MOLECULAR INVESTIGATION INTO REGULATORY REGIONS OF THE LDLR GENE INVOLVED IN LIPOPROTEIN METABOLISM

C. L. SCHOLTZ

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Supervisor: Prof. MJ Kotze

Co-supervisor: Prof. G George

University of Stellenbosch

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature :

Date :

SUMMARY

The advent of the new millennium saw the complete sequencing of the entire human genome. Only approximately 30 000 genes, much less than was initially predicted, have been identified to be responsible for the genetic diversity in humans. This discovery has prompted a shift in the approach to disease research, since one gene can be involved in numerous diseases. This phenomenon seems to be especially true for the low-density lipoprotein receptor (LDLR) gene. Various substances beside sterols can induce transcription of the LDLR gene.

Non-communicable diseases (e.g. hypertension) are common in the developing world and contribute significantly to mortality rates. The finding that a promoter variant (-175 g→t) in the LDLR gene is associated with elevated diastolic blood pressure may explain the phenomenon of high LDL-cholesterol levels in hypertensive individuals. Studies have demonstrated that the lowering of cholesterol, especially LDL-cholesterol, can reduce the incidence of hypertension. The -175 g→t variant is located in a newly described cis-acting regulatory element which contains a putative binