 volumes of 3M sodium acetate (pH4.5-5.5) and the DNA precipitated with 2-4 volumes of ethanol at -70°C for 15 minutes. The pelleted DNA was washed once with 70% ethanol and redissolved in 7μl distilled water. Sequencing was carried out using a modification of the dideoxy method (Sanger et al 1977), based on that of Green at al (1989) using a modified T7 DNA
polymerase (Sequenase, USB). Annealing was carried out in a total volume of 10μl using the primer and template in a molar ratio of approximately 25:1. 1μl of primer and 2μl Sequenase buffer were added to the denatured DNA and placed in a beaker of water in a water bath at 65°C for 2 minutes. The beaker was then removed from the water bath and allowed to cool slowly to just below 30°C when annealing was complete. The annealing reaction was then placed on ice and used within 4 hours. A "labelling mixture" was made for 4 reactions: 4μl 0.1M dithiothreitol, 8μl diluted labelling mix, 2μl (10μCi/μl) [α-35S]dATP and 8μl 1:8 diluted Sequenase (T7 DNA polymerase) enzyme. 5.5μl of this "labelling mixture" was added to 10μl of template-primer and incubated for 2-5 minutes at room temperature. Once incubation was complete, 3.5μl of the labelling reaction was added to separate 1.5ml tubes containing 2.5μl of ddATP, 2.5μl ddCTP, 2.5μl ddGTP and 2.5μl ddTTP termination mixes respectively. The solutions were centrifuged to mix simultaneously and incubated for 3-5 minutes at 37°C. Each termination reaction was stopped by the addition of 4μl of the formamide dye supplied with the Sequenase kit and the tubes placed on ice. The samples were heated to 75-80°C for 2 minutes and loaded onto a 4mm, 6% polyacrylamide urea gel which was run as described in section 2.2.6b. The gel was autoradiographed at room temperature for between 48 hours and one week, depending upon the strength of the signal, using Hyperfilm βmax (Amersham UK).

5x Sequenase buffer:  200mM Tris-HCl pH 7.5
100mM MgCl₂
250mM NaCl

Labelling mix:  7.5μM dGTP
ddA,C,G orT termination mix: 80μM dGTP
80μM dATP
80μM dCTP
80μM dTTP
50mM NaCl
8.0μM ddATP,ddCTP,ddGTP or ddTTP

Enzyme dilution buffer: 10mM Tris-HCl pH7.5
5mM DTT
0.5mg/ml BSA

2.2.11 Bandshift Assay

a Preparation of nuclear extracts

Nuclear extracts were prepared by the method previously described by Lee et al 1988. HepG2 cells were cultured in 15cm diameter dishes, as in 2.2.1b, to 80% confluency, as estimated by viewing under an inverted light microscope. Cells were untreated or grown in the presence of IL-6 (1000 units/ml) and/or TGF-β (11.5 ng/ml) for 24 hours prior to extraction. Cells were harvested by scraping (Falcon cell scraper), washed in 30 volumes of PBS, and pelleted by centrifugation. Packed cell volume (PCV) was determined by comparison with known volumes. One PCV of buffer A was added and
the cells allowed to swell on ice for 15 minutes. Cells were lysed by repeatedly passing
the cell suspension through a 25g needle (five times). The cell suspension was examined
under a microscope to ensure >80% cell lysis using trypan blue dye exclusion (only
lysed cells taking up the dye). The sample was centrifuged for 20 minutes and the crude
nuclear pellet resuspended in two thirds PCV of buffer B and incubated on ice for 30
minutes with agitation. The suspension was centrifuged in an Eppendorf centrifuge for
a further 20 minutes and the supernatant removed and dialysed against buffer C for 2
hours with one change of buffer. Aliquots of the extracts were snap frozen in liquid
nitrogen and stored at -70°C. Protein concentration of the nuclear extracts was
determined spectrophotometrically as described in 2.2.13.

Buffer A: 10mM Hepes pH7.9/4°C

1.5mM MgCl₂
10mM KCl
0.5mM dithiothreitol

Buffer B: 20mM Hepes pH7.9/4°C

25%(v/v) glycerol
0.42M NaCl
1.5mM MgCl₂
0.2mM EDTA
0.5mM dithiothreitol
0.5mM phenylmethysulphonylfluoride (PMSF)

Buffer C: 20mM Hepes pH7.9/4°C
20% (v/v) glycerol
0.1M KCl
0.2mM EDTA
0.5mM dithiothreitol
0.5mM PMSF

Dithiothreitol and PMSF were added fresh.

**b Preparation of oligonucleotides**

Two double stranded 26-mer oligonucleotides were designed to represent both β-fibrinogen C-148 and T-148 allele sequences (i.e. one base difference) with additional overhanging 5' ends of 3 cytosine or guanine residues to facilitate radiolabelling.

C-148 allele

\[
5' \text{CCCACATCTTCCCAGCAAAGCTTATTTAC} 3' \\
3' \text{TGTAGAAGGGTCGTTTTGAATAAATGGG5'}
\]

T-148 allele

\[
5' \text{CCCACATCTTCCCAGCAAAACTTATTTAC} 3' \\
3' \text{TGTAGAAGGGTCGTTTTGAATAAATGGG5'}
\]

10μg of each complementary, single stranded oligonucleotide were annealed together in medium salt buffer (2.2.11c) in a total volume of 20μl by heating to 95°C in a beaker of water for 10 minutes and allowing to cool slowly to room temperature in the same beaker. The annealed oligonucleotides were run on a 3% agarose/TAE/EtBr gel alongside the single stranded oligonucleotides to check for complete annealing.
c Bandshift assay

Annealed oligonucleotides to be used in bandshift assays were labelled using a "filling-in" method. Two microlitres of the appropriate annealed oligonucleotide (20ng) were added to a labelling mixture of 2µl of 10x medium salt buffer, 1.5µl 2mM dGTP and 10.5µl dH₂O. Three microlitres of ³²P α-dCTP (800Ci/mmol) and 1µl of Klenow enzyme (1U/µl) were added and incubated at 37°C for 30 minutes. The labelled oligonucleotide was separated from unincorporated nucleotides using a Sephadex G25 column spun at 1000rpm for 3 minutes, producing the probe with minimal contamination.

For each bandshift assay, 3µg of poly dI/dC, 4-6µg of crude nuclear extract and any competitor oligonucleotide were mixed in Parker buffer in a total volume of 20µl and incubated on ice for 10 minutes. Then 15,000 cpm of radiolabelled double-stranded oligonucleotide were added and incubated for 10 minutes at room temperature. The entire sample was loaded onto a 7% non-denaturing polyacrylamide gel and run for 3-4 hours at 150V, at 4°C and then dried onto DE81 paper and autoradiographed overnight.

10x Medium salt buffer:
0.5M NaCl
0.1M Tris-HCl pH7.5
0.1M MgCl₂
0.01M dithiothreitol

2x Parker buffer:
8% Ficoll
20mM Hepes
50mM KCl
2.2.12 Construction of β-fibrinogen promoter transient expression plasmids

a Ligation

The vector pE3 (kindly provided by L.Buluwela, Charing Cross and Westminster Medical School) was linearized with the enzyme SphI which generates a sticky-end. This was followed by digestion with the blunt-end generating enzyme SmaI. The PCR amplified β-fibrinogen promoter sequences from individuals homozygous for the C-148 and T-148 alleles were purified away from PCR primers and nucleotides using GeneClean. PCR products were then digested with MslI and NlaIII (see fig 1) and then ligated into the SphI/SmaI digested vector. Ligations were set up in 15μl volumes with molar ratios of 1:3 for vector:insert, 1.5μl 10x ligase buffer and 1 unit of T4 DNA ligase (Boehringer). The ligation reactions were incubated at 15°C overnight. A background control was also set up containing prepared vector but no insert.

10x ligase buffer:

- 10mM ATP
- 600mM Tris-HCl pH 7.5
- 50mM MgCl₂
- 10mM dithiothreitol
Cloning of the beta-fibrinogen promoter fragment

Insert can only be cloned in the correct orientation because of the blunt and sticky end created in the cloning site.
Competent *E. Coli* XL1-blue cells were kindly supplied by Dr F Green. One millilitre of sterile water and 17.4 μl of β-mercaptoethanol were left to cool on ice. Ten microlitres of the β-mercaptoethanol solution was transferred to a Falcon 2063 tube and 100 μl of competent cells added. The sample was left on ice for 3 minutes and then half the ligation mixture was added and mixed with a pipette tip. The sample was left on ice for 40 minutes and then heat shocked for 3 minutes at 42°C. Cells were chilled on ice for a few minutes and 500 μl SOB broth was added and incubated at 37°C for at least 30 minutes to allow expression of antibiotic resistance genes. The broth was then plated out on L-agar plates with 50 μg/ml of ampicillin added to select for transformed cells. The plates were incubated at 37°C overnight. A transformation control of uncut pE3 was also included. Background controls of linearized vector alone and ligated, linearized vector were included.

Single colonies were picked and grown in 3mls SOB broth media (+AMP) overnight. A loopful of this broth was streaked out on an L-agar plate, grown overnight and stored at 4°C until required. Plasmid DNA was extracted from the rest of the broth using the miniprep method. One hundred nanogrammes of each plasmid was run on a midi agarose gel alongside uncut pE3 vector. A number of the plasmids migrated more slowly than the pE3 vector suggesting that they contained an insert. These plasmids were PCR amplified with an oligonucleotide complementary to part of the CAT coding sequence and either the C\(^{148}\) or T\(^{148}\) upper strand oligonucleotides used in the bandshift assay to determine whether or not they contained an insert. The insert could only ligate into the vector in one direction and the PCR would only work if the orientation of the
The β-fibrinogen promoter inserts in the constructs used in the CAT assays were sequenced in full (2.2.10) to ensure that the sequences were identical except for the C/T base change at position -148.

2.2.13 Transfection of HepG2 cells

The calcium phosphate transfection protocol described here was kindly provided by L. Buluwela and T. Kalamati (Dept. Biochemistry Charing Cross and Westminster Medical School). Optimal conditions for the amount of DNA added and length of glycerol shock had been previously determined for HepG2 cells by Dr Sally Dawson (PhD Thesis, University of London 1992). The constructs to be transfected were extracted and purified from bacterial cultures using the Circle prep kit (BIO 101 INC., California) as described in section 2.2.4b. DNA was stored at -20°C in ddH₂O.

The pSV-β-galactosidase reporter construct (Promega) was used as a positive control for monitoring transfection efficiencies of HepG2 cells. The SV40 early promoter and enhancer drive transcription of the bacterial lacZ gene which, in turn, is translated into the β-galactosidase enzyme. The β-galactosidase enzyme activity can then be assayed in cell extracts from transfected cells using a spectrophotometric assay (Silhavy 1972).

HepG2 cells were seeded at an approximate density of 1.5x10⁶ on 10cm tissue culture treated dishes in 10mls Optimem medium (Life Technologies, Paisley, Scotland) supplemented with 10% foetal calf serum (FCS). Optimem medium gives a pH of 7.1 which is favourable for transfection. Twenty four hours later the medium was changed for fresh Optimem/FCS. Between five and seven hours after the medium change, the
calcium phosphate/DNA precipitate was made.

**a Preparation of calcium phosphate/DNA precipitate**

Ten microgrammes of the DNA to be transfected were pipetted into a Falcon 2063 tube (5µg of either the C\(^{148}\) or the T\(^{148}\) allele constructs, plus 5µg of the internal control plasmid pSV-\(\beta\)-galactosidase, 10µg pSV-\(\beta\) galactosidase, 10µg pRSVCAT or 5µg pE3 plasmid). This was made up to 240µl with sterile water and then 240µl of buffer A was added. The tube was capped and left at room temperature for at least 10 minutes. The tube was held horizontally while 480µl of buffer B was carefully pipetted onto the side of the tube. Buffers A and B were not allowed to mix while the cap was replaced. The tube was maintained in a horizontal position until it was mixed by vortexing for a few seconds, so that the precipitate was sheared as it formed. The tube was then left standing upright for 15 minutes at room temperature. A separate CaPO\(_4\)/DNA precipitate was prepared for each dish to be transfected.

**Buffer A:**

- 0.5M CaCl\(_2\)
- 0.1M Hepes
- pH 7.05-7.12 with NaOH

**Buffer B:**

- 0.28M NaCl
- 0.05M Hepes
- 0.75mM NaH\(_2\)PO\(_4\)
- 0.75Mm NaPO\(_4\)
- pH 7.05-7.12 with NaOH
b Transfection of cells

The DNA precipitate was added to the medium above the cells dropwise while gently rocking the plates. The precipitate was visible under the microscope close to the surface of the medium. Cells were grown overnight at 37°C in a humidified 5% CO\textsubscript{2} incubator after which time the precipitate had settled onto the surface of the cells. Early next morning (16-18 hours after adding the precipitate) the culture medium was removed and replaced with 2mls of filter sterilized 15% glycerol/Optimem which was left in contact with the cells for exactly 2.5 minutes. Five millilitres of sterile PBS were then added to dilute the glycerol. This was then removed and the cells washed once with PBS. Ten millilitres of Optimem/FCS medium were added and the cells allowed to grow for a further 36-48 hours before preparing the protein extracts for assay of CAT and \(\beta\)-galactosidase.

c Protein extraction from cells for CAT and \(\beta\)-galactosidase assays

Each dish of cells was washed with 5mls PBS (Mg\textsuperscript{2+} and Ca\textsuperscript{2+} free) buffer three times and then incubated with 750µl TEN buffer for 5 mins at room temperature. The cells were scraped using a rubber policeman and the resulting suspension was transferred to a microfuge tube and spun for 10 mins. The pellet was washed with 400µl TEN buffer and resuspended vigorously by vortexing in 150µl of 0.25M Tris-HCl, pH 8.0. The cell suspension was then frozen in dry ice/ethanol and thawed at 37°C four times, incubating 5 mins per cycle (i.e. 5mins freezing; 5mins thawing). After freezing and thawing the cell suspension was spun for 10mins to remove nuclei and membranes and the supernatant containing the cell extract was frozen on dry ice/methanol and stored at
-70°C until the assays were performed. Protein content of the extracts was estimated by measuring the absorbance of a 1:200 dilution of the extract at 230nm and 260nm and performing the calculation below:

\[
187 \times A_{230\text{nm}} - 81.7 \times A_{260\text{nm}} = \mu g \text{ protein/ml} \times \text{dilution factor}
\]

TEN buffer:  
40mM Tris pH 7.8  
1mM EDTA  
0.15M NaCl

2.2.14 Assays of CAT and \(\beta\)-galactosidase enzymes from transfection experiments

a CAT ELISA

The CAT ELISA (enzyme linked immunosorbent assay) was carried out using a commercial kit (Boehringer Mannheim). This CAT ELISA is based on the sandwich-enzyme-immunoassay principle. Briefly anti-CAT antibodies are bound adsorptively to the walls of microtiter plate-strips which are postcoated with a blocking reagent and are supplied with the kit. 50\(\mu\)g of protein from cell extracts were added to the wells allowing any CAT contained in the extracts to bind specifically to the coated modules. A digoxigenin-labelled anti-CAT (anti-CAT-DIG) antibody was bound to the fixed CAT and then the bound anti-CAT-DIG was detected by a peroxidase-labelled anti-DIG-
antibody (anti-DIG-POD) and visualized by the colour reaction resulting from the action of the peroxidase on the ABTS substrate. The absorbance of the reactions was then read using a Titertek plate reader at 405nm. Standard curves using known amounts of a standard CAT enzyme were prepared each time the assay was performed.

b β-galactosidase enzyme assay

The β-galactosidase enzyme assay was carried out using a commercial kit (Promega). Diluted samples of the cell extracts from transfection experiments were added to an equal volume of 2x assay buffer supplied with the kit containing the enzyme substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) in a microtitre plate. The samples were incubated at 37°C for 30 mins, during which time the β-galactosidase hydrolyzed the substrate to o-nitrophenyl which is yellow. The absorbance of the reactions was measured using a Titertek plate reader at 405nm. Standard curves using known amounts of β-galactosidase were prepared each time the assay was performed.
3 Fibrinogen gene expression: results

3.1 Effects of lipid-loading and LPS on IL-6 production by monocyte-macrophages

In order to investigate the possibility that IL-6 produced by the lipid-laden macrophages or "foam cells" in atherosclerotic plaques may contribute to the elevated levels of plasma fibrinogen associated with coronary heart disease, fresh human peripheral blood monocytes were isolated and cultured in vitro. Conditioned medium from monocyte-macrophages incubated with acetylated LDL (AcLDL), lipopolysaccharide (LPS) or both, was incubated with HepG2 hepatoma cells and the effect on fibrinogen mRNA production was measured. These results have been published in Lane et al 1991.

Northern blots were performed on HepG2 RNA (positive control) and human skin fibroblast (HSF) RNA (negative control) to test the specificity of the cDNA probes. The ApoE cDNA probe hybridised to a mRNA band of the expected size, 1.2 kb. The β-fibrinogen cDNA probe gave a major band of approximately 1.6 kb and a minor band size of 1.85 kb again as expected. The two species of β-fibrinogen mRNA are due to heterogeneity in the length of 3' non-coding sequences (1.3, Chung et al 1983). The actin cDNA probe hybridised to a mRNA band of 1.2 kb and was used as a standard for slot blots to enable differences in loading of RNA to be accounted for. The apoE and β-fibrinogen probes were used to detect ApoE and β-fibrinogen mRNAs on slot blots of mRNA extracted from monocyte-macrophages. After probing with the ApoE or β-fibrinogen cDNAs the slot blots were stripped and rehybridised with actin cDNA. Values are expressed in arbitrary densitometric units, and are the ratio of the area under the curve of the specific mRNA of interest to that of control actin specific mRNA. All
measurements were made within the linear response range of X-ray film, each measurement being the average of three dilutions in the linear range.

ApoE mRNA was detectable in unstimulated 17day-old monocyte-macrophages and was increased approximately 4-fold on loading with AcLDL (Fig 1). The ApoE protein in the conditioned medium was determined by an enzyme-linked immunosorbent assay (ELISA) by Dr Fabienne Nigon, Paris, France (Rouis et al 1990). ApoE protein in the conditioned medium from cells treated with AcLDL increased 10 to 50-fold (these increases were seen in three separate experiments) and treatment with LPS in addition to AcLDL suppressed this stimulation by almost a third in the experiment shown (Fig 2) and by 10% in another experiment.

In a representative experiment, conditioned medium from monocyte-macrophages stimulated with LPS after increasing time in culture was assayed for IL-1β, TNFα and IL-6 by Dr David Chantry and Mr Andrew Jackson, Charing Cross Sunley Research Centre, London. The IL-1 and TNF were assayed using an ELISA (Turner et al 1990) and the IL-6 was measured by its ability to stimulate proliferation of an IL-6 dependent hybridoma cell-line B9 (Aarden et al 1981). Cytokine production began to decrease 24 hours after placing the cells in culture, was reduced by 50% of initial levels by 48h and continued to decrease with increasing time in culture (Fig 3). Medium from untreated 17-day-old monocyte-macrophages and from cells treated with AcLDL alone, LPS and AcLDL and LPS alone contained no detectable IL-1β, IL-6 and TNF.
Fig 1 A representative experiment showing levels of ApoE mRNA in monocyte-macrophages cultured for 17 days and then treated as indicated. Levels were determined by densitometric scanning of autoradiographs of slot blots.

Fig 2 A representative experiment showing ApoE protein secretion into the culture medium by monocyte-macrophages cultured for 17 days and then treated as indicated. Levels were determined by an ELISA assay.
Fig 3 Effects of time in culture on peripheral monocyte-macrophage cytokine production in response to LPS. Cells were isolated from fresh whole blood as described, and the medium was assayed for IL-1β, TNF and IL-6. Assays were done in duplicate and the intra-assay variation was under 10% of absolute values in all cases.
3.2 Effects of monocyte-macrophage conditioned medium on fibrinogen production by human hepatoma cells.

To confirm that the monocyte-macrophages stopped producing cytokines after a time in culture and to ensure that the absence of any IL-6 activity in the bioassay was not due to any inhibitory factors in the conditioned medium which may have affected the B9 hybridoma cells, HepG2 cells were treated with conditioned medium from untreated and LPS treated, 3-day-old monocyte-macrophages and with conditioned medium from untreated and AcLDL loaded 17-day-old monocyte-macrophages. Recombinant human IL-6 was used to treat HepG2 cells as a positive control for β-fibrinogen mRNA production and a polyclonal anti-IL-6 antibody was used to block IL-6 stimulation of β-fibrinogen mRNA production and thus ensure that any increase effected by conditioned medium was due to IL-6 in the medium. Treatment of HepG2 cells with recombinant human IL-6 caused a 12-fold increase over control levels of fibrinogen mRNA, normalised to actin. By comparison, a 1:10 dilution of conditioned medium from untreated 3-day-old monocyte-macrophages caused a 4-fold stimulation of fibrinogen mRNA above those seen in control HepG2 cells. This effect was blocked by anti-IL-6 antibody. Conditioned medium from LPS stimulated 3-day-old monocyte-macrophages caused a 6-fold stimulation of fibrinogen mRNA levels in HepG2 cells. Conditioned medium from 17-day-old monocyte-macrophages had no effect on fibrinogen mRNA levels whether or not the monocyte-macrophages had been AcLDL loaded (Fig 4). These results are from a single experiment and confirm the results seen in the bioassays (3.1) that in vitro monocyte-macrophages have a limited time of cytokine production so that at 17 days, even after lipid-loading, no cytokine production is detectable.
Fig 4 Effect of recombinant human IL-6 and monocyte-macrophage conditioned medium, in the presence or absence of an antibody to IL-6, on expression of fibrinogen mRNA in HepG2 cells. Levels were determined by densitometric scanning of autoradiographs of slot blots; rhIL6, recombinant human IL-6; 3MCM, monocyte conditioned medium from 3-day-old monocytes; 3MCM + LPS, monocyte conditioned medium from LPS treated 3-day-old monocytes; 17MCM, monocyte conditioned medium from 17-day-old monocytes; and 17MCM + AcLDL, monocyte conditioned medium from AcLDL treated 17-day-old monocytes.

3.3 Effect of TGF-β on IL-6 induced expression of β-fibrinogen mRNA

TGF-β is implicated both in the development of the atherosclerotic plaque (1.2) and in the regulation of some of the acute phase proteins and interacts with many other cytokines. In view of this, the effect of TGF-β on β-fibrinogen mRNA expression in unstimulated HepG2 cells and in IL-6 stimulated cells was investigated. These results
were published in Turner et al 1990.

HepG2 cells were stimulated for 24h with human recombinant IL-6 alone, TGF-β alone and IL-6 and TGF-β together. β-fibrinogen mRNA levels were determined by slot blots and normalised to actin. TGF-β alone reduced basal β-fibrinogen mRNA production in HepG2 cells. IL-6 increased β-fibrinogen mRNA production approximately 2-fold above basal levels. TGF-β inhibited this IL-6 induced increase in a dose-dependent fashion to below basal levels at 10ng/ml (Figs 5 and 6). These results were from a single experiment but were confirmed in northern blots from further experiments in collaboration with Dr Martin Turner and Dr David Chantry (Turner et al 1990).
Fig 5 Effect of TGF-β on rhIL-6 induced expression of β-fibrinogen mRNA. Autoradiograph of slot blot probed with 32P-labelled β-fibrinogen cDNA probe. 1a 10ng/ml TGF-β; 1b 1ng/ml TGF-β; 1c 0.1ng/ml TGF-β; 2a 10ng/ml TGF-β + 1000U/ml rhIL-6; 2b 1ng/ml TGF-β + 1000U rhIL-6; 2c 0.1ng/ml TGF-β + 1000U rhIL-6; 3a untreated; 3b 1000U/ml rhIL-6.
Fig 6

Effect of TGF-β on rhIL-6 induced expression of β-fibrinogen mRNA. Levels were determined by densitometric scanning of autoradiographs of slot blots (see Fig5).

1- rhIL-6 1000U/ml; 2-TGF-β 0.1ng/ml; 3-TGF-β 1ng/ml; 4-TGF-β 10ng/ml; 5-TGF-β 10ng/ml + rhIL-6 1000U/ml; 6-TGF-β 1ng/ml + rhIL-6 1000U/ml; 7-TGF-β 0.1ng/ml + rhIL-6 1000U/ml; 8-untreated.
3.4 Effect of the C/T\textsuperscript{-148} polymorphism and interaction with IL-6 and TGF-β.

The C/T\textsuperscript{-148} polymorphism, which is associated with inter-individual differences in plasma fibrinogen levels (1.7), is close to the region conferring IL-6 responsiveness on the β-fibrinogen promoter (Huber et al 1990). Examination of the surrounding sequence revealed a similarity to a consensus sequence for a TGF-β responsive element (Kerr et al 1990) (Fig 7).

**Human β-fibrinogen promoter**

-149 GCTTTGCTGG

**Consensus sequence**

GNNTTGGtGa

(N = any nucleotide; capital letters = invariant nucleotides; small letters = preferred nucleotide)

The previous results suggested an interaction between IL-6 and TGF-β in controlling β-fibrinogen mRNA levels. Because of the proximity of the putative TGF-β and IL-6 responsive elements to the C/T\textsuperscript{-148} polymorphism, the effect of the base change on the binding of hepatoma cell nuclear proteins to this region of DNA was investigated under basal and cytokine-stimulated conditions. Double-stranded 29-mer oligonucleotides containing either C or T at position -148 were used as probes in band-shift assays with nuclear extracts from Hep3B and HepG2 cells, untreated and treated with IL-6 and/or TGF-β.
HUMAN BETA-FIBRINOGEN GENE - 5'-flanking region and first exon

**KEY**
- ► Transcription start site
- ► Translation start site
* ▼ Oligos used in bandshift assays

**C/T**

-170
 AGTTGTATGA  CAAATTAATA  AGCTTGTGCTG  GGAAGATGTT  GCTTTAAATGA

-120
 TAAAAATGGTT  CAGCCAAACAA  GTGAAACCAAA  AATTAATAT  TAAGCTAAGGA

-70
 AAGGTAACCA  TTTCTGAAGT  CATTGCTAGC  AGAGGACTCA  GATATATATA

-20
 GGATTGAAGAA  TCTCTCAGTT  AAGTCTACAT  GAAAAGGATG  GTTTCCTTGGAA

GCTTCCACAA  ACTTAAAAACC  ATGAAACATC  TATTATTGCT  ACTATTGTGT

GTTTTTCTAG  TTTAAGCTCA  AGGTGTCAAC  GACAATGAGG  AG

**TATA box**

**CAAT box**

**TGF-beta consensus sequence**

**IL-6 response element**

**C/EBP binding site**

**HNF-1**

-1 +1 _ +30
 GGATTGAAGA  TGTGTGAGTT  AAGTGTAGAT  GAAAAGGATG  GTTTTGTTGGA

+80
 GGTTGGAGAA  AGTTAAAAGG  ATGAAAGATG  TATTATTGCT  AGTATTGTGT

GTTTTTGTAG  TTAAGCTCA  AGGTGTCAAC  GACAATGAGG  AG
Incubation of the double-stranded oligonucleotides corresponding to the two allele sequences with Hep3B and HepG2 nuclear protein extracts showed a number of protein/oligonucleotide complexes which migrated more slowly through the polyacrylamide gel than the free oligonucleotide. These complexes fell into five groups labelled A-E (Fig 8a). The A complexes, a group of three bands, were observed in both HepG2 and Hep3B nuclear extracts. The relative intensities of these bands were altered according to the different cytokine treatment, and were different on incubation with the different allele sequences. Incubation of HepG2 untreated and IL-6 stimulated extracts with the C allele resulted in the top band of the group being more intense in relation to the other two bands (lanes 3 and 4). On treatment with TGF-β and IL-6, the top band was of equal, if not greater, intensity than the second band of the group (lane 6). On incubation with the T allele, the top band was the weaker of the three, except under IL-6 treatment (lane 10), where it was of equal intensity to the second band of the group. There was a very faint fourth band, below the group of three, but this did not
Fig 8a

<table>
<thead>
<tr>
<th>Lanes</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+7</td>
<td>oligo alone</td>
</tr>
<tr>
<td>2+8</td>
<td>+Hep3B extract</td>
</tr>
<tr>
<td>3+9</td>
<td>+HepG2 extract</td>
</tr>
<tr>
<td>4+10</td>
<td>+HepG2/IL-6 extract</td>
</tr>
<tr>
<td>5+11</td>
<td>+HepG2/TGF-beta extract</td>
</tr>
<tr>
<td>6+12</td>
<td>+HepG2/IL-6+TGF-beta extract</td>
</tr>
</tbody>
</table>

Unbound oligonucleotide

A
B
C
D
E

1 2 3 4 5 6 7 8 9 10 11 12
<table>
<thead>
<tr>
<th>Lanes</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+8</td>
<td>oligo alone</td>
</tr>
<tr>
<td>2+9</td>
<td>Hep3B extract</td>
</tr>
<tr>
<td>3+10</td>
<td>Hep3B extract</td>
</tr>
<tr>
<td></td>
<td>+100x unlabelled C148</td>
</tr>
<tr>
<td>4+11</td>
<td>Hep3B extract</td>
</tr>
<tr>
<td></td>
<td>+100x unlabelled T148</td>
</tr>
<tr>
<td>5+12</td>
<td>HepG2 extract</td>
</tr>
<tr>
<td>6+13</td>
<td>HepG2 extract</td>
</tr>
<tr>
<td></td>
<td>+100x unlabelled C148</td>
</tr>
<tr>
<td>7+14</td>
<td>HepG2 extract</td>
</tr>
<tr>
<td></td>
<td>+100x unlabelled T148</td>
</tr>
</tbody>
</table>

Unbound oligonucleotide

Fig 8b
vary with the different alleles or according to cytokine treatment. This group of bands was competed away with both the C and T unlabelled alleles, suggesting that the differences between the two alleles are due to differences in strength of binding of protein to DNA (Fig 8b). The group of bands labelled C was observed in HepG2 and Hep3B extracts incubated with both alleles. The intensity of these bands appeared to change with cytokine treatment, but this may have been due to small differences in concentration of the nuclear extracts. The bands were better resolved in HepG2 extracts incubated with the T allele (lanes 9-12). These bands were also competed away with both unlabelled C and T alleles. Band B was only observed in HepG2 extracts incubated with the T allele. This band was visible in the Hep3B extracts incubated with the T allele and much more faintly on incubation with the C allele. This band was competed away with both unlabelled C and T alleles. Band E was observed on incubation with both alleles, but was more intense on incubation with the T allele. This band appeared to be competed away with both unlabelled alleles, but as it migrated close to the labelled T allele alone, it was difficult to distinguish from the unbound labelled oligonucleotide. These results were consistent when repeated in a second bandshift experiment, as were the competition experiment results (not shown).

3.6 Effect on CAT expression in HepG2 cells

Although there appeared to be allele specific differences in the binding of nuclear proteins to these sequences, the bandshift assay cannot show conclusively whether or not the protein binding differences affect transcription. It was therefore decided to proceed with assaying the effect of the different alleles on promoter strength directly. In order to investigate the possibility that the C/T148 polymorphism affected the
expression of β-fibrinogen, constructs were made containing both allele sequences
cloned 5' of the chloramphenicol acetyl transferase (CAT) reporter gene. These
constructs contained the β-fibrinogen promoter sequence from +11 to -270 cloned from
a PCR product into a promoterless pE3 CAT vector (Fig 1 Methods). The two 280bp
inserts were sequenced to ensure complete identity apart from the C/T-148 base change
(data not shown). The CAT gene is a widely used reporter gene and allows comparison
of promoter activity through the easy detection of the gene product since it is not found
in eukaryotic cells. In this instance, levels of CAT were detected using a commercial
ELISA kit (2.2.14a). The CAT constructs were co-transfected with a pSV-β-
galactosidase reporter plasmid (Promega) which was designed as a positive control
vector for monitoring transfection efficiencies of mammalian cells. The SV40 early
promoter and enhancer are used to drive transcription of the bacterial lacZ gene which,
in turn, is translated into the β-galactosidase enzyme. The β-galactosidase enzyme
activity was then assayed in cell extracts from transfected cells using a
spectrophotometric assay (Silhavy 1972). Control experiments were performed in
triplicate. Mock transfections, where cells were treated in the same way as the other
transfections but had no DNA added, were performed to determine background levels
for the CAT and β-galactosidase assays. Cells were transfected with 10μg of the
promoterless pE3 vector alone to ensure that this did not result in CAT expression.
Cells were transfected with 10μg of a pRSVCAT plasmid, which contained the strong
Rous sarcoma virus promoter to drive CAT expression, as a positive control for CAT
expression, and with 10μg of the pSV β-galactosidase plasmid as a positive control for
β-galactosidase expression. The results of these control experiments are shown in Table
1.
The β-galactosidase vector appeared to be toxic at 10 μg (i.e. in the positive controls), since the β-galactosidase activity is not much higher than the background level seen in the mock transfections. In co-transfections it was used at 5 μg with 5 μg of the C¹⁴³ or T⁻¹⁴³ pE3 constructs, the average β-galactosidase activity was approximately 1.1x10⁴ U/50 μg cell extract compared to 6.5x10⁴ U/50 μg cell extract seen in the positive control. CAT expression is expressed in arbitrary units and is the ratio of the amount of CAT in the extracts to the β-galactosidase activity.

Table 1 Mean absorbance at 405/50 μg cell extract for CAT ELISA and β-galactosidase activity (Ux10⁵/50 μg cell extract) - results from control experiments for transfections.

<table>
<thead>
<tr>
<th>Control (n)</th>
<th>pgCAT/50 μg cell extract</th>
<th>Ux10⁵/50 μg cell extract β-galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock transfection (3)</td>
<td>0</td>
<td>4.5</td>
</tr>
<tr>
<td>pE3 (3)</td>
<td>0</td>
<td>4.6</td>
</tr>
<tr>
<td>pSVβ-galactosidase (3)</td>
<td>-</td>
<td>6.5</td>
</tr>
<tr>
<td>pRSV CAT (3)</td>
<td>33.5</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2 Expression of CAT protein from HepG2 cells transfected with constructs containing the C\textsuperscript{-148} and T\textsuperscript{-148} allele sequences.

<table>
<thead>
<tr>
<th></th>
<th>C\textsuperscript{-148} allele</th>
<th>T\textsuperscript{-148} allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ux10\textsuperscript{4}/50\mu g cell extract</td>
<td>CAT pg/50\mu g cell extract</td>
</tr>
<tr>
<td></td>
<td>β-galactosidase activity</td>
<td>CAT expression mean±se</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>32.5*</td>
</tr>
<tr>
<td></td>
<td>.31</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>.53</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Large value may due to carry over from other wells in microtitre plate, when this value was removed and the t-test repeated, there was still no significant difference between the alleles.
There was a large variability in the amount of CAT expression between different experiments for each of the constructs, particularly in transfections where the C-tg construct was used. This may have been due to possible toxic effects of the pSVβ-galactosidase vector, even though only 5μg of this was used in the co-transfections, and the resulting β-galactosidase activity was greater than that seen in the β-galactosidase positive control (transfected with 10μg pSVβ-galactosidase plasmid). However, comparison of the mean CAT activity from the two alleles using Student’s t-test, showed that the differences were not significant (p = 0.371).

Owing to financial and time constraints, it was not possible at this time to repeat the CAT assays in the presence of cytokines. These experiments will be discussed further in the 'Future studies' section of the Conclusions.
4 Fibrinogen gene expression: discussion

4.1 Effects of IL-6 and TGF-β on β-fibrinogen mRNA levels

4.1.1 Effects of lipid-loading and LPS on IL-6 production by monocyte-macrophages.

The aim of these experiments was initially to determine if the elevated fibrinogen levels associated with IHD may be due to IL-6 production by foam cells in the atherosclerotic plaque. Elevated levels of fibrinogen have been shown to be a risk factor for IHD (Wilhelmsen 1984, Stone 1985, Meade 1986, Kannel 1987). Since IL-6 has been shown to be a regulator of the acute-phase response (Fuller 1985, Gauldie 1987, Castell 1988, Jambou 1988, Morrone 1988, Castell 1989), in particular fibrinogen, factors which may contribute to circulating levels of this cytokine are of interest. However, these results suggest that after somewhere between 3 and 17 days in culture human monocyte-macrophages lose the ability to secrete cytokines, even after LPS stimulation. After 17 days in culture they are immunologically inactive but can be induced to form foam cells by 24 hour incubation with AcLDL. This is accompanied by an increase in apoE mRNA levels as well as a large increase in apoE secretion into the medium.

In HepG2 cells treated with conditioned medium from monocyte-macrophages, the increase in fibrinogen mRNA levels was due to the action of IL-6, since the increase was prevented by a neutralising polyclonal antibody specific for IL-6. However, this increase was only induced by conditioned medium from 3-day old macrophages, either with or without treatment with LPS. Conditioned medium from human monocyte-
macrophages after 17 days in culture, had no effect on fibrinogen mRNA, irrespective of cholesterol loading. This was consistent with the observation that monocyte-macrophages cultured for 17 days are incapable of producing IL-6, even under the effect of LPS. In the *in vivo* situation as compared to that *in vitro*, there are a large number of cellular and humoral factors which could affect the monocyte-macrophages and foam cells in the atherosclerotic plaque (Aqel et al 1985, Niculescu et al 1985, Seifert et al 1989). More recently, Libby et al have investigated macrophage function directly in atheromatous and non-atheromatous carotid and coronary vessels (Libby et al 1992). This work showed selective activation of macrophages within the plaque compared to the situation in the normal vessel wall. ApoE protein was shown to be associated immunohistochemically with macrophages in atherosclerotic plaques whereas nonatherosclerotic vessels showed no immunoreactive apoE. Levels of IL-6 and IL-1 did not vary between atheromatous plaques and non-atheromatous vessels. This suggests that the in vitro results in this thesis were reflecting those found in vivo. ApoE synthesized by cultured cholesteryl ester-loaded macrophages associates with co-incubated HDL and may therefore potentiate the movement of cholesterol from cells to HDL (Gordon et al 1983) and its subsequent removal by associating with cholesteryl ester-rich HDL-subfractions from foam cells and then acting as a recognition signal for removal from the circulation through the hepatic apoE receptor (Mazzone 1987). Although this would appear to be protective, it apparently does not prevent the formation of foam cells and progression of lesion formation. There have also been recent reports that there are proliferating monocytes in the atherosclerotic plaque at all stages both in rabbit (Rosenfeld and Ross 1990) and in human atherosclerotic lesions (Gordon et al 1990). Macrophage action on LDL produces oxidised LDL which has been shown to be chemotactic for human monocytes (Quinn et al 1987). Therefore,
although the results presented in this thesis (3.1, 3.2) suggest that it is unlikely that cytokine production by foam cells in the atherosclerotic plaque is a major contributing factor to the elevated fibrinogen levels associated with IHD, the foam cells may be acting to attract proliferating, cytokine-producing monocytes to the plaque.

The effect of IL-6 on HepG2 cells was to increase levels of \( \beta \)-fibrinogen mRNA, suggesting that this cytokine was acting pre-translationally. TGF-\( \beta \) is known to regulate the expression of several genes, and it has also been shown to control negative acute-phase genes post-transcriptionally (Morrone et al 1989). TGF-\( \beta \) is also implicated in the development of the atherosclerotic plaque, as discussed in the introduction, and has been shown to upregulate plasminogen activator inhibitor-1 (PAI-1) mediated by TGF-\( \beta \) inducible elements in the PAI-1 promoter (Westerhausen et al 1991). This property may be involved in the deposition of fibrin found to be associated with the plaque and plaque development (Duguid et al 1948, Wolf 1967, Smith 1979, Naito 1989) by inhibiting fibrinolysis. TGF-\( \beta \) has also been implicated in fibrinogen regulation and was found to inhibit IL-6 induced increases in \( \alpha \)-fibrinogen mRNA in Hep3B cells (Mackiewicz et al 1990). TGF-\( \beta \) has been shown to be involved in the regulation of other genes at the transcriptional level (Kerr et al 1990) and because of the evidence for its effect on fibrinogen regulation, the effect of TGF-\( \beta \) on the IL-6 induced increase in \( \beta \)-fibrinogen mRNA levels observed in HepG2 cells was investigated. The results of these experiments showed that TGF-\( \beta \) did inhibit the IL-6 induced increase of \( \beta \)-fibrinogen mRNA, and suggested that TGF-\( \beta \) was acting pre-translationally.
4.1.2 Effect of C/T\(^{148}\) polymorphism on response of fibrinogen expression to IL-6 and TGF-β.

There are two polymorphisms in the 5' flanking region of the β-fibrinogen gene, the G/A\(^{455}\) and the C/T\(^{148}\) polymorphisms. The G/A\(^{455}\) polymorphism has been shown to alter the binding of a liver nuclear protein and is in almost complete linkage disequilibrium with the C/T\(^{148}\) polymorphism, that is, the G\(^{455}\) allele is almost invariably associated with the C\(^{148}\) allele and the A\(^{455}\) with the T\(^{148}\) allele (Thomas, Green, Humphries unpublished data). This could mean that the C/T\(^{148}\) polymorphism is simply a marker for the G/A\(^{455}\), or that it could be having an effect itself. As mentioned in the introduction, the C/T\(^{148}\) polymorphism is very close to an IL-6 responsive element in the 5' flanking region of the β-fibrinogen gene (Fig 1). Further investigation of this site revealed similarity to a TGF-β inhibitory element consensus sequence \(^{9}\)Kerr et al 1990) at position -149 to -140, GCTTTGCTGG, the C/T\(^{148}\) polymorphism lying within this sequence (Fig 1). It is therefore possible that the polymorphism may disrupt any putative transcriptional interaction between IL-6 and TGF-β. IL-6 and TGF-β may be altering expression or binding properties of nuclear DNA-binding proteins which act to regulate expression of the β-fibrinogen gene through response elements in the promoter or elsewhere in the gene. It therefore seemed reasonable to investigate the influence of IL-6 and TGF-β on binding of hepatoma cell nuclear proteins in the region of the C/T\(^{148}\) polymorphism in the β-fibrinogen promoter.

The bandshift assay method was used to test this hypothesis. In this method double-stranded oligonucleotides containing the C\(^{148}\) or T\(^{148}\) allele sequences were radiolabelled and incubated with nuclear extracts made from HepG2 cells which had been untreated
or had been stimulated with IL-6, TGF-β or IL-6 and TGF-β together. The oligonucleotide sequences used contained both the TGF-β response element consensus sequence and the IL-6 response element which overlap (see results Fig 7). The results of the bandshift assay suggest the C/T<sup>148</sup> polymorphism does alter the binding of at least one nuclear protein, and that the binding of HepG2 cell nuclear proteins did appear to alter under the effects of IL-6 and TGF-β, although these results must be viewed with some reservation since the differences seen according to cytokine treatment may have been due to small differences in the amount of nuclear extract used. However, there were differences in the pattern of binding of the group A bands, the relative intensities of which altered with cytokine treatment and on binding to the different allele sequences. The protein which formed the top band of the group bound more strongly to the C<sup>148</sup> allele on treatment of the HepG2 cells with IL-6 compared with untreated cells, and this binding seemed to be reduced to a lesser extent by the action of TGF-β in conjunction with IL-6 in the presence of the C<sup>148</sup> allele compared to the T<sup>148</sup> allele. This suggests that the T<sup>148</sup> allele binds this IL-6-induced nuclear protein more weakly than does the C allele and that TGF-β has a greater effect on the T allele compared with the C. However, these results are not entirely consistent with the observation that the T allele is associated with higher plasma fibrinogen levels, and might therefore have been expected to have the stronger response to IL-6, and the weaker response to TGF-β, unless the protein concerned is a transcriptional repressor. It is not possible to tell from these experiments which proteins are binding to which response elements and, although IL-6 is known to induce the IL-6RE binding protein (Ito et al 1989, Hattori et al 1990 Hocke et al 1992) the possibility remains that some of these proteins may be repressors rather than activators. Band B, seen only in the presence of the T allele, may be a degradation product or monomer of the protein.
forming the top band of group A, and appears to increase in the presence of IL-6. If this protein is involved in the IL-6 response, then this may account for the association of the T allele with higher fibrinogen levels. Again, it is not possible to identify the binding proteins from these results. However, the intensity of binding of some of the nuclear proteins did vary according to treatment with IL-6 or TGF-β, which suggests that TGF-β may influence β-fibrinogen gene expression at the level of transcription and that there may be interaction between IL-6 and TGF-β at this level.

Untreated Hep3B cell nuclear extracts gave bands, similar in intensity and pattern to those seen in HepG2 cell extracts on treatment with IL-6, which suggests that Hep3B cells may already be in an 'acute phase state'. Previous experiments to compare response of Hep3B cells and HepG2 cells to IL-6 showed that Hep3B cell mRNA production responded less strongly to IL-6 stimulation (data not shown), which would be expected if they were already in an 'acute phase state'. However, as for the HepG2 nuclear extracts, nuclear protein binding to either allele was competed out with both unlabelled alleles, suggesting that the allele-specific bands may result from differences in strength of binding rather than differences in specificity of binding.

In this case, where the nuclear protein binding is complex, the band-shift assay technique cannot provide conclusive evidence of affinity differences and must be interpreted with caution. Affinity differences may be measured by titrating the concentration of competitor DNA to see which protein-DNA complexes are competed away first i.e. which DNA-protein complexes have the greater affinity for each other.

Only one type of nuclear extract was used in these experiments and it is possible that
nuclear proteins extracted by a different method, i.e. at a different salt concentration, may have produced different results. Results observed previously using these particular extracts in studies of the PAI-1 gene promoter could not be repeated when new extracts were prepared using a different protocol, in a different laboratory, suggesting that nuclear extracts vary according to the preparation method (personal communication, Per Eriksson, Karolinska Hospital, Stockholm). Three other considerations in the preparation of nuclear extracts are that, firstly, the relevant proteins may have been inefficiently extracted from the nuclei. Secondly, excessive amounts of non-specific inhibitory proteins may have been extracted. Thirdly, although a large amount of protease inhibitor was added when the extracts were prepared, it is a possibility that the extracts may have started to degrade. This may account for the rather poorly defined bands seen in competition experiments, and comparisons of extracts thawed once with extract aliquots frozen and thawed a number of times suggested that the extracts are not very stable (data not shown).

Incubation time of the oligonucleotide sequences with the extracts may also affect the banding pattern produced, and further experiments increasing and decreasing incubation time may reveal binding proteins not observed at the incubation time used in these experiments. In initial experiments, salmon sperm DNA was used to reduce the amount of non-specific protein-DNA complex. Under these conditions, no bands were seen at all. However, when the synthetic polymer poly(dI-dC).poly(dI-dC) was used alone, more specific protein-DNA complexes were formed.

Finally, the conditions under which the gel electrophoresis is performed may also influence the results observed. Because the mobility of the protein-DNA complex
through the gel is largely determined by the size and charge of the protein, and by the conformation of the protein-DNA complex, changes in pH, temperature and acrylamide:bisacrylamide ratio can significantly alter mobility of the DNA-protein complexes (Current protocols 1992).

4.2 Effect on CAT expression in HepG2 cells

The bandshift assay technique can provide evidence for interaction with likely transcription factors, however it does not provide direct evidence of transcriptional function. Therefore, the relative promoter strengths of the C-148 and T-148 alleles were analysed using the CAT reporter gene system. HepG2 cells were transfected separately with constructs containing both the allele sequences cloned in front of a promoterless CAT gene. The differences between the C-148 and T-148 promoter sequences were not expected to be large and it was therefore important that this experiment was carefully controlled to ensure that there was no CAT expression from the promoterless pE3 plasmid. Co-transfection of a β-galactosidase vector allowed transfection efficiency to be measured to allow meaningful comparison of promoter strength between the C-148 and T-148 sequences. Co-transfection of the β-galactosidase vector with the C-148 and T-148 constructs means that each transfection (i.e. each culture dish) can be regarded as a separate experiment. The results of the previous bandshift assays suggested that there was an effect of IL-6 and TGF-β on binding of nuclear proteins to this region of the promoter sequence. Although transfection of HepG2 cells with constructs containing the different alleles showed no significant difference in the CAT reporter gene activity, this result would be expected since the transfections were carried out in the absence of added IL-6 and TGF-β. Any differences would be expected to be relatively small, since
the polymorphisms themselves are not associated with large differences in plasma fibrinogen levels. The C/T\textsuperscript{148} polymorphism is close to an IL-6 responsive element, and so it is reasonable to expect that it is involved in the response to IL-6. If a large difference had been seen without IL-6 stimulation, the polymorphism might be expected to be associated with a much larger difference in plasma fibrinogen levels in different genotypes. This will be discussed further in ‘future plans’.

Recent work by Dalmon et al (1993) supports this result and may also help to explain some of the bandshift patterns. This work showed that there was a complex interaction between nuclear proteins within the β-fibrinogen IL-6-responsive element. HNF-1 and C/EBP have been shown to be amongst the most prominent transcription factors in the hepatocyte (Baumhueter et al 1990, Landschultz 1988) and have been shown to control β-fibrinogen gene expression (Huber et al 1990). Apart from the IL-6 responsive element, there is a binding site for members of the C/EBP family in several hepatic gene promoters, and C/EBPβ and δ have been shown to be posttranslationally modified by IL-6 stimulation (Akira et al 1992, Kinoshita et al 1992, Williams et al 1991). Dalmon et al showed that interaction between HNF-1, C/EBP and an IL-6-responsive element-binding protein (IL-6 RE-BP) (Ito et al 1989, Hattori et al 1990, Hocke et al 1992) was necessary for a response to IL-6 stimulation. In these studies, the HNF-1 protein-DNA interaction was shown to be unresponsive to, but necessary for, IL-6 stimulation and was proposed to have a key role in assembly of the transcriptional apparatus. A C/EBP binding site between -125 and -133 in the β-fibrinogen promoter also appeared to be necessary in eliciting a full IL-6 response, in conjunction with the IL-6 responsive element (Fig 1).
Functional organization of IL-6 responsive element in the human beta-fibrinogen gene

(adapted from Dalmon et al 1993)
Interestingly, the region of the promoter of the β-fibrinogen gene examined in the studies of Dalmon et al only extended as far as position -148, that is within the putative TGF-β response consensus sequence, and so interaction with this element may have been missed. Dalmon et al (1993) utilised the HindIII site at -150 as a cloning site for their promoter constructs; the C/T\(^{-148}\) lies within this site and therefore, since only the C\(^{-148}\) allele will cut with HindIII, any effect of this polymorphism would not have been detected. There is another polymorphism in the 5′ flanking region of the β-fibrinogen gene at position -455, G/A\(^{455}\), which is also associated with plasma fibrinogen levels and is in almost complete linkage disequilibrium with the C/T\(^{-148}\) polymorphism. This polymorphism also altered the binding of a nuclear protein. The interaction between the different elements in the promoter of the β-fibrinogen gene shown by the work of Dalmon et al (1993) make it reasonable to suspect that there may also be interaction between the region of the promoter in which the G/A\(^{455}\) polymorphism lies, the region including the C/T\(^{-148}\) polymorphism, and the HNF-1, IL-6 and C/EBP sites. Dalmon et al (1993) also report that in bandshift studies they have carried out using oligonucleotide sequences from -195 to -156 of the α\(_2\)-macroglobulin promoter, known to contain an IL-6 response element, and nuclear extract from IL-6 stimulated HepG2 cells, they observed a specific retarded complex. This was not observed using a single or dimerized β-fibrinogen CTGGGAA motif, the IL-6 response element. They suggest that this could be due to influences of nucleotides 5′ of this element on the affinity for the IL-6-responsive element binding protein. Comparison of the arrangement of IL-6 response elements in relation to other regulatory sequences on other gene promoters shows that there are differences in the organization of these elements. This diversity of organization has been suggested to reflect the different transcriptional requirements in vivo (Dalmon et al 1993). In particular, comparison of synthesis of fibrinogen and C-
reactive protein shows that both are dependent upon HNF-1 and C/EBP binding. However, there are no intervening sequences between the HNF-1 binding site and the TATA box in the fibrinogen promoter, whereas, in the C-reactive protein promoter, there is a C/EBP binding site between the HNF-1 binding site and the TATA box. The arrangement in the fibrinogen promoter produces an active promoter under basal conditions, which would be in accordance with the relatively high synthesis of fibrinogen under basal conditions. In the C-reactive protein promoter, C/EBP may be interfering with HNF-1 under basal conditions (the synthesis of C-reactive protein is poor under basal conditions), but co-operates after IL-6 stimulation and therefore produces an inducible transcriptional switch (Dalmon et al 1993). These observations, together with those presented in this thesis, make it tempting to speculate that there is also interaction between TGF-β and other cis-acting elements and trans-acting factors controlling expression of the β-fibrinogen gene - a subject requiring further study.

4.3 Conclusions

The C/T<sup>148</sup> polymorphism of the β-fibrinogen promoter which is associated with inter-individual differences in plasma fibrinogen level, appears to alter the binding of nuclear proteins in this region of the DNA. However, transient expression assays failed to show a difference in promoter strength under basal conditions. Based on these results and those of Dalmon et al (1993), the action of this polymorphism may be to affect β-fibrinogen expression by altering the interaction of a number of cis- and trans-acting elements, including nuclear proteins induced by TGF-β and/or IL-6. These may also be affected by the G/A<sup>435</sup> polymorphism which is in linkage disequilibrium with the C/T<sup>148</sup> polymorphism and itself alters binding of a nuclear protein to DNA. Further
work is needed on these polymorphisms and their interactions before a definitive conclusion can be reached.
5 Factor VII genetic polymorphism: results

Statistical analysis on the Warfarin, dietary inventory and Camberley studies was carried out by Helen Wilkes and Vera Ogden at the MRC Epidemiology and Medical Care Unit (EMCU), St Bartholomew’s Medical College, London.

Factor VIIc was measured by a one-stage assay using reconstituted FVII deficient plasma at the MRC EMCU, Northwick Park Hospital, except in the ECTIM study where congenitally deficient plasma was used and samples measured at a central laboratory in Paris (INSERM U258). These assays will be discussed in more detail in Chapter 5.

The genotype of the factor VII Arg/Gln\textsubscript{533} polymorphism was determined by MspI digestion of PCR amplified DNA (Methods Fig 1). Allele frequencies in the following samples were determined by gene counting. Ninety-five percent confidence intervals (95\% CI) for population allele frequencies were calculated from sample allele frequencies according to Colton 1974, based on the approximation of the binomial and normal distributions when n is large. The critical ratio with continuity correction (zc) was calculated to test the significance of the difference between two allele frequencies, as described by Colton 1974 (also based on the binomial-normal distribution approximation). \( \chi^2 \) analysis was used to test whether genotype distributions conformed to Hardy-Weinberg equilibrium. Multiple linear regression analysis was used to determine the effect of environmental variables on FVIIc levels and to adjust for confounding factors. Analysis of variance was used on adjusted FVIIc levels to test for differences between genotype groups and for interaction with environmental factors.
Variables were log_{10} transformed where appropriate to improve skewness and kurtosis of distribution prior to statistical analysis. Statistical significance was taken as \( p \leq 0.05 \). Statistical analysis was carried out using SPSS PC+ and Microsoft Excel computer programmes.

5.1 TPT study

The Arg/Gln\(_{353}\) polymorphism is located on the periphery of the FVII molecule and it is possible that the amino acid change may affect the interaction between the Gla domain and tissue factor, or the \( \gamma \)-carboxylation of this region during processing of the FVII molecule (1.3i). Warfarin is an inhibitor of \( \gamma \)-carboxylation (1.4) and therefore this study was undertaken to examine possible differences in an individual’s response to warfarin treatment according to genotype. The sample size required to detect a difference of 1mg in warfarin dose between the different genotype groups was calculated to be 300.

Blood for DNA extraction was obtained from samples from white European middle-aged men (45-65 years) entered into the TPT study. These were men who were selected to be at high risk of CHD using a scoring system for each risk factor associated with CHD derived from the Northwick Park Heart Study. The study is divided into four main groups by treatment, firstly warfarin, secondly warfarin and aspirin, thirdly aspirin alone and lastly placebo. The samples used here were taken from men who were in the group being treated with warfarin.
5.1.1 Association between Arg/Gln\textsubscript{353} genotype and factor VIIc levels.

The frequency of the Gln353 allele was 0.09 (95% CI 0.07-0.12), similar to that found in a previous study of men screened for entry into the TPT study (Green et al 1991) (see Table 4). The FVII Arg-Gln353 genotype distribution in this sample did not differ significantly from that expected for a sample drawn from a population in Hardy-Weinberg equilibrium. The average of two measurements of FVIIc (screening and entry) was calculated for each genotyped subject and this value was used in subsequent calculations. FVIIc values were log transformed to give a normal distribution. The association between the Arg/Gln\textsubscript{353} genotype and baseline FVIIc level was examined in the group of 285 men from the TPT, the null hypothesis to be tested being that mean FVIIc in the Arg/Arg genotype group was not different from that in the Arg/Gln genotype group (no individual was found to be a Gln\textsubscript{353} homozygote in this sample).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Mean FVIIc (%standard)</th>
<th>95% CI for mean FVIIc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg/Arg\textsubscript{353}</td>
<td>236</td>
<td>124</td>
<td>121-127</td>
</tr>
<tr>
<td>Arg/Gln\textsubscript{353}</td>
<td>49</td>
<td>102</td>
<td>96-108</td>
</tr>
</tbody>
</table>

The mean FVIIc level (Table 1) of the Arg/Arg\textsubscript{353} genotype group was found to be
significantly higher by 21% (p < 0.0001) than the Arg/Gln<sub>353</sub> genotype group using a 2-tailed t-test of equality of means. This therefore means that the null hypothesis can be rejected, and that FVIIc levels in the Arg/Arg<sub>353</sub> group are different from those in the Arg/Gln<sub>353</sub> group.

5.1.2 Association between Arg/Gln<sub>353</sub> and response to warfarin treatment

In the TPT study, men in the warfarin group were treated with varying amounts of warfarin until a stable dose was reached at which FVIIc levels were reduced to approximately 70% of standard (corresponding to an international normalized ratio of 1.5). Stable dose was defined as the mean of the first three successive doses of warfarin after the man was classified as being 'stable' for this analysis (Meade et al. 1988).

The null hypothesis to be tested was that there was no difference in the stable dose of warfarin required to reduce FVIIc levels to 70% of standard between men of Arg/Arg<sub>353</sub> genotype and men of Arg/Gln<sub>353</sub> genotype. There appeared to be no relationship between FVIIc level at entry to the TPT study and the amount of warfarin required to reach stable dose, i.e. a higher FVIIc plasma level at entry did not necessarily mean that a larger dose of warfarin was required to reduce the level of FVIIc to 70% of standard, and therefore it was not necessary to adjust amount of warfarin required to reach stable dose for baseline FVIIc.
TABLE 2 Median (95% CI) stable dose of warfarin according to genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Median (mg)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg/Arg353</td>
<td>238</td>
<td>4.0</td>
<td>(3.5, 4.20)</td>
</tr>
<tr>
<td>Gln/Gln353</td>
<td>49</td>
<td>3.6</td>
<td>(3.5, 4.29)</td>
</tr>
</tbody>
</table>

The difference in mean loge of the stable dose of warfarin was estimated as 0.0513 with a 95% CI of (-0.074, 0.1823) (Table 2), and since this includes zero the null hypothesis was accepted at the 5% significance level. Therefore there is no evidence to suggest that Arg/Gln353 genotype significantly affects the stable dose of warfarin required.

5.2 Ethnic group study

Within the UK, individuals of different ethnic origin are known to differ in their risk of CHD (1.4), with the incidence being low in individuals of Afro-Caribbean origin (Marmot et al 1984) and high in individuals from the Indian sub-continent (Balarajan et al 1984, McKeigue and Marmot 1988). These different ethnic groups are also known to exhibit differences in factor VIIc and triglyceride levels (Meade et al 1978, Miller et al 1988), with Afro-Caribbeans having lower mean factor VIIc and triglyceride levels than Europeans (Meade et al 1987).

5.2.1 Association between Arg/Gln353 genotype and plasma factor VIIc levels.

In order to investigate whether ethnic differences in factor VIIc level are due to
different frequencies of the Arg_{353} and Gln_{353} alleles, population samples of Europeans, Afro-Caribbeans and Gujaratis were genotyped for the Arg/Gln_{353} polymorphism. These samples were obtained from individuals recruited for a study on ethnic differences in insulin and plasma C-peptide (Cruickshank 1991). Ethnic group was determined by grandparental origin with at least three grandparents being from one ethnic group. Factor VII coagulant activity measurements were obtained on 68 white Europeans, 78 Afro-Caribbeans and 93 Gujaratis.
<table>
<thead>
<tr>
<th>ETHNIC GROUP</th>
<th>SEX M</th>
<th>SEX F</th>
<th>AGE (years)</th>
<th>BMI (kg/m²)</th>
<th>WHIP</th>
<th>TRIGS (mmol/l)</th>
<th>FACTOR VIIc % standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europeans</td>
<td>60</td>
<td>63</td>
<td>62 (7.18)</td>
<td>26.15 (4.27)</td>
<td>0.88 (0.08)</td>
<td>1.57 (1.03)</td>
<td>135.41 (57.23)</td>
</tr>
<tr>
<td></td>
<td>[123]</td>
<td></td>
<td>[119]</td>
<td>[117]</td>
<td>[113]</td>
<td>[65]</td>
<td>[68]</td>
</tr>
<tr>
<td>Afro-Carib</td>
<td>60</td>
<td>63</td>
<td>57 (5.59)</td>
<td>27.47 (4.36)</td>
<td>0.89 (0.07)</td>
<td>1.21 (0.57)*</td>
<td>111.41 (32.09)**</td>
</tr>
<tr>
<td></td>
<td>[123]</td>
<td></td>
<td>[114]</td>
<td>[114]</td>
<td>[112]</td>
<td>[86]</td>
<td>[78]</td>
</tr>
<tr>
<td>Gujaratis</td>
<td>76</td>
<td>66</td>
<td>59 (7.16)</td>
<td>26.01 (4.10)</td>
<td>0.92 (0.08)</td>
<td>1.72 (0.87)</td>
<td>123.26 (45.24)</td>
</tr>
<tr>
<td></td>
<td>[142]</td>
<td></td>
<td>[140]</td>
<td>[139]</td>
<td>[137]</td>
<td>[89]</td>
<td>[93]</td>
</tr>
</tbody>
</table>

BMI.....Body/Mass Index; TRIGS.....Triglyceride level; WHIP.....Waist/Hip Ratio  *significantly lower than Europeans (p = .009) and Gujaratis (p = .000)
**significantly lower than Europeans (p = .003) and Gujaratis (p = .030)
Not all measurements were obtained on all individuals, accounting for discrepancies in number of individuals shown.
Triglyceride measurements were obtained on 65 white Europeans, 86 Afro-Caribbeans and 89 Gujaratis. Measurements were not obtained on all individuals, accounting for the discrepancies in numbers of individuals analysed. These results have been published in Lane et al 1992.

Table 3 shows relevant characteristics of the individuals in the three samples studied. There were no significant differences between the three ethnic groups in proportions of males to females, but one way analysis of variance and t-tests showed that there were significant differences in mean age, body mass index (BMI) and waist/hip ration (WHIP). The Europeans were significantly older than both the Afro-Caribbeans and the Gujaratis (p < 0.001 and p = 0.011 respectively), and the Gujaratis were significantly older than the Afro-Caribbeans (p = 0.001). Afro-Caribbeans had a higher BMI than the Europeans and the Gujaratis (p = 0.02 and p = 0.007 respectively). None of these characteristics were significantly correlated with Factor VIIc in this study.

The factor VII Arg-Gln<sub>353</sub> genotype distribution in the three samples did not differ significantly from that expected for a sample drawn from a population in Hardy-Weinberg equilibrium. The frequency of the allele coding for Gln<sub>353</sub> (absence of cutting site for MspI enzyme) varied among the population samples (Table 4). The frequency in Europeans was 0.09 and in Afro-Caribbeans was 0.08, similar to that in a sample of healthy men from the U.K. (Green et al 1991). In the Gujarati sample the frequency was 0.25, significantly greater than in the other ethnic groups (p < 0.001) (Table 4).
TABLE 4 Frequency of Gln\textsubscript{353} allele [n] according to ethnic group.

<table>
<thead>
<tr>
<th>ETHNIC GROUP</th>
<th>FREQUENCY OF GLN\textsubscript{353} ALLELE</th>
<th>95% CONFIDENCE INTERVALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPT</td>
<td>0.10 [284]</td>
<td>0.08 to 0.13</td>
</tr>
<tr>
<td>EUROPEANS</td>
<td>0.09 [84]</td>
<td>0.05 to 0.13</td>
</tr>
<tr>
<td>AFRO-CARIBBEANS</td>
<td>0.08 [108]</td>
<td>0.04 to 0.12</td>
</tr>
<tr>
<td>GUJARATIS\textsuperscript{*}</td>
<td>0.25 [126]</td>
<td>0.20 to 0.30</td>
</tr>
</tbody>
</table>

* Frequency of Gln\textsubscript{353} allele significantly different from European sample (p<0.001)

All three groups showed an association between lower factor VIIc levels and the presence of the allele coding for Gln\textsubscript{353}. However this association was significant only in the Gujarati sample (p= <0.001) (Table 5) by analysis of variance of factor VIIc levels after adjustment for age, BMI, WHIP, sex and triglycerides.
### TABLE 5 Mean factor VIIc levels** (95% confidence intervals) by Arg/Gln⁸⁵₃ genotype by ethnic group.

<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>TPT*</th>
<th>AFRO-CARIB</th>
<th>GUJARATIS</th>
<th>EUROPEANS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[n]</td>
<td>[n]</td>
<td>[n]</td>
<td>[n]</td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>102</td>
<td>109</td>
<td>141</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>(26.79)</td>
<td>(101 to 118)</td>
<td>(127 to 156)</td>
<td>(117-139)</td>
</tr>
<tr>
<td></td>
<td>[228]</td>
<td>[44]</td>
<td>[25]</td>
<td>[22]</td>
</tr>
<tr>
<td>Arg/Gln</td>
<td>75</td>
<td>94</td>
<td>107</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>(18.73)</td>
<td>(74 to 120)</td>
<td>(99 to 116)</td>
<td>(23-572)</td>
</tr>
<tr>
<td></td>
<td>[54]</td>
<td>[5]</td>
<td>[24]</td>
<td>[2]</td>
</tr>
<tr>
<td>Gln/Gln</td>
<td>56</td>
<td></td>
<td>86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(8.80)</td>
<td></td>
<td>(67 to 110)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[2]</td>
<td></td>
<td>[4]</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>97</td>
<td>107</td>
<td>120</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>(27.78)</td>
<td>(100 to 116)</td>
<td>(111 to 129)</td>
<td>(117 to 137)</td>
</tr>
<tr>
<td></td>
<td>[284]</td>
<td>[49]</td>
<td>[53]</td>
<td>[24]</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>0.228</td>
<td>&lt;0.001</td>
<td>0.49</td>
</tr>
</tbody>
</table>

* p value from one way analysis of variance

** Log₁₀ transformed factor VIIc levels adjusted for age, BMI, WHIP, sex and log₁₀ transformed triglycerides by multiple regression.

Factor VIIc levels are expressed as % of standard.

See also footnote for Table 3.

### 5.2.2 Arg/Gln⁸⁵₃ genotype-specific relationship with plasma triglyceride levels.

The correlation between triglycerides and factor VIIc was examined separately in Gujarati individuals homozygous for the Arg⁸⁵₃ allele and those heterozygous for the polymorphism. In the whole sample of Gujarati individuals (n=59) plasma factor VIIc level showed a positive correlation with plasma triglyceride level (r=0.20, p=0.07; Table 4). Taking the homozygotes and heterozygotes for the Arg⁸⁵₃ allele separately there was a positive relationship between triglycerides and factor VIIc in individuals
lacking the Gln_{353} allele but in individuals carrying the Gln_{353} allele the relationship was absent, although the numbers in each group were comparable (Table 6). In the whole European sample (n=31) the correlation between triglycerides and factor VIIc was 0.48 (p=0.003, 95% C.I. 0.14 to 0.89) and 0.53 (p=0.005, 95% C.I. 0.14 to 1.04) in Arg_{353} homozygotes (n=22). In the whole Afro-Caribbean sample (n=54) the correlation was 0.08 (p=0.282, 95% C.I. -0.19 to 0.36) and in the Arg_{353} homozygotes (n=47) the correlation was 0.19 (p=0.099, 95% C.I. -0.10 to 0.49). There were too few heterozygotes in either the European or Afro-Caribbean samples (n=2; n=6 respectively), on whom we had both FVIIc and triglyceride levels, to give a statistically meaningful correlation.

TABLE 6 Pearson product moment correlations between factor VIIc and triglyceride levels according to genotype in Gujaratis.

<table>
<thead>
<tr>
<th>GLN_{353} GENOTYPE [n]</th>
<th>CORRELATION COEFFICIENT (P VALUE)</th>
<th>95% CONFIDENCE INTERVALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL [59]</td>
<td>0.20 (0.07)</td>
<td>0.17 to 0.46</td>
</tr>
<tr>
<td>ARG_{353}/ARG_{353} [25]</td>
<td>0.23 (0.13)</td>
<td>-0.18 to 0.65</td>
</tr>
<tr>
<td>ARG_{353}/GLN_{353} [24]</td>
<td>0.001 (0.5)</td>
<td>-0.42 to 0.42</td>
</tr>
</tbody>
</table>

See also footnote for Table 3
5.3 Dietary inventory study

Because of the genotype-specific correlation between FVIIc and triglyceride level found in the study of different ethnic groups, a small sample of men who had been included in a weighed dietary inventory study were genotyped for the Arg/Gln\(_{353}\) polymorphism. The sample of middle-aged European men was originally obtained from the register of a GP practice in Wembley, north-west London. Diet was measured by 5-day weighed inventory as part of a study to search for an association between fat intake and FVIIc (Miller et al 1989). Plasma FVIIc and plasma triglyceride measurements were done before and after completion of the dietary inventory study and again at two years later when individuals were recalled to obtain blood samples for DNA extraction. Dietary fat intake per unit height was measured after completion of the 5-day period of the dietary inventory.

5.3.1 Association between Arg/Gln\(_{353}\) genotype and plasma FVIIc levels.

A total of 90 men were genotyped and the frequency of the Gln\(_{353}\) was found to be 0.12 (95% CI 0.07-0.17), similar to that found in the previous study and in the original TPT study. The distribution of Arg/Gln\(_{353}\) genotype did not differ significantly from that drawn from a population in Hardy-Weinberg equilibrium. There was a significant association between plasma FVIIc level and genotype using the post-diet and follow-up measurements, but not using the pre-diet measurements. However the Arg\(_{353}\) homozygotes did have higher plasma FVIIc levels than the heterozygotes in this group (Table 7).
TABLE 7 Mean (95%CI) of variables according to genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>FVIIc (%standard) [n]</th>
<th>Trigs (mmol/l) [n]</th>
<th>Fat/H² (SD) [n]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-diet*</td>
<td>Post-diet**</td>
<td>Follow-up*</td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>109 (103-115) [71]</td>
<td>107 (102-113) [71]</td>
<td>117 (111-123) [71]</td>
</tr>
<tr>
<td>Arg/Gln + Gln/Gln</td>
<td>98 (88-109) [19]</td>
<td>89 (80-99) [18]</td>
<td>93 (84-104) [19]</td>
</tr>
<tr>
<td>All</td>
<td>106 (101-112) [90]</td>
<td>103 (99-108) [89]</td>
<td>111 (106-117) [90]</td>
</tr>
</tbody>
</table>

* p = .107  **, ▲ means significantly different by ANOVA (p = 0.002, p < 0.0001 respectively)
5.3.2 FVIIc genotype-specific relationship with plasma triglyceride levels.

There was no difference in correlation between triglycerides and FVIIc in the different genotype groups. This was the case whether pre-, post- or follow-up measurements were used.

5.4 Camberley G.P. practice samples from NPHSII

Samples were obtained from white European men aged between 40 and 64 years from a GP practice in Camberley who were being recruited by Dr George Miller and colleagues to participate in the second Northwick Park Heart Study (NPHSII). Both FVIIag and FVIIc were measured in this sample, and the association between Arg/Gln\textsuperscript{353} genotype and both of these variables was examined. The genotype-specific effect of triglycerides and cholesterol on FVIIag and FVIIc was also examined. Table 10 shows the general characteristics of the population sample according to Arg/Gln\textsubscript{353} genotype. The frequency of the Gln\textsubscript{353} allele was 0.09 (95% CI 0.07-0.11), similar to that in the previous studies.

5.4.1 Association between Arg/Gln\textsubscript{353} genotype and plasma factor VII antigen (ag) and coagulant activity (c) levels.

The association between Arg/Gln\textsubscript{353} genotype, FVIIag and FVIIc was examined by analysis of variance. There was a significant association between genotype and both FVIIag and FVIIc. F-values were respectively 50.2 (p<0.0001) and 47.6 (p<0.0001). (See Table 8).
TABLE 8 Mean (CI) of variables according to genotype

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (CI) [n]</th>
<th>Arg/Arg&lt;sub&gt;353&lt;/sub&gt; [301]</th>
<th>Arg/Gln&lt;sub&gt;353&lt;/sub&gt; and Gln/Gln&lt;sub&gt;353&lt;/sub&gt; [63]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>54.67 (54.33-55.01)</td>
<td>54.03 (53.31-54.75)</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.59 (26.22-26.96)</td>
<td>26.34 (25.42-27.26)</td>
<td></td>
</tr>
<tr>
<td>Chol (mMol/l)</td>
<td>5.77 (5.65-5.89)</td>
<td>6.00 (5.74-6.25)</td>
<td></td>
</tr>
<tr>
<td>Trig (mMol/l)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.66 (1.56-1.77)</td>
<td>1.81 (1.59-2.06)</td>
<td></td>
</tr>
<tr>
<td>Factor VIIc (% standard)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>97.8 (95.1-100.3)</td>
<td>78.2* (73.8-82.9)</td>
<td></td>
</tr>
<tr>
<td>Factor VIIa</td>
<td>98.3 (95.6-101.1)</td>
<td>77.7* (73.9-81.9)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Geometric means, * means significantly different by ANOVA p<0.0001, CI=95% confidence interval

5.4.2 Arg/Gln<sub>353</sub> genotype-specific relationship with plasma triglyceride levels.

The relationship between cholesterol and FVIIa and FVIIc, and triglycerides and FVIIa and FVIIc in the different genotypes was examined using step-wise regression and testing for a significant interaction between genotype and cholesterol and genotype and triglycerides in determining FVIIa and FVIIc levels. In other words, the correlation between FVIIa or FVIIc with triglyceride level or cholesterol level in the different genotype groups was different except for the correlation between FVIIc and cholesterol, which did not vary. Results are shown in table 9.
TABLE 9 Slope (SE) of regression of FVIIc and FVIIag on plasma cholesterol and triglyceride levels and standardised regression coefficient (SRE)*according to FVII genotype.

<table>
<thead>
<tr>
<th></th>
<th>ALL</th>
<th>ARG/ARG</th>
<th>ARG/GLN+GLN/GLN</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVIIc - Triglyceride(^1)</td>
<td>slope 0.115 (0.022)</td>
<td>0.143 (0.024)</td>
<td>-0.015 (0.055)</td>
</tr>
<tr>
<td></td>
<td>SRE 0.062 (0.012)</td>
<td>0.077 (0.012)</td>
<td>-0.008 (0.030)</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>= 0.8</td>
</tr>
<tr>
<td>FVIIc - Cholesterol(^2)</td>
<td>slope 0.068 (0.012)</td>
<td>0.068 (0.013)</td>
<td>0.069 (0.028)</td>
</tr>
<tr>
<td></td>
<td>SRE 0.069 (0.012)</td>
<td>0.069 (0.013)</td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>FVIIag - Triglyceride(^3)</td>
<td>slope 0.163 (0.022)</td>
<td>0.181 (0.024)</td>
<td>0.082 (0.048)</td>
</tr>
<tr>
<td></td>
<td>SRE 0.088 (0.012)</td>
<td>0.098 (0.013)</td>
<td>0.044 (0.026)</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>= 0.09</td>
</tr>
<tr>
<td>FVIIag - Cholesterol(^2)</td>
<td>slope 0.078 (0.012)</td>
<td>0.090 (0.013)</td>
<td>0.029 (0.026)</td>
</tr>
<tr>
<td></td>
<td>SRE 0.080 (0.012)</td>
<td>0.091 (0.013)</td>
<td>0.029 (0.026)</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.0001</td>
<td>&lt; 0.001</td>
<td>= 0.26</td>
</tr>
</tbody>
</table>

1 interaction genotype x Trig p = 0.007
2 interaction genotype x cholesterol p = 0.05
3 interaction genotype x Trig p = 0.083

*SRE is the change in FVIIc or FVIIag to accompany a one standard deviation change in the independent variable (i.e. triglyceride or cholesterol level).

Serum cholesterol and plasma triglyceride levels were significantly correlated with both FVIIag and FVIIc (see Table 9). There was a significant interaction between triglycerides and genotype in determining both FVIIag and FVIIc levels. There was a significant interaction between cholesterol and genotype in determining FVIIag but not for FVIIc. In other words there was a significant correlation between triglycerides and FVIIag and FVIIc in the Arg/Arg homozygotes, but this was absent in individuals carrying the Gln allele as shown by the essentially zero slope (Table 9). The correlation between FVIIag and cholesterol was significant in the Arg/Arg homozygotes but absent in those individuals carrying the Gln allele. Although there was a significant relationship between FVIIc and cholesterol, this did not differ in the two genotype groups.
5.5 ECTIM multi-centre study

The ECTIM study (Etude Cas Témoin sur l'Infarctus du Myocarde - Case-Control Study on Myocardial Infarction) is a large multi-centre study including samples from Belfast, Lille, Strasbourg and Toulouse. The aims of the study are both to compare the frequency of DNA polymorphisms in patients who have had a myocardial infarction and controls, and in different centres with differing risks of MI, and to study the association between these polymorphisms and measured levels of risk factors for MI i.e. plasma lipoprotein levels and coagulation factor levels. All the individuals in the study were men, the control sample being drawn at random from GP practices in the four areas and the patients being aged between 25 and 64 who had had a myocardial infarction according to MONICA category I (Löwel 1990). A number of variables were measured on these individuals, including FVIIc, fibrinogen, triglycerides, alcohol consumption, medication to include oral and intravenous anticoagulants, lipid-lowering drugs, cholesterol, LDL, VLDL etc. FVIIc was measured in a one-stage assay using congenitally FVII deficient plasma in a central laboratory.

Mean levels of some of these variables and significant differences between centres and patient and control groups are shown in Table 10. There were considerable differences in triglyceride level between groups, but FVIIc was significantly different between the patients and controls, although the Strasbourg controls had significantly higher FVIIc levels than the Toulouse controls. A total of 991 samples were genotyped and the frequencies of the Gln allele in the samples from different centres and in patients and controls is shown in table 11. There were no significant differences in frequency of the Gln353 allele between control groups in the different centres, between patient groups in
the different centres or between patient and control groups in the different centres. The FVII Arg/Gln<sub>353</sub> genotype distribution in the patient and control groups from the different centres did not differ significantly from that expected for a sample drawn from a population in Hardy-Weinberg equilibrium.
TABLE 10 Mean (SD) of variables according to centre and group

<table>
<thead>
<tr>
<th>Centre</th>
<th>Group (n)</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Trig (mmol/l)</th>
<th>FVIIc (%standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belfast</td>
<td>Control (152)</td>
<td>54§ (8.07)</td>
<td>25.54 (3.28)</td>
<td>1.45* (1.34-1.56)</td>
<td>118 (23)</td>
</tr>
<tr>
<td></td>
<td>Patient (189)</td>
<td>54 (7.99)</td>
<td>26.18 (3.68)</td>
<td>1.83•• (1.71-1.95)</td>
<td>116 (22)</td>
</tr>
<tr>
<td>Lille</td>
<td>Control (94)</td>
<td>55§§ (7.23)</td>
<td>25.80 (3.78)</td>
<td>1.42 (1.29-1.57)</td>
<td>118 (28)</td>
</tr>
<tr>
<td></td>
<td>Patient (32)</td>
<td>54 (8.68)</td>
<td>27.42 (4.82)</td>
<td>1.87†† (1.58-2.20)</td>
<td>120 (27)</td>
</tr>
<tr>
<td>Strasbourg</td>
<td>Control (145)</td>
<td>53 (8.71)</td>
<td>27.21† (3.49)</td>
<td>1.61• (1.46-1.77)</td>
<td>122 (23) □</td>
</tr>
<tr>
<td></td>
<td>Patient (90)</td>
<td>54 (8.06)</td>
<td>27.20 (2.94)</td>
<td>1.49• (1.37-1.63)</td>
<td>110 (23)</td>
</tr>
<tr>
<td>Toulouse</td>
<td>Control (133)</td>
<td>52 (8.50)</td>
<td>26.56‡ (3.89)</td>
<td>1.18 (1.08-1.28)</td>
<td>114 (22)</td>
</tr>
<tr>
<td></td>
<td>Patient (98)</td>
<td>54 (8.17)</td>
<td>26.50 (3.34)</td>
<td>1.22 (1.13-1.32)</td>
<td>113 (24)</td>
</tr>
</tbody>
</table>

† Log transformed values (95% CI)
§ Bel higher than Tou p = .002
§§Lille higher than Tou p = .003
†§Str higher than Bel and Str p < .001 and = .004 respectively
‡Tou higher than Bel p = .018
* Bel higher than Tou p < .001
** Bel higher than Str and Tou p = .001 and < .001 respectively
††Lille higher than Str and Tou p = .013 and < .001 respectively
■ Str higher than Tou p < .001
● Str higher than Tou p = .001
□ Str higher than Tou p = .002
### TABLE 11 Frequency of the Gln^gg allele (95% CI) [n] in the ECTIM study according to centre in patient and control groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Belfast</th>
<th>Lille</th>
<th>Strasbourg</th>
<th>Toulouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0.10 (0.07-0.13) [152]</td>
<td>0.10 (0.06-0.14) [94]</td>
<td>0.14 (0.10-0.18) [145]</td>
<td>0.11 (0.07-0.15) [133]</td>
</tr>
<tr>
<td>Patients</td>
<td>0.09 (0.06-0.12) [189]</td>
<td>0.14 (0.06-0.23) [32]</td>
<td>0.13 (0.08-0.18) [90]</td>
<td>0.09 (0.05-0.13) [98]</td>
</tr>
</tbody>
</table>

No of individuals in each sample in brackets.

#### 5.5.1 Association between Arg/Gln^gg genotype and plasma factor VIIc levels.

The association between FVIIc and Arg/Gln^gg genotype was examined separately in patients and controls in each centre by analysis of variance after those individuals taking oral anticoagulants had been excluded. There was a significant association between FVIIc and genotype in patient and control groups in all centres, except for the control sample from the Lille centre (p=0.2) although the sample trend was apparent here, i.e. Arg^gg^ homozygotes having the highest plasma FVIIc levels and the Gln^gg^ homozygotes having the lowest. The results are shown in tables 12 and 13.
TABLE 12 Mean FVIIc levels (SD) by Arg/Gln<sup>353</sup> genotype according to centre in control groups

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg/Arg</td>
<td>122 (20) [122]</td>
<td>120 (28) [77]</td>
<td>125 (22) [108]</td>
<td>117 (22) [103]</td>
</tr>
<tr>
<td>Arg/Gln</td>
<td>103 (18) [30]</td>
<td>106 (22) [16]</td>
<td>116 (23) [33]</td>
<td>103 (17) [29]</td>
</tr>
<tr>
<td>All</td>
<td>118 (23) [152]</td>
<td>118 (28) [94]</td>
<td>122 (23) [145]</td>
<td>114 (22) [133]</td>
</tr>
<tr>
<td>p</td>
<td>&lt;.0001</td>
<td>0.2</td>
<td>.005</td>
<td>.006</td>
</tr>
</tbody>
</table>
TABLE 13 Mean FVIIc Levels (SD) by Arg/Gln$^{363}$ genotype according to centre for patient groups

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg/Arg</td>
<td>118 (22) [155]</td>
<td>126 (24) [24]</td>
<td>114 (22) [108]</td>
<td>115 (24) [83]</td>
</tr>
<tr>
<td>Arg/Gln</td>
<td>105 (18) [34]</td>
<td>110 (26) [7]</td>
<td>100 (25) [24]</td>
<td>99 (24) [14]</td>
</tr>
<tr>
<td>All</td>
<td>116 (22) [189]</td>
<td>120 (27) [32]</td>
<td>110 (23) [90]</td>
<td>113 (24) [98]</td>
</tr>
<tr>
<td>p</td>
<td>.002</td>
<td>.02</td>
<td>.01</td>
<td>.05</td>
</tr>
</tbody>
</table>
5.5.2 Arg/Gln$_{353}$ genotype-specific correlation with plasma triglyceride levels.

The correlation between triglycerides and FVIIc in the different genotype groups was examined separately in controls and patients according to centre. The results are shown in Tables 14 and 15. The Belfast sample showed similar trends to those seen in the ethnic study and the Camberley study (4.2, 4.4), i.e. stronger correlation in the Arg$_{353}$ homozygotes compared with the other genotype groups, but these were very different from the results seen in the other centres, and there were no significant differences in the correlation between FVIIc and triglycerides in the different genotype groups in controls or patients in any of the centres.
TABLE 14 Pearson product moment correlations between factor VIIc and triglyceride levels according to genotype in controls in the different centres

<table>
<thead>
<tr>
<th>Centre</th>
<th>Genotype</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arg/Arg</td>
<td>Arg/Gln +</td>
<td>Gln/Gln</td>
</tr>
<tr>
<td>Belfast</td>
<td>0.34 (122)</td>
<td>0.26 (30)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p = 0.079</td>
<td></td>
</tr>
<tr>
<td>Lille</td>
<td>0.13 (77)</td>
<td>0.52 (17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p = 0.137</td>
<td>p = 0.016</td>
<td></td>
</tr>
<tr>
<td>Strasbourg</td>
<td>0.42 (108)</td>
<td>0.52 (37)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Toulouse</td>
<td>0.31 (103)</td>
<td>0.48 (30)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p = 0.003</td>
<td></td>
</tr>
</tbody>
</table>

Correlation coefficients are in bold typeface and number of individuals in brackets

TABLE 15 Pearson product moment correlations between factor VIIc and triglyceride levels according to genotype in patients in the different centres

<table>
<thead>
<tr>
<th>Centre</th>
<th>Genotype</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arg/Arg</td>
<td>Arg/Gln +</td>
<td>Gln/Gln</td>
</tr>
<tr>
<td>Belfast</td>
<td>0.34 (155)</td>
<td>0.09 (34)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p = 0.300</td>
<td></td>
</tr>
<tr>
<td>Lille</td>
<td>0.08 (24)</td>
<td>0.56 (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p = 0.351</td>
<td>p = 0.081</td>
<td></td>
</tr>
<tr>
<td>Strasbourg</td>
<td>0.14 (65)</td>
<td>0.19 (25)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p =0.137</td>
<td>p = 0.182</td>
<td></td>
</tr>
<tr>
<td>Toulouse</td>
<td>0.23 (83)</td>
<td>0.76 (15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p = 0.017</td>
<td>p &lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>
6. Factor VII genetic polymorphism: discussion

The Arg/Gln<sub>353</sub> amino acid substitution was found to be a common polymorphism, due to a G to A base change in exon 8 of the gene, which altered a cutting site for the restriction enzyme MspI (Green et al 1991). An initial study showed that the frequency of the allele coding for the factor VII Gln<sub>353</sub> was 0.1 in a sample of healthy men from the UK, and carriers for the Gln<sub>353</sub> allele had levels of Factor VIIc 22% lower than the sample mean (Green et al 1991). Individuals homozygous for the Gln<sub>353</sub> allele had both low factor VII coagulant activity and low factor VII antigen levels suggesting that the amino acid substitution may alter the conformation of the protein, leading to reduced secretion of the protein from the liver or increased catabolism. This thesis describes further population studies, carried out to confirm this association independently and to determine whether other measured variables influence the association, i.e. to investigate possible gene-environment interactions.

6.1 Association between warfarin treatment response and Arg/Gln<sub>353</sub> genotype.

The samples used in this study were from white European middle-aged men (45-65 years) entered into the TPT study as described in the Results section 4.1. The samples used here were taken from men who were in the group being treated with warfarin.

Warfarin inhibits the action of the vitamin K-dependent coagulation factors, by preventing the conversion of the glutamic acid (Glu) residues in these coagulation factors to γ-carboxyglutamic acid (Gla). This γ-carboxylation is due to the action of a vitamin K-dependent γ-carboxylase. The function of the Gla domain of FVII is known;
its action is to bind calcium ions and it has been shown to be essential for the
interaction with cell surface tissue factor (TF) (Sakai 1990). Recent studies by
Wildgoose et al (1992) suggest that the FVII gla domain does not interact directly with
TF but rather, by binding calcium ions, induces a calcium-dependent conformational
change in FVII/VIIa that allows it to interact with the extracellular domain of TF.
Because the location of the Arg/Gln amino acid change was found to be on the
periphery of the FVII molecule, its lowering effect on FVII coagulant activity may be
due to some disruption of the interaction between FVII and lipid surfaces or cofactors.
If this amino acid change affected the interaction between the Gla domain and tissue
factor, or affected the γ-carboxylation during processing of the FVII molecule, then it
would be reasonable to expect that there would be differences in an individual’s
response to warfarin treatment according to genotype.

The results of the warfarin study confirmed the association between the Arg/Gln polymorphism and plasma FVIIc levels (p < 0.0001), with Arg/Arg homozygotes
having plasma FVII levels 21% higher than those individuals with the Gln allele.
There was found to be no relation between the plasma FVIIc level at entry and the
amount of warfarin required to reach approximately 70% of standard FVIIc levels,
which had been one potential confounding factor considered in setting up the study. If
there had been a correlation between baseline plasma FVIIc and warfarin dose, any
differences in the amount of warfarin required according to genotype group may simply
have been due to the differences in FVIIc associated with genotype rather than any
effect of warfarin action. On examination of the warfarin dose required according to
genotype there was no significant difference between the Arg/Arg homozygotes and
the Arg/Gln heterozygotes, suggesting that the Arg/Gln polymorphism does not
affect the action of warfarin on FVIIc, that is, it is unlikely to interfere directly with either γ-carboxylation or the action of warfarin on γ-carboxylation of FVII. Major determinants of warfarin dose are likely to be inter-individual differences in absorption and/or catabolism, in common with other orally administered drugs, and it is highly unlikely that such differences would be related to FVII genotype.

6.2 Association between Arg/Gln<sub>353</sub> genotype and factor VIIc levels in different ethnic groups and the correlation between FVIIc and triglyceride levels.

Both the original study by Green et al (1991) and the previous study in this thesis showed a similar frequency of the Gln<sub>353</sub> allele and confirmed the association between the polymorphism and plasma levels of FVIIc, with a similar reduction of approximately 20% in FVIIc levels in individuals carrying the Gln<sub>353</sub> allele. To investigate further that this amino acid substitution was having a functional effect, rather than acting as a marker for a functional mutation elsewhere in the FVII gene, the allele frequency and the association between the Arg/Gln<sub>353</sub> polymorphism and plasma FVIIc levels was examined in different ethnic groups. The ethnic groups used were white Europeans, Gujarati Indians and Afro-Caribbeans as described in 4.2. These groups will have different environmental factors due to diet and lifestyle, and also different genetic backgrounds. Different populations may have a different array of genotypes at loci that interact with the effects of the locus of interest. Allele frequency of many RFLPs has been shown to vary in different populations (reviewed in Humphries 1988, Hixson 1991). The genetic differences between ethnic groups might therefore be expected to be larger than those between individuals from similar ethnic background, and so any association between the polymorphism and FVIIc levels may breakdown in some groups
If the polymorphism is simply a marker for a functional change elsewhere in the FVII gene.

Afro-Caribbeans are known to have lower levels of FVIIc and reduced incidence of IHD in comparison with other ethnic groups (Marmot 1984, Meade 1988), while the Gujaratis had higher FVII. One aim of this study was to investigate whether differences in plasma levels of FVIIc amongst the ethnic groups could be accounted for by differences in the frequency of the Gln$^{353}$ allele. Individuals heterozygous for the Gln$^{353}$ allele had levels of factor VIIc below that of individuals lacking the allele in all three ethnic groups, although this association only reached statistical significance in the Gujaratis, probably because of the small sample size in the Afro-Caribbean or European groups and an increased frequency of the Gln$^{353}$ allele in the Gujaratis. The consistency of the lowering effect on factor VIIc levels associated with the Gln$^{353}$ allele in individuals from these different ethnic backgrounds suggests that the Arg-Gln$^{353}$ substitution itself is having a direct effect on factor VIIc levels, and is not simply an RFLP in linkage disequilibrium with a functional sequence change elsewhere in the gene.

The lower levels of factor VIIc observed in the Afro-Caribbean group could not be accounted for by a higher frequency of the Gln$^{353}$ allele compared with that in the other population samples. Plasma triglyceride levels are correlated with factor VIIc levels (Miller et al 1985, Simpson et al 1983, Miller et al 1986, Mitropoulos et al 1987a, Mitropoulos et al 1987b, Miller et al 1986, Mitropoulos et al 1989, Miller et al 1989, Carvalho de Sousa et al 1989, Mitropoulos and Esnouf 1990) and in one report with factor VII antigen levels (Carvalho de Sousa 1989). However, there was no correlation
between plasma factor VIIc and triglyceride levels in this group, probably because of the narrow range of plasma triglyceride concentrations as shown by the low standard deviation (Results, table 3). It seems likely, however, that the low mean plasma triglyceride level may account for the low factor VIIc levels in the Afro-Caribbean sample compared with those in the European or Gujarati group. Afro-Caribbeans had a lower WHIP ratio than the Gujaratis, their triglyceride levels and dietary fat intake were the lowest of the three groups (Thompson and Cruickshank 1990) so that these facts together may be major determinants of their low factor VIIc levels. There is also the possibility that additional genetic variation in the factor VII gene, in other regulatory genes, or other unidentified environmental factors, may determine the low factor VIIc levels among the Afro-Caribbeans.

The frequency of the Gln\textsubscript{353} allele was highest in the Gujaratis (0.25), although the mean factor VIIc level was not correspondingly low. In the Gujarati group as a whole there was a positive relationship between triglycerides and factor VIIc, as expected. However, this was confined entirely to the individuals lacking the Gln\textsubscript{353} allele; in the group carrying this allele there was no evidence for any positive correlation of triglycerides with levels of factor VIIc. Presumably because of the small sample size, the confidence intervals for the correlation coefficients in these two groups are broad and overlap with each other and with zero (Arg\textsubscript{353} homozygotes -0.18 to 0.65; Arg\textsubscript{353} heterozygotes -0.42 to 0.42). Due to the higher frequency of the Gln\textsubscript{353} allele in the Gujaratis, around forty percent of individuals carry this allele compared with around twenty percent in the other ethnic groups. Since there was essentially no relationship between plasma triglycerides and factor VIIc levels in these individuals, although strictly, the difference between the two groups was not statistically significant, this may
explain why the mean factor VIIc levels are lower than might be expected given the high levels of triglycerides. Correlation between factor VIIc and triglycerides is greater in the European sample as a whole than in the Gujarati sample. This may be explained by the higher frequency in the Europeans of the Arg_{353} allele, which is associated with a higher correlation between plasma factor VIIc and triglycerides.

Dietary manipulation studies have shown that factor VIIc levels are directly correlated with dietary fat intake and plasma triglyceride levels (Mitropoulos et al 1989, Miller et al 1989, Carvalho de Sousa et al 1989). It is possible that the Factor VIIc levels in individuals homozygous for the Arg_{353} allele are more responsive to dietary fat than those of carriers of the Gln_{353} allele, because of the observed genotype-specific interaction, but this would need to be tested in a longitudinal study.

6.3 Dietary inventory study Arg/Gln_{353} genotype-specific relationship with plasma triglyceride levels.

Individuals used in this study were a sample of middle-aged European men obtained from the register of a GP practice in Wembley, north-west London. Diet was measured by 5-day weighed inventory as part of a study to search for an association between fat intake and FVIIc (Miller et al 1989). Plasma FVIIc and triglyceride levels were measured before and after completion of the dietary inventory study, and approximately two years later, together with the amount of dietary fat intake as a ratio of the amount of dietary fat taken in the period of the inventory to individual height (fat/Ht).

Plasma FVIIc was found to be significantly associated with Arg/Gln_{353} genotype
(p<0.001), with carriers of the Arg<sub>353</sub> allele having approximately 20% higher FVIIc levels than carriers of the Gln<sub>353</sub> allele. The correlation between plasma triglyceride level and FVIIc was examined in Arg<sub>353</sub> homozygotes and in pooled Gln<sub>353</sub> heterozygotes and homozygotes. There was found to be no difference in the correlation between the genotype groups in the relationship between plasma FVIIc and triglyceride level. This result may have been explained by a narrow range of plasma triglyceride levels, which would have effectively masked any genotype-specific relationship between plasma FVIIc and triglycerides, however, this was not the case. The sample size was larger than that for the Gujarati sample examined in the previous study, where a genotype-specific interaction was detected. Small sample size cannot therefore account for the lack of a genotype-specific relationship between plasma FVIIc and triglycerides in this study. This makes the results from this study puzzling, especially in the light of the results from the Camberley G.P. practice study discussed next and it remains a possibility that the genotype-specific correlations observed in the ethnic study are misleading. It is also possible that the genotype-specific correlation seen in the Gujarati sample in the ethnic study may have been more prominent because of the dietary fat intake of this group, which is mainly derived from ghee or clarified butter, which is saturated and contains a high level of free radicals.

6.4 Camberley study-association between the Arg/Gln<sub>353</sub> polymorphism and FVIIc and FVIIa<sub>g</sub> levels and their relationship with triglycerides and cholesterol.

The previous study confirmed the association between plasma FVIIc levels and Arg/Gln<sub>353</sub> genotype but did not show the genotype-specific correlation of FVIIc and triglyceride levels. These relationships were then investigated in the sample of 364
healthy middle-aged men from the Camberley G.P. practice (from the second NPHS) on whom plasma FVIIag, FVIIc, triglyceride and serum cholesterol levels were available. The association between plasma FVIIc and Arg/Gln353 genotype was again confirmed, and a strong association between plasma FVIIag and genotype was detected. Both these associations were highly significant (p < 0.0001) and carriers of the Gln353 allele had approximately 20% lower levels of both FVIIag and FVIIc.

Overall plasma FVIIag and FVIIc were weakly (but highly significantly) positively correlated with serum cholesterol and triglycerides, with the correlation being slightly stronger with FVIIag. There were differences in the correlation of both triglycerides and cholesterol with FVIIag according to genotype group, and there were similar differences in the correlation between FVIIc and triglycerides, but not cholesterol. A hypothesis for the genotype-specific effect of plasma triglyceride level on FVIIc is that the presence of Gln instead of Arg at amino acid 353 interferes with the interaction of FVII with lipid surfaces, in particular unesterified fatty acids (UFAs) released from triglyceride-rich lipoprotein particles through the action of lipoprotein lipase (LPL) (Mitropoulos and Esnouf 1991) and therefore affects its activation or activity. The amino acid, arginine, has a basic side chain and is positively charged at physiological pH. Glutamine contains a terminal amide group and has no charge at physiological pH (fig 3). This difference in charge may therefore result in a reduced attraction between the Gln353 variant of FVII and the contact surface provided by the UFAs resulting from the action of LPL on VLDL.
However, the results from the Camberley study show that this genotype-specific effect extends to FVIIag as well. The genotype-specific correlation between FVIIc and triglycerides was exactly as predicted from the hypothesis that the Arg/Gln<sup>155</sup> amino acid substitution affects the interaction between FVIIc and lipid surfaces. What is perhaps more interesting is that although there appears to be no genotype-specific difference in correlation between FVIIc and cholesterol (although there is an overall highly significant correlation between FVIIc and cholesterol), there is one between FVIIag and cholesterol, as well as the genotype-specific difference in correlation with triglycerides. The blood samples on which the measurements of plasma levels of the variables were carried out were taken in the morning. They were non-fasting samples, the subjects having been asked to have only a light breakfast and no recommendations given as to timing of the last meal the previous evening. Plasma triglyceride concentration peaks 3-5 hours after fat ingestion and returns to fasting levels 8-10 hours
later (Grundy and Mok 1976). This means that the fat intake the previous evening will influence FVIIc levels, which will peak after the plasma triglyceride level has peaked, and will not therefore return to fasting levels until after 8-10 hours. In a study to determine FVII activation by postprandial triglyceridaemia, it was found that FVIIc level was positively associated with VLDL triglyceride concentration about 3 hours later (Miller et al 1991). If the Arg/Gln<sup>353</sup> polymorphism affects the interaction between UFAs and FVII, then the genotype-specific correlation between FVIIc and triglycerides would be expected. However, because it is the triglycerides that are proposed to directly affect FVIIc, a genotype-specific correlation with cholesterol would not be expected.

Persistently increased plasma lipoprotein particle concentration is thought to trigger a higher flux through the coagulation system, probably through contact activation, increasing production of FVIIag as a result of increased thrombin production and the positive feedback action of prothrombin activation peptide, fragment 1.2 (F1.2) in stimulating synthesis of the vitamin K-dependent coagulation factors by the liver (Mitropoulos 1987a and b). The plasma triglyceride and serum cholesterol level is a reflection of habitual dietary fat intake. The genotype-specific difference in correlation between FVIIag and plasma triglyceride level may therefore also be explained by the effect of the Arg/Gln<sub>353</sub> polymorphism on FVII activation. However, the differences in correlation between genotypes are less marked with FVIIag than FVIIc, probably because the positive feedback of F1.2 will stimulate synthesis of both alleles, although the Gln<sub>353</sub> allele will be present at a lower concentration because of its postulated effect on secretion and/or catabolism. These data suggest that cholesterol is indirectly increasing FVIIc activity in this way, whereas plasma triglyceride level directly affects
the FVII coagulant activity.

6.5 ECTIM study

The ECTIM study (Etude Cas Témoin sur l’Infarctus du Myocarde - Case-Control Study on Myocardial Infarction ) is a large multi-centre study including samples from Belfast, Lille, Strasbourg and Toulouse, centres at differing risk of CHD. The ratio of CHD incidence between Belfast and Toulouse is approximately 2.5 to 1. The aims of this study were both to compare the frequency of DNA polymorphisms in patients who have had a myocardial infarction and controls to examine possible association of the Arg/Gln\textsubscript{353} polymorphism with risk of IHD, and to study the association between these polymorphisms and measured levels of risk factors for MI i.e. plasma lipoprotein levels and coagulation factor levels. All the individuals in the study were men, the control sample being drawn at random from GP practices in the four areas and the patients being aged between 25 and 64 who had had a myocardial infarction according to MONICA category I (Löwel 1990).

There were no observed differences in the frequency of the Arg\textsubscript{353} allele between patient and control groups and so it appears that the Arg/Gln\textsubscript{353} allele does not affect an individuals risk of IHD. However, if the polymorphism proves to be causative in determining plasma level of FVIIc, itself a risk factor, it follows that the polymorphism must have some effect on risk, albeit perhaps small.

The association between FVIIc and genotype and the genotype-specific correlation between FVIIc and triglycerides was examined in the four centres included in the study.
The results show that the association was present in all the centres and in both patient
and control groups, apart from the Lille controls, although the number of samples in
this group was by far the smallest. There appeared to be no evidence of the genotype-
specific correlation between FVIIc and triglycerides observed in the other studies,
which were done on much smaller numbers of individuals. One reason for this may be
because the ECTIM study was the only study presented in this thesis in which the blood
samples on which measurement of the variables was carried out were fasting samples.
Therefore, postprandial hypertriglyceridaemia would not influence FVIIc and a
genotype-specific difference in correlation between FVIIc and triglycerides would not
be expected. However, another difference between this study and all the previous
studies is that the FVIIc assays differed. The ECMU (MRC Epidemiology and Medical
Care Unit at Northwick Park Hospital (now at St.Bartholomew's Hospital Medical
School) assay, which was used in all except the ECTIM study, was developed before
the discovery of the vitamin K-dependent coagulation factor Protein C. The importance
of Protein C in the haemostatic process was discovered when a deficiency of this
protein was found to cause thromboembolic disorders (Griffin et al 1981). Protein C
is activated by thrombin in conjunction with the endothelial cell thrombin receptor
thrombomodulin. Thrombomodulin acts to enhance thrombin activation of Protein C
approximately 30,000-fold. Activated Protein C, together with its cofactor Protein S,
inactivates FV and FVIII and increases levels of plasminogen activator by inhibiting the
activation of its inhibitor PAI-1. Presence of Protein C in plasma prolongs the clotting
time by inactivating FV and FVIII and enhancing fibrinolysis (for review see Bouma
1988). The ECMU assay uses normal bovine plasma, which has had all the vitamin K-
dependent clotting factors removed by barium sulphate precipitation. Factor II, factor
IX and factor X are added back to the plasma and FVII coagulant activity is then
measured in sample plasma using this mixture. The assay used in the ECTIM study however uses human congenitally FVII deficient plasma, which contains all of the vitamin K-dependent clotting factors including Protein C (except for FVII). Because of the inhibitory effects of Protein C and Protein S, this makes the assay far less sensitive to circulating activated FVII than the ECMU assay. The congenitally FVII deficient plasma will support the coagulant activity of activated FVII but, because of the presence of Protein C, dampens the autoactivation of FVII. This means that the range of FVIIc which can be detected with this assay is narrower than for the ECMU assay, a property which probably accounts for the lack of genotype-specific differences in the correlation between plasma FVIIc and triglycerides in the ECTIM study.

6.6 Conclusions

The Arg/Gln$^{353}$ polymorphism is associated with differences in both FVII antigen levels and FVII coagulant activity. FVIIc was strongly associated with the Arg/Gln$^{353}$ polymorphism in all the different population samples studied, including those from different ethnic groups, from both men and women, and from both MI patients and healthy people. In addition, this association has recently been observed in a study of healthy women in the USA (Elaine Meilahn and colleagues, personal communication). Two of the studies described in this thesis also showed a genotype-specific correlation between both FVIIag and FVIIc and triglycerides, and in the case of FVIIag this was also evident with serum cholesterol levels. Together these results strongly suggest that the Arg/Gln$^{353}$ amino acid substitution is affecting production of the FVII molecule and its activity in the presence of lipids. The diversity of population samples and the consistency of the results, together with the fact that an amino acid substitution occurs,
make it highly unlikely that the Arg/Gln333 polymorphism is not causing the observed differences and is merely a marker for another change elsewhere in the gene, although this can still not be ruled out entirely.
7 Final conclusions

Both fibrinogen and FVII levels are strong risk factors for IHD. The risk associated with elevated levels of these plasma proteins is greater than that for serum cholesterol level, and the difference in risk is even more pronounced for fatal events, as shown in the NPHS (Meade et al 1986). The studies presented in this thesis are primarily concerned with gene-environment interaction, with IL-6 in the case of the fibrinogen work and triglycerides in the case of the FVII studies. If the polymorphisms in these genes are proved to be predictors of interaction with environmental factors, which may to be the case, they would be more useful in predicting risk than a single measure of the risk factor in question, i.e. plasma fibrinogen or plasma FVIIc, because of the intra-individual variation in levels of these factors.

It may be useful to know an individuals Arg/Gln\textsubscript{353} genotype in order to identify those individuals who would benefit from low fat diets. Table 1 shows the results from the Ethnic study (5.2) presented in a different way, that is, with the genotype groups divided according to whether the plasma triglyceride level was above or below the mean. These data show that it is only the Arg\textsubscript{353} homozygotes with a plasma triglyceride level above the mean who have appreciably raised plasma FVIIc. Therefore, if there proves to be a causal relationship between triglycerides and FVII activation, it is this group who would derive the greatest benefit from advice to adopt a lower dietary fat intake. However, since most people will be Arg\textsubscript{353} homozygotes, a low fat diet would be beneficial in the majority of cases.
<table>
<thead>
<tr>
<th>Factor VII genotype</th>
<th>Trig &lt; 1.24 mmol/l</th>
<th>Trig &gt; 1.24 mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>95% CI</td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>108</td>
<td>97-120</td>
</tr>
<tr>
<td>Arg/Gln + Gln/Gln</td>
<td>101</td>
<td>86-118</td>
</tr>
</tbody>
</table>

**TABLE 1**
One of the environmental factors which may affect IL-6 level and therefore fibrinogen level is smoking. The effect of smoking in raising plasma fibrinogen levels is thought to be mediated through lung macrophage IL-6 production, and in a study on smokers and non-smokers who had been genotyped for the C/T-148 polymorphism, the major effect of smoking was seen in the carriers of the T allele. However, because smoking is associated with many other effects, such as emphysema, increased blood pressure and cancer, it would not be appropriate to advise anyone, albeit a C-allele carrier, that smoking would not affect their risk of IHD.

In the future, knowledge of the molecular mechanisms whereby genetic polymorphism determines an individual’s response to his or her environment, may enable advice to be given on alteration of lifestyle, pharmacological intervention or perhaps even gene therapy, for disease prevention.
Future studies

Fibrinogen gene expression

No difference was detected in promoter strength between the C-148 and the T-148 alleles in the CAT assays, but this was without cytokine stimulation. Since the evidence is that cytokines are involved in the control of fibrinogen expression and particularly in this region of the DNA sequence, CAT assays in the presence of IL-6 and TGF-β would be the next obvious set of experiments to perform. Once the effect of these alleles was determined in the presence of cytokine stimulation, any interaction between the C/T-148 polymorphism and the G/A-455 could be investigated. This would involve making transient expression constructs to test the four possible combinations of the two base changes in the fibrinogen promoter, and assay of their activation in the presence and absence of cytokines. It would also be interesting to repeat the bandshift assays using different extracts, antibodies to known transcription factors in so-called ‘supershift’ assays, together with DNase I footprinting and methylation interference assays, to try and characterise further the nuclear proteins involved in binding to this region of the DNA.

Factor VII genetic polymorphism

The evidence that the Arg/Gln133 amino acid substitution is having a direct effect on production and activity in the presence of lipid is becoming increasingly clear. Population studies can only act as an indication of the possible effects of a polymorphism, but no definite conclusions as to mechanisms can be drawn from them.
Future studies will use molecular biology techniques to elucidate the mechanism of this effect. The substitution of glutamine for arginine at amino acid 353 may affect the synthesis or secretion of FVII or it may affect the kinetic properties of FVII and the interaction of FVII with plasma lipid components.

In the next year I plan to investigate the effect of the Arg/Gln_353 on synthesis and secretion of FVII using a high efficiency cell expression system which allows rapid transient expression of the FVII protein by transfecting constructs containing cDNA coding for the two forms of FVII into cells. FVII production from the transfected cells could be directly monitored by measuring FVII antigen secreted into the culture medium. The synthesis and secretion of the two forms of the FVII protein could be compared by ^{35}S-methionine pulse-chase experiments on the transfected cells. Subcellular fractionation at different time points would enable the progress of the FVII through the cell to be traced.

Kinetic studies would require large amounts of the FVII protein. This could be achieved by producing stably transformed cells producing the Arg_353 and Gln_353 forms of the FVII protein. Interaction with triglycerides or unesterified fatty acids (UFA) released from VLDL by LPL (Mitropoulos et al 1992) could be investigated by assembling a suitable assay system. A 'soluble' tissue factor (TF) has recently been reported (Neuenschwander and Morrissey 1992) that does not support autoactivation of FVII and is therefore specific for FVIIa. A suitable assay system would therefore include 'soluble' TF, ^{125}I-labelled Arg or Gln_353, purified FXII, and VLDL particles with or without LPL.
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MODULATION OF CYTOKINE PRODUCTION AND FUNCTION
BY TRANSFORMING GROWTH FACTOR BETA

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INTRODUCTION

TGFβ1, a 25 kDa disulphide linked homodimeric protein, is the prototype of a structurally and functionally related family of proteins which have diverse effects upon growth and development. Activated macrophages, B and T lymphocytes can synthesise TGFβ1 and exogenous TGFβ1 inhibits T lymphocyte and thymocyte proliferation, IL-2 receptor expression, B cell proliferation and immunoglobulin production, and the generation of NK, LAK and CTL function (reviewed by Wahl et al., 1989).

We have examined the modulation of cytokine production by recombinant human TGFβ1, and show TGFβ1 inhibits the production of IL-1 and TNF but induces the production of IL-6. The inhibitory effect of TGFβ1 is dependent on the inducing stimulus, as the induction of IL-1 and TNF by phorbol esters is not inhibited by TGFβ1. Furthermore we have investigated the effect of the combination of TGFβ1 and IL-6 on thymocyte proliferation and acute phase protein gene expression in a liver cell line.

RESULTS

We examined the effect of TGFβ1 by itself or in combination with agents known to induce the production of IL-1, TNF and IL-6. The results of these experiments are summarised in Table 1. PBM cultured with medium alone produced little IL-1β, TNFα or IL-6 protein, and there was no enhancement of IL-1β and TNFα protein.
<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>TNF (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
<th>IL-6 U/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No TGFβ</td>
<td>TGFβ (10 ng/ml)</td>
<td>No TGFβ</td>
</tr>
<tr>
<td>Untreated</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Poly I: C</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>1000</td>
</tr>
<tr>
<td>TNF</td>
<td>ND</td>
<td>ND</td>
<td>660</td>
</tr>
<tr>
<td>IFNY</td>
<td>250</td>
<td>240 (-4%)</td>
<td>400</td>
</tr>
<tr>
<td>LPS</td>
<td>1750</td>
<td>1250 (-29%)</td>
<td>2200</td>
</tr>
<tr>
<td>TNF/IFNY</td>
<td>ND</td>
<td>ND</td>
<td>2200</td>
</tr>
<tr>
<td>LPS/IFNY</td>
<td>2380</td>
<td>1375 (-43%)</td>
<td>1740</td>
</tr>
<tr>
<td>PMA</td>
<td>2200</td>
<td>2100 (-4%)</td>
<td>4120</td>
</tr>
<tr>
<td>PHA</td>
<td>1200</td>
<td>750 (-48%)</td>
<td>840</td>
</tr>
<tr>
<td>PHA/PMA</td>
<td>4290</td>
<td>4320 (+1%)</td>
<td>3960</td>
</tr>
</tbody>
</table>

Table 1. The inhibitory effects of TGFβ are stimulus and cytokine specific. PBM were cultured at 1 × 10^6 cells/ml in RPMI 1640 containing 10% FCS and with or without TGFβ 10 ng/ml were stimulated with the following agents: PMA 50 ng/ml, PHA 1 µg/ml, IFNY 1000 U/ml, TNFa 2500 U/ml, LPS 10 µg/ml, Poly I:C 100 µg/ml, in the indicated combinations. Conditioned medium was collected and assayed for IL-6 using B9 cells, IL-1β using the Cistron IL-1β ELISA and TNFa using an ELISA.
when TGFβ1 was included at 10ng/ml (400 pM). IL-6 levels were elevated significantly above background in the presence of 10ng/ml TGFβ1 alone. Combinations of stimuli which included the phorbol ester, PMA were potent inducers of all three cytokines and were not susceptible to inhibition by TGFβ1. Treatment of PBM with IFNγ by itself, caused a small but significant elevation in the production of TNFα, IL-1β and IL-6 and this was not inhibited by the addition of TGFβ1. Of the other stimuli tested, the production of IL-1β and TNFα was inhibited in the presence of TGFβ1, in contrast there was a marginal increase in the amount of IL-6 produced, however this effect was not synergistic.

![Graph](image-url)

**Fig. 1.** TGFβ1 and TGFβ2 inhibit IL-6 stimulated thymocyte proliferation. Thymocytes were cultured with 10,000 (squares), 1000, (circles) and 100 U/ml (circles) of IL-6 in the presence of increasing concentrations of TGFβ1 and TGFβ2. Proliferation was determined after 72 hours by tritiated thymidine uptake.
The ability of TGFβ₁ to induce IL-6 was unexpected, therefore to evaluate the interaction between IL-6 and TGFβ₁ further we examined the effect of the combination of these cytokines on thymocyte proliferation. IL-6 has been previously shown to enhance thymocyte proliferation (Helle et al., 1989), while TGFβ₁ has been shown to be a potent inhibitor of IL-1 in this assay (Chantry et al., 1989). Thymocyte proliferation was stimulated in a dose dependent manner by recombinant human IL-6 and inhibited in a dose dependent manner by both recombinant human TGFβ₁ and TGFβ₂ (Fig. 1).

![Fig. 1](image)

**Fig. 1.** TGFβ₁ and TGFβ₂ inhibit IL-6 stimulated fibrinogen gene expression. Confluent cultures of hepG2 cells were treated for 16 hours with cytokines and levels of α, β and γ fibrinogen mRNA determined by northern blotting. The ethidium bromide staining of ribosomal RNA and confirms equal loading in each well. Lane 1 untreated, lane 2 1000 u/ml IL-6, lane 3 1000 u/ml IL-6 + TGFβ₁ 10 ng/ml, lane 4, 1000 u/ml IL-6 + TGFβ₁ 0.1 ng/ml, lane 5 1000 u/ml IL-6 + TGFβ₂ 10 ng/ml, lane 6 1000 u/ml IL-6 + TGFβ₂ 0.1 ng/ml.
We next studied the interaction between IL-6 and TGFβ1 and TGFβ2 in the induction of the acute phase protein genes for fibrinogen in the hepG2 liver cell line. Northern blotting experiments were used to analyse changes in mRNA levels encoding the three fibrinogen genes. IL-6 mediated changes in fibrinogen gene expression were found to be blocked by both TGFβ1 and TGFβ2 in a dose dependent manner (Fig. 2).

**DISCUSSION**

Our results are consistent with the concept that TGFβ cannot be considered as a solely immunosuppressive molecule. Thus the inhibitory effects of TGFβ on cytokine production are specific in the sense that TNF and IL-1 are inhibited while the production of IL-6 is augmented by TGFβ. TGFβ has also been shown to induce PDGF in a variety of cell lines (Pierce et al., 1989). Interestingly, both IL-1 and TNF are potent inducers of IL-6 and PDGF. Thus TGFβ appears to have properties in common with both IL-1 and TNF.

TGFβ may also modulate IL-6 biological activities in a selective manner, thus TGFβ does not interfere with the IL-6 bioassay, but exerts an inhibitory effect on IL-6 driven thymocyte proliferation and hepG2 fibrinogen gene expression. Other activities of IL-6 and TGFβ may be co-operative such as inhibition of endothelial cell proliferation (May et al., 1989). Recent data suggests a role for TGFβ in the induction of IgA (Coffman et al., 1989) and it remains to be established whether IL-6 plays a role in this response.

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Cytokine production by cholesterol-loaded human peripheral monocyte-macrophages: the effect on fibrinogen mRNA levels in a hepatoma cell-line (HepG2)

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Key words: Cytokine; Human monocyte; Fibrinogen; Cholesterol; HepG2 cell

Conditioned medium from human monocyte-macrophages incubated under various conditions was tested for its ability to stimulate fibrinogen mRNA levels in the hepatoma cell line HepG2. Recombinant human interleukin-6 (IL-6) stimulated fibrinogen mRNA levels 4.4-fold over control levels; this response was blocked by an anti-IL-6 antibody. Conditioned medium from 3-day-cultured monocyte-macrophages produced a slight stimulation of fibrinogen synthesis in HepG2 cells which was enhanced when the monocyte-macrophages had been treated with lipopolysaccharide (LPS). This stimulation was blocked by the anti IL-6 antibody. The cytokines, interleukin-1 (IL-1) and tumour necrosis factor (TNF) were also detected in the conditioned medium from the 3-day-cultured monocyte-macrophages. Monocyte-macrophages were cultured for 17 days and then incubated with acetylated low density lipoprotein (AcLDL) for 48 h. Such cells were ‘foamy’ in appearance and showed a 4-fold increase in apoE mRNA and a 10 to 50-fold increase in apoE secretion. This increase in apoE production was suppressed by almost a third when cells were coincubated with AcLDL and LPS. Conditioned medium from these 17-day-cultured AcLDL-treated human monocyte-macrophages did not stimulate fibrinogen mRNA synthesis in HepG2 cells, nor did the conditioned medium contain detectable levels of cytokines. These results suggest that cytokine production from foam cells in the atherosclerotic lesion is unlikely to be a major contributing factor in determining the elevated fibrinogen levels seen in the plasma of patients with IHD.

Introduction

Several prospective studies have shown a direct association between plasma fibrinogen concentration and subsequent incidence of ischaemic heart disease (IHD) [1–4] and stroke. The Northwick Park Heart Study [1] showed that an elevation of one standard deviation in fibrinogen levels (0.6 g/l) was associated with an 84% increase in the risk of IHD within the next 5 years. Fibrinogen is one of the acute-phase proteins produced by the liver in response to injury [5]. Recent studies have shown that several cytokines, including IL-6, TGF-β, IL-1β and TNF, act on hepatocytes and regulate the synthesis of acute-phase proteins. IL-6 has been shown to be a major regulator of acute-phase proteins and shares functional and immunological identity with hepatocyte stimulating factor (HSF) [6–8]. Monocyte-derived macrophages, which synthesise a wide range of secretory products, including IL-6 [9], play a central role in atherogenesis [10,11]. On accumulation of cholesteryl esters macrophages become lipid-laden ‘foam cells’ which form a large part of the atherosclerotic plaque [12]. Macrophages are thought
to participate in the process of transformation of atheroma to fibrous plaque by removal of lipids [13], and to act as scavenger cells in lipoprotein metabolism and clear lipids from the area of lesion formation in atherogenesis [14].

After more than 2 weeks in culture these cells are known to show maximum expression of the scavenger receptor and exhibit other macrophage-like properties [15,16]. At this stage they can be induced to form foam cells by incubation with acetylated LDL [17,18]. Apolipoprotein E is a major secretory product of macrophages and since apoE secretion is stimulated when macrophages ingest large quantities of cholesterol it can be used as an indicator of foam cell formation [19]. Lipopolysaccharide (LPS), a glycolipid component of the outer membrane of gram negative bacteria, is capable of activating macrophages [20]. Since bacterial toxins like LPS may be able to penetrate the arterial wall during infection, this agent or others like it may play a role in atherogenesis [20]. In order to investigate the possibility that secretion of IL-6 from monocyte-derived foam cells in atherosclerotic plaques may be elevated levels of fibrinogen associated with coronary heart disease, fresh human peripheral blood monocytes were isolated and cultured in vitro. Conditioned medium from untreated monocyte-macrophages and monocyte-macrophages incubated with AcLDL, LPS or both, was incubated with HepG2 hepatoma cells and the effect on fibrinogen mRNA production was measured.

Materials and Methods

Cells

Monocyte-macrophages. Monocytes were isolated from 25 ml of fresh anticoagulated blood from healthy volunteer donors using Ficoll/Hypaque centrifugation [21]. The cells were seeded in RPMI 1640 medium supplemented with 10% foetal calf serum in 24-well plates at a density of 1.5·10⁶/well. After incubation for 2 h at 37°C, non-adherent cells (mainly lymphocytes) were removed by washing with HBSS and fresh medium was added. 24 hours after seeding, the cells were washed again. Experiments were performed 3 or 17 days after seeding, in the latter case when the remaining adherent cells had assumed features characteristic of macrophages and were capable of taking up modified LDL.

HepG2 cells. HepG2 cells were obtained from the American Type Culture Collection and maintained in MEM (Gibco) supplemented with 10% foetal calf serum (FCS) (Gibco), 2 mM sodium pyruvate, 1 × non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C in a humidified incubator equilibrated with 5% CO₂ and 95% air. Cells were seeded at a density of approx. 6.6·10⁵ cells/10 cm² well.

Human Detroit foreskin fibroblasts. Human Detroit foreskin fibroblasts were obtained from Flow Laboratories and maintained in DMEM/10% FCS.

AcLDL and LPS treatment

Monocyte-macrophages to be treated with AcLDL were cultured for 17 days and washed with PBS. These cells were then incubated with LDL (gift from Dr. Arvin Jaddah, Hammersmith Hospital) treated with acetic anhydride as described by Basu et al. [22] at 50 μg/ml for 48 h. Monocyte-macrophages to be treated with LPS (from Salmonella typhimurium, Sigma, Poole, U.K.) were cultured for either 3 or 17 days, washed with PBS and incubated for 48 h with LPS at a concentration of 100 μg/ml. Cells which were to be left untreated had medium replaced at 17 days with normal tissue culture medium. The conditioned medium was removed and stored in aliquots at −20°C until used to treat hepatoma cells.

Hepatocyte treatment with recombinant human IL-6 (rhIL-6) and anti-IL-6 antibody

At the time of these experiments there were no reports of treatment of HepG2 cells with recombinant human IL-6. The dose of IL-6 was calculated to give a maximal response, since the reported Kₐ of the IL-6 receptor is 0.1–1 nm depending on the cell type and a dose of 1000 U/ml IL-6 is approx. 400 pmol and therefore within the range of the receptor Kₐ [23]. When almost confluent, HepG2 cells were incubated for 24 h with 1 ml of medium containing rhIL-6 at 1000 U/ml (Gift from D. Novick, Weizmann Institute, Israel). Cells to be treated with anti IL-6 antibody were incubated for 24 h with medium containing 80 μl of 1:10 dilution of polyclonal anti-IL-6 antibody (gift from L. Aarden, Leiden, The Netherlands). Where both rhIL-6 and anti-IL-6 were used, appropriate amounts were preincubated together in 100 μl of medium for 1 h at room temperature before diluting into 1 ml of medium and incubating with the cells. Control cells were treated in the same way as test cells, omitting additives.

Hepatocyte treatment with conditioned medium

HepG2 cells were treated with a 1 in 10 dilution in fresh medium of conditioned medium from treated and untreated monocyte-macrophages. The dilution of conditioned medium used was determined in a previous dose response experiment (not shown). After 48 h the medium was removed and RNA extracted from the cells using NP-40 extraction.

RNA preparation

Nonidet P-40 extraction. For slot blots RNA was extracted from monocyte-macrophages by the NP-40 lysis method [24]. The RNA was extracted with equal
volumes of TE-saturated phenol, phenol: chloroform and chloroform and then precipitated overnight at 20°C after adding 1/10th volume 3 M sodium acetate and 2 vols. absolute ethanol. Finally the RNA was pellet was vacuum dried and resuspended in 30 μl sterile water.

_Guanidinium isothiocyanate (GT) extraction._ Total cellular RNA from HepG2 and fibroblast cells was isolated by the guanidinium isothiocyanate-caesium chloride method [25]. The final RNA pellet was vacuum dried and dissolved in 200 μl sterile water. Total RNA was quantitated by measuring its absorbance at 260 nm, and stored at a concentration of 1 mg/ml at −20°C until RNA analysis.

**RNA analysis**

Human skin fibroblast and HepG2 total RNA were used as negative and positive controls respectively for both apoE and fibrinogen cDNA.

_Northern blotting._ Total RNA extracted using GT was denatured by treatment with 50% formamide at 65°C for 10 min then separated by electrophoresis through a 1% agarose-formaldehyde gel in MOPS running buffer [26]. RNA was transferred to a nylon membrane (Hybond N, Amersham) according to the manufacturer’s instructions.

_Slot blotting._ Specific mRNAs were quantitated by slot blotting onto nylon membranes (as for northern blotting) using 6 doubling dilutions of 20 μg total RNA from GT extraction or all RNA extracted by NP-40 extraction.

_Hybridisation._ Human apo E cDNA and human β-fibrinogen cDNA probes were used to detect apo E specific mRNA and β-fibrinogen specific mRNA, respectively. The apo E cDNA probe is a 930 bp Xho II fragment in a pKT218 vector and contains most of the coding region [27]. The β-fibrinogen cDNA probe is a 700 bp PstI fragment from pFB5 [28]. A mouse α-actin cDNA probe was used as a control [29].

All probes were labelled with 32P dCTP (Amersham U.K., specific activity 10 mCi/ml) by the random priming method [30] to specific activities of approx. 5 · 10^5 cpm/μg DNA. Northern and slot blots were prehybridised for 2–6 h at 42°C in 4 ml of hybridisation solution: 50% formamide, 5 × SSC (1 × SSC: 150 mM sodium chloride, 15 mM sodium citrate, pH 7.5), 0.1 M phosphate buffer (pH 7.0), 2 × Denhardt’s solution (100 × Denhardt’s: 2% bovine serum albumin (BSA), 2% polyvinylpyrrolidone, 2% Ficoll) and 1 ml denatured salmon sperm DNA (1% w/v). Filters were hybridised in 4 ml fresh hybridisation solution containing 4 · 10^6 counts per min (cpm) of 32P-labelled probe. Filters were washed at 65°C in 3 × SSC for 30 min, then 1 × SSC for 30 min, and finally in 0.1 × SSC for 15–30 min if necessary. Membranes were exposed to Konica X-ray film with 2 intensifying screens for 16–72 h. Blots were stripped of probes by incubation with 5 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.1 × Denhardt’s solution for 2 h at 65°C before rehybridising with other probes. Autoradiographs were analyzed by densitometric scanning using a Joyce-Loebl Chromoscan 3 and the relative amount of specific mRNA was given by the area under the curve. The background absorbance registered by the Chromoscan from each autoradiograph was adjusted to zero before scanning.

Results are expressed in arbitrary densitometric units, and are the ratio of the area under the curve of the specific mRNA of interest to that of control actin specific mRNA to ensure that any differences in loading of mRNA onto the slot blot were taken into account. All measurements were made within the linear response range of X-ray film, each measurement being the average of three dilutions in the linear range.

**Assay of proteins**

II-1 and TNF proteins were assayed using an enzyme linked immunosorbent assay (ELISA) [31]. The apo E content of the supernatants from monocyte-macrophage cultures was determined by an ELISA assay [32]. II-6 was measured by its ability to stimulate proliferation of II-6 dependent hybridoma cell-line B9 as described by Aarden et al. [33]. All assays were done in duplicate and intra-assay variation was under 10% of absolute values in all cases.

**Results**

Northern blots were performed on HepG2 mRNA (positive control) and human skin fibroblast (HSF) mRNA (negative control) to test the specificity of the cDNA probes. The Apo E cDNA probe hybridised to a mRNA band of the expected size, 1.2 kb (not shown) [27]. The β-fibrinogen cDNA probe gave a major band of approx. 1.6 kb and a minor band size of 1.85 kb again as expected (not shown) [28], two species of β-fibrinogen mRNA being due to heterogeneity in the length of 3' non-coding sequences [34].

Apo E mRNA was detectable in unstimulated monocyte-macrophages and was increased approx. 4-fold on loading with AcLDL (Fig. 1a). Similar results were seen in another previous experiment (data not shown). As expected [35,36] Apo E protein in the conditioned medium from cells treated with AcLDL increased to 50-fold (these increases were seen in three experiments) and treatment with LPS in addition to AcLDL suppressed this stimulation by almost a third in the experiment shown (Fig. 1b) and by 10% in another previous experiment.

Conditioned medium from monocyte-macrophages stimulated with LPS after increasing time in culture was assayed for II-1β, TNFα II-6. Cytokine production began to decrease 24 h after placing cells in culture,
Fig. 1. (a) A representative experiment showing levels of ApoE mRNA in monocyte-macrophages cultured for 17 days and then treated as indicated. Levels were determined by densitometric scanning of autoradiographs of slot blots. (b) A representative experiment showing ApoE protein secretion into the culture medium by monocyte-macrophages cultured for 17 days and then treated as indicated. Levels were determined by an ELISA assay.

was reduced to 50% of that in control cells by 48 h and continued to decrease with increasing time in culture (Fig. 2). Medium from untreated 17-day-old monocyte-macrophages and from cells treated with AcLDL alone, LPS and AcLDL and LPS alone contained no detectable IL-1β, IL-6 and TNF (not shown).

Treatment of HepG2 cells with recombinant human IL-6 caused a 12-fold increase over control levels of fibrinogen mRNA, normalised to actin. By comparison, a 1:10 dilution of conditioned medium from untreated 3-day-old monocyte-macrophages caused a 4-fold stimulation of fibrinogen mRNA levels above those seen in control HepG2 cells. This effect was blocked by anti IL-6 antibody (Fig. 3). Conditioned medium from LPS stimulated 3-day-old monocyte-macrophages caused a 6-fold stimulation of fibrinogen mRNA levels in HepG2 cells. Conditioned medium from 17-day-old monocyte-macrophages had no effect on fibrinogen mRNA levels whether or not the monocyte-macrophages had been AcLDL-loaded (Fig. 3). These results are from a single experiment which was performed simply to confirm the results seen from the bioassays carried out on conditioned medium (Fig. 2). However these cells were still metabolically active since AcLDL-loading produced cells of a foamy appearance with a 10- to 50-fold increase in ApoE production (Fig. 1b).

Discussion

The aim of this study was to determine if the elevated fibrinogen levels associated with IHD may be due to IL-6 production by foam cells in the atherosclerotic plaque. Our observations suggest that after 2 to 3 days in culture human monocyte-macrophages become immunologically inactive even after LPS stimu-

![Fig. 2. Effects of time in culture on peripheral monocyte-macrophages cytokine production in response to LPS. Assays were done in duplicate and the intra-assay variation was under 10% of absolute values in all cases.](image)

![Fig. 3. Effect of recombinant human IL-6 and monocyte-macrophage conditioned medium, in the presence or absence of an antibody to IL-6, on expression of fibrinogen mRNA in HepG2 cells. Levels were determined by densitometric scanning of autoradiographs of six blots. rhIL6, recombinant human IL-6; 3MCM, monocyte conditioned medium from 3-day-old monocytes; 3MCM + LPS, monocyte conditioned medium from LPS treated 3-day-old monocytes; 17MCM, monocyte conditioned medium from 17-day-old monocytes; 17MCM + AcLDL, monocyte conditioned medium from AcLDL treated 17-day-old monocytes.](image)
ation. After 17 days in culture they are immunologically inactive but can be induced to form foam cells by 24 h incubation with AcLDL with an accompanying increase in apoE mRNA levels and a large increase in apoE secretion into the medium.

In HepG2 cells treated with conditioned medium from monocyte-macrophages, the increase in fibrinogen mRNA levels was due to the action of IL-6 since the increase was prevented by a neutralising polyclonal antibody specific for IL-6. After 17 days in culture, conditioned medium from human monocyte-macrophages had no effect on fibrinogen mRNA, irrespective of cholesterol loading. This is consistent with our observations that monocyte-macrophages cultured for 17 days are incapable of producing the cytokines IL-1, IL-6 and TNF even after the transition to foam cells achieved by loading with AcLDL and irrespective of LPS stimulation.

In the in vivo situation as compared with that in vitro, there are a large number of cellular and humoral factors which could affect the monocyte/macrophages and foam cells in the atherosclerotic plaque [37–39]. There have also been recent reports that there are proliferating monocytes in the atherosclerotic plaque [40] and in human atherosclerotic lesions [41]. Therefore, although these experiments suggest that it is unlikely that cytokine production by foam cells in the atherosclerotic plaque is a major contributing factor to the elevated fibrinogen levels associated with IHD, the foam cells may be acting to attract proliferating, cytokine-producing monocytes to the plaque.

Acknowledgements

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References

Genetic and environmental determinants of factor VII coagulant activity in ethnic groups at differing risk of coronary heart disease

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Summary

It has been shown previously that individuals possessing the Gln^55 allele of factor VII have significantly lower factor VIIc levels. In this population based study of Europeans, Afro-Caribbeans and Gujarati Indians, the Gln^55 allele was associated with lower factor VIIc in all groups, carriers having factor VIIc levels 20–25% below the group mean. Although the Afro-Caribbeans had the lowest factor VIIc levels, the frequency of the Gln^55 allele was not different from the European sample. However, in the Gujaratis, the frequency of the Gln^55 allele was significantly higher than in the Europeans (0.25 compared to 0.09, P < 0.001). Factor VIIc is known to be positively correlated with plasma triglyceride levels, although the Gujaratis, having the highest mean triglyceride levels, did not have the highest mean factor VIIc levels. On examination of the relationship between triglycerides and factor VIIc in the Gujaratis there was a correlation (r = 0.23, P = 0.13) in individuals homozygous for the factor VII Arg^55 allele, but no correlation (r = 0.001, P = 0.5) among Gln^55 carriers. This striking difference suggests that the effect of triglycerides on factor VIIc is genotype specific and thus provides an example of gene–environment interaction. The high frequency of the Gln^55 allele, with its associated lack of relationship between triglyceride and factor VIIc levels, may explain the lower than expected factor VIIc levels in the Gujaratis.

Key words: Factor VII coagulant activity; Triglycerides; Ischaemic heart disease; Ethnic group; Genetic variation; Thrombosis

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Introduction

Factor VII is a vitamin K dependent coagulation factor secreted by the liver as a single chain glycoprotein (M_r 50 000). It is cleaved in the
blood by factor Xa and a number of other proteases to the activated form factor VIIa [1]. Factor VIIa is composed of two polypeptide chains held together by a disulphide bond and in the presence of tissue factor and Ca\(^{2+}\), converts factor X to factor Xa [2].

The Northwick Park Heart Study showed that raised factor VII coagulant activity (Factor VIIc) is a powerful independent risk factor for coronary heart disease (CHD) [3]. Hypertriglyceridaemia is associated with increased coagulability [4,5] and there is good evidence to suggest that triglycerides have an effect on factor VII levels and factor VII activation [4,6]. The large, negatively charged, triglyceride rich lipoprotein particles, very low density lipoproteins (VLDLs) and chylomicrons are thought to activate the intrinsic coagulation pathway via contact surface activation and therefore ultimately to catalyse factor VII activation [7-11]. The fractional catabolic rate of factor VII is also reduced by its binding to lipoprotein particles which would result in raised plasma levels of factor VIIc, enhancing the effect on activation [12]. It has also been suggested that in prolonged hyperlipidaemia, where sustained factor VII activation causes an increase in procoagulant activity, hepatic production of factor VII and other vitamin-K dependent clotting factors is stimulated by the resulting increase in the levels of prothrombin fragment F1.2 [13].

We have previously reported a strong association between an \(Mspl\) polymorphism of the factor VII gene and plasma factor VIIc levels [14]. This \(Mspl\) polymorphism is caused by a single base change, a G to A substitution in the codon for amino acid 353, which leads to arginine (Arg) being replaced by glutamine (Gln) in the protein product of the \(M2\) allele (designated Gln\(^{353}\)) [14]. In a sample of 284 men from the UK the frequency of the allele coding for the factor VII Gln\(^{353}\) is 0.1 and carriers for the Gln\(^{353}\) allele have levels of factor VIIc 22% lower than the sample mean [14]. Individuals homozygous for the Gln\(^{353}\) allele have both low factor VIIc and low factor VII antigen levels suggesting that the amino acid substitution may alter the conformation of the protein, leading to reduced secretion of the protein from the liver or increased catabolism [14].

Within the UK, individuals of different ethnic origin are known to differ in their risk of CHD, with the incidence being low in individuals of Afro-Caribbean origin [15] and high in individuals from the Indian sub-continent [16,17]. These different ethnic groups are also known to exhibit differences in factor VIIc and triglyceride levels [18,19], with Afro-Caribbeans having a lower mean factor VIIc level than Europeans [18]. Initially, we analysed the association of the Gln\(^{353}\) allele with factor VIIc levels in three samples of individuals of European, Afro-Caribbean and Gujarati Indian origin. We then examined the possibility that the ethnic differences in factor VIIc level were due to different frequencies of the Gln\(^{353}\) allele and analysed the relationship of plasma factor VIIc with triglyceride levels in the Gujarati Indians in different factor VII genotype groups.

**Methods**

**Subjects**

The population samples were recruited from general practices in the Brent and Harrow areas of north-west London as part of a cardiovascular and glucose-tolerance survey [20]. These were divided by sex and ethnic group. Ethnic groups consisted of local white Europeans (63 women, 60 men), Afro-Caribbeans (63 women, 60 men) and Gujarati Indians (66 women, 76 men). Assignment to any one ethnic group was determined by at least three grandparents of the individual being in that group. Individuals of more mixed descent were not included and all Afro-Caribbeans and Gujaratis were first generation migrants who had been resident for more than 15 years [20].

Factor VII coagulant activity measurements were obtained on 68 white Europeans, 78 Afro-Caribbeans and 93 Gujaratis. Triglyceride measurements were obtained on 65 white Europeans, 86 Afro-Caribbeans and 89 Gujaratis. Blood for DNA extraction was obtained from 90 white Europeans, 113 Afro-Caribbeans and 130 Gujaratis. Not all measurements were obtained on all individuals, accounting for the discrepancies in numbers of individuals analysed.

**FVII assays**

Factor VII coagulant activity (factor VIIc) was
measured by a one-stage biological assay [21]. The mean factor VIIc levels were higher in all three samples in the current study than in the previously reported sample (TPT; 14) (Table 2). This may be because the subjects in the current study came from an inner city area and the assay was done at varying times after sample collection.

Triglyceride assay
Plasma triglyceride concentrations were measured in whole plasma by an automated enzymic assay using Peridochrom GPO-PAP reagents (Boehringer) and a Centrifichem centrifugal analyser (Baker Instruments).

DNA procedures
DNA was extracted from whole blood by a rapid small scale method [22]. Briefly, 100 µl whole blood was mixed with 400 µl 0.17 M NH₄Cl and incubated at room temperature for 20 min. After spinning in a microfuge, the pellet was washed 2–3 times with cold 0.9% NaCl, resuspended in 200 µl 0.05 M NaOH, boiled for 10 min and neutralised with 25 µl 1 M Tris (pH 8.0).

Enzymatic amplification of DNA was performed by polymerase chain reaction (PCR) using 10 µl of the DNA extract and thermostable Taq polymerase (Perkin Elmer-Cetus) according to the manufacturer’s instructions. Oligonucleotide primers for PCR were obtained from OSWEL DNA Service (University of Edinburgh, UK). The PCR reactions were performed in an Intelligent Heating Block (Cambio, Cambridge, UK). The oligonucleotide primers and cycle time and temperatures for MspI genotype analysis have been described previously [14] but are reiterated here. The nucleotide sequences (5’–3’) of the PCR primers were GGGAGACTCCCCAAATA-TCAC and ACGCAGCTTGGCTTTTCTC. The initial cycle consisted of three steps, 95°C for 5 min, 55°C for 1 min and 72°C for 2 min, with 30 subsequent cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. Aliquots of 10 µl of each PCR reaction were digested with 15 units of MspI at 37°C overnight. DNA fragments were separated by electrophoresis through 2% agarose gels, in 40 mM Tris-acetate, 1 mM EDTA (pH 7.7) containing 0.5 µg/ml ethidium bromide and visualised by means of ultra-violet light.

The PCR-amplified region was 312 bp long and contained the variable MspI site and a nearby constant MspI site to act as an internal positive control for MspI digestion (Fig. 1). MspI digestion yielded a constant band of 40 bp irrespective of genotype. The common M1 allele (presence of cutting site, coding for the Arg353) gave bands of 205 bp and 67 bp and the M2 allele (absence of cutting site, coding for the Gln353) gave a band of 272 bp as described previously [14] (Fig. 1).

Statistical analysis
Mean values of variables were compared between different ethnic groups by t-test and analysis of variance. A chi-squared test was applied to each
population sample to compare the observed numbers of each genotype with those expected for a population in Hardy-Weinberg equilibrium. Allele frequencies in different population samples were compared by gene counting and chi-squared analysis.

Distribution of factor VIIc and triglyceride levels was examined in each ethnic group and log_{10} transformed to reduce the skewness and kurtosis of the distribution, prior to statistical analysis.

Plasma factor VIIc levels are known to be higher in females than in males [23,24] and to vary with a number of factors including triglycerides and age [4,6,24]. Log transformed factor VIIc levels were therefore adjusted for sex, triglycerides, age, BMI and waist/hip ratio (WHIP) by multiple linear regression analysis in each group. Mean factor VIIc levels of individuals of different Arg-Gln353 polymorphism genotype were then compared within each ethnic group by one-way analysis of variance.

The relationship between plasma factor VIIc levels (adjusted for sex) and triglyceride levels in individuals of different Gln353 polymorphism genotype was examined in the Gujarati sample by correlation. In the European and the Afro-Caribbean samples this analysis was performed only on Arg353 homozygotes as there were insufficient individuals having the Gln353 allele. In all statistical tests, P < 0.05 was taken as indicating statistical significance.

Results

Table 1 shows relevant characteristics of the individuals in the three samples studied. There were no significant differences between the three ethnic groups in proportions of males to females, but there were significant differences in mean age, BMI and WHIP. The Europeans were significantly older than both the Afro-Caribbeans and the Gujaratis (P < 0.001 and P = 0.011, respectively) and the Gujaratis were significantly older than the Afro-Caribbeans (P = 0.001). Afro-Caribbeans had a higher BMI than the Europeans and the Gujaratis (P = 0.02 and P = 0.007, respectively). None of these characteristics were significantly correlated with factor VIIc.

The genotype of the factor VII Arg-Gln353 polymorphism was determined by Mspl digestion of PCR amplified DNA (Fig. 1). The factor VII Arg-Gln353 genotype distribution in the three samples did not differ significantly from that expected for a sample drawn from a population in Hardy-Weinberg equilibrium. The frequency of the allele coding for Gln353 (absence of cutting site for Mspl enzyme) varied among the population samples (Table 2). The frequency in Europeans was 0.09 and in Afro-Caribbeans was 0.08, similar

<table>
<thead>
<tr>
<th>Ethnici group</th>
<th>Sex</th>
<th>Age (years)</th>
<th>BMI (kg/m^2)</th>
<th>WHIP</th>
<th>TRIGS (mmol/l)</th>
<th>Factor VIIc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europeans</td>
<td>60</td>
<td>63</td>
<td>62 (7.18)</td>
<td>26.15 (4.27)</td>
<td>0.88 (0.08)</td>
<td>1.57 (1.03)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[119]</td>
<td>[117]</td>
<td>[113]</td>
<td>[65]</td>
</tr>
<tr>
<td>Afro-Carib.</td>
<td>60</td>
<td>63</td>
<td>57 (5.59)</td>
<td>27.47 (4.36)</td>
<td>0.89 (0.07)</td>
<td>1.21 (0.57)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[114]</td>
<td>[114]</td>
<td>[112]</td>
<td>[86]</td>
</tr>
<tr>
<td>Gujaratis</td>
<td>76</td>
<td>66</td>
<td>59 (7.16)</td>
<td>26.01 (4.10)</td>
<td>0.92 (0.08)</td>
<td>1.72 (0.87)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[140]</td>
<td>[139]</td>
<td>[137]</td>
<td>[89]</td>
</tr>
</tbody>
</table>

*BMI, body/mass index; WHIP, waist/hip ratio; TRIGS, triglyceride level.
*Significantly lower than Europeans (P = 0.009) and Gujaratis (P = 0.000); **significantly lower than Europeans (P = 0.003) and Gujaratis (P = 0.030).
to that in a sample of healthy men from the UK (TPT, 14). In the Gujarati sample the frequency was 0.25, significantly greater than in the other ethnic groups (P < 0.001) (Table 2).

All three groups showed an association between lower factor VIIc levels and the presence of the allele coding for Gln353. However, this association was significant only in the Gujarati sample (P < 0.001) (Table 2) by analysis of variance of factor VIIc levels after adjustment for age, BMI, WHIP, sex and triglycerides. This association between genotype and plasma factor VIIc levels is shown in Table 3.

The correlation between triglycerides and factor VIIc was examined separately in Gujarati individuals homozygous for the Arg353 allele and those heterozygous for the polymorphism. In the whole sample of Gujarati individuals (n = 59), plasma factor VIIc level showed a strong correlation with plasma triglyceride level (r = 0.20, P < 0.07; Table 4). Taking the homozygotes and heterozygotes for the Arg353 allele separately there was a positive relationship between triglycerides and factor VIIc in individuals lacking the Gln353 allele but in individuals carrying the Gln353 allele the relationship was absent (Table 4). In the whole European sample (n = 31) the correlation between triglycerides and factor VIIc was 0.48 (P = 0.003, 95% C.I. 0.14–0.89) and 0.53 (P = 0.005, 95% C.I. 0.14–1.04) in Arg353 homozygotes (n = 22). In the whole Afro-Caribbean sample (n = 54) the correlation was 0.08 (P = 0.282, 95% C.I. –0.19 to 0.36) and in the Arg353 homozygotes (n = 47) the correlation was 0.19 (P = 0.099, 95% C.I. –0.10 to 0.49). There were too few heterozygotes in either the European or Afro-Caribbean samples (n = 2; n = 6, respectively), on whom we had both plasma factor VIIc and triglyceride levels, to give a statistically meaningful correlation.

### Table 2

<table>
<thead>
<tr>
<th>Ethnic group</th>
<th>Frequency of Gln353 allele [n]</th>
<th>95% Confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPT</td>
<td>0.10 [284]</td>
<td>0.08–0.13</td>
</tr>
<tr>
<td>Europeans</td>
<td>0.09 [84]</td>
<td>0.05–0.13</td>
</tr>
<tr>
<td>Afro-Caribbeans</td>
<td>0.08 [108]</td>
<td>0.04–0.12</td>
</tr>
<tr>
<td>Gujaratis*</td>
<td>0.25 [126]</td>
<td>0.20–0.30</td>
</tr>
</tbody>
</table>

*Frequency of Gln353 allele significantly different from European sample: P < 0.001

### Table 3

MEAN FACTOR VIIc LEVELS (95% CONFIDENCE INTERVALS) BY Arg/Gln GENOTYPE BY ETHNIC GROUP

Note: not all measurements were obtained on all individuals, accounting for discrepancies in numbers of individuals shown.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TPT</th>
<th>Afro-Carib.</th>
<th>Gujaratis</th>
</tr>
</thead>
<tbody>
<tr>
<td>[n]</td>
<td>[n]</td>
<td>[n]</td>
<td>[n]</td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>102 (26.79)</td>
<td>109 (21.08)</td>
<td>141 (22.09)</td>
</tr>
<tr>
<td>[228]</td>
<td>[44]</td>
<td>[25]</td>
<td></td>
</tr>
<tr>
<td>Arg/Gln</td>
<td>75 (18.73)</td>
<td>94 (21.24)</td>
<td>107 (21.08)</td>
</tr>
<tr>
<td>[54]</td>
<td>[9]</td>
<td>[24]</td>
<td></td>
</tr>
<tr>
<td>Gln/Gln</td>
<td>56 (8.80)</td>
<td>—</td>
<td>86 (17.53)</td>
</tr>
<tr>
<td>[2]</td>
<td>[4]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>97 (27.78)</td>
<td>107 (21.08)</td>
<td>120 (21.08)</td>
</tr>
<tr>
<td>[284]</td>
<td>[49]</td>
<td>[53]</td>
<td></td>
</tr>
</tbody>
</table>

P <0.001 0.228 <0.001

*Factor VIIc levels are expressed as percentage of standard.

**Sample from Thrombosis Prevention Trial [16], all factor VIIc levels were unadjusted and untransformed and therefore standard deviations are shown.

^Analysis of variance of log10 transformed factor VIIc levels adjusted for age, BMI, WHIP, sex and log10 transformed triglycerides by multiple regression.

### Table 4

PEARSON PRODUCT MOMENT CORRELATIONS BETWEEN FACTOR VIIc AND TRIGLYCERIDE LEVELS ACCORDING TO GENOTYPE IN GUJARATIS

Note: not all measurements were obtained on all individuals, accounting for discrepancies in numbers of individuals shown.

<table>
<thead>
<tr>
<th>Gln353 genotype [n]</th>
<th>Correlation coefficient (P value)</th>
<th>95% Confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>All [59]</td>
<td>0.20 (0.07)</td>
<td>0.17–0.46</td>
</tr>
<tr>
<td>Arg353/Gln353 [25]</td>
<td>0.23 (0.13)</td>
<td>–0.18–0.65</td>
</tr>
<tr>
<td>Arg353/Gln353 [24]</td>
<td>0.001 (0.5)</td>
<td>–0.42–0.42</td>
</tr>
</tbody>
</table>
Discussion

In our previous study in a group of men of European origin the factor VII Gln353 allele was associated with a lowering effect on factor VIIc levels [15]. In confirmation of this in the present study, individuals heterozygous for the Gln353 allele had levels of factor VIIc below that of individuals lacking the allele in all three ethnic groups. This association was not statistically significant in the Afro-Caribbean or European groups because of the small sample size. The consistency of the lowering effect on factor VIIc levels associated with the Gln353 allele in individuals from these different ethnic backgrounds suggests that the Arg-Gln353 substitution itself is having a direct effect on factor VIIc levels and is not simply an RFLP in linkage disequilibrium with a functional sequence change elsewhere in the gene. Experiments to confirm this hypothesis are in progress, by in vitro expression of the allele coding for Gln353.

Although the mechanism for the effect of the Arg-Gln353 substitution is not clear, a recent study of an Arg-Gln substitution at amino acid 304 which is spatially distant from the active site, showed that this caused reduced factor VIIc activity and reduced affinity for tissue factor [26]. Although the Arg-Gln353 substitution is close to the active site serine (aminoacid 344) in the primary sequence, a three dimensional model of factor VII suggests that aminoacid 353 is on the opposite surface of the molecule to the active site (E.G.D. Tuddenham, pers. commun.). The peripheral location of aminoacid 353 suggests that the Arg-Gln353 substitution may not directly affect enzyme activity; however, it could influence interactions of factor VII with lipid surfaces or cofactors, thereby affecting factor VII activation or activity indirectly. However, since levels of both factor VIIc and antigen are low in homozygotes for the Gln353 allele [14], it seems that the aminoacid substitution affects either secretion or catabolism of the factor VII protein, rather than any specific functional attribute of the molecule.

Contrary to our expectation, the low levels of factor VIIc observed in the Afro-Caribbean group could not be accounted for by a higher frequency of the Gln353 allele compared with that in the other population samples. There was no relationship between plasma factor VIIc and triglyceride levels in this group which was probably due to the narrow range of plasma triglyceride concentrations as shown by the low standard deviation (Table 1). These data suggest that additional factors are involved in determining the low factor VIIc levels in the Afro-Caribbean sample compared with those in the European or Gujarati group. These could include environmental differences such as dietary fat intake (a factor known to influence factor VIIc levels [4-13] or additional genetic variation in the factor VII gene or other genes involved in regulation of factor VIIc levels. Afro-Caribbeans had a lower WHIP ratio than the Gujaratis, their triglyceride levels and dietary fat intake were the lowest of the three groups [25] so that these facts together may be major determinants of their low factor VIIc levels.

The frequency of the Gln353 allele was highest in the Gujaratis, although the mean factor VIIc level was not correspondingly low. The Gujarati sample had the highest triglyceride levels of the three groups and high triglycerides are associated with high factor VIIc [4,5,7-12] and in one report with high factor VII antigen levels [12]. In the Gujarati group as a whole there was a positive relationship between triglycerides and factor VIIc, as expected. However, this was confined entirely to the individuals lacking the Gln353 allele; in the group carrying this allele there was no evidence for any positive effect of triglycerides on levels of factor VIIc. Because of the small sample size, the confidence intervals for the correlation coefficients in these two groups are broad (Arg353 homozygotes -0.18 to 0.65; Arg353 heterozygotes -0.42 to 0.42). Due to the higher frequency of the Gln353 allele in the Gujaratis, around 40% of individuals carry this allele compared with around 20% in the other ethnic groups. Since there is essentially no relationship between plasma triglycerides and factor VIIc levels in these individuals, this may explain why the mean factor VIIc levels are lower than predicted given the high levels of triglycerides. However, preliminary data from a larger sample of European individuals (unpublished) confirms this genotype-specific correlation of factor VIIc with
triglycerides. Correlation between factor VIIc and triglycerides is greater in the European sample as a whole than in the Gujarati sample. This may be explained by the higher frequency of the Arg353 allele, which is associated with a higher correlation between plasma factor VIIc and triglycerides, in the Europeans.

Dietary manipulation studies have shown that factor VIIc levels are directly correlated with dietary fat intake and plasma triglyceride levels [10-12,14]. It is possible that the factor VIIc levels in individuals homozygous for the Arg353 allele are more responsive to dietary fat than those of carriers of the Gln353 allele; however, this remains to be tested in a longitudinal study. This may be useful in determining those individuals who would benefit from dietary or drug intervention to lower triglyceride levels and concommitantly plasma factor VIIc levels. The elucidation at the molecular level of the mechanism of the interaction between factor VII genotype and triglycerides in determining factor VIIc levels will further our understanding of how genetic and environmental factors may interact to determine thrombotic risk.

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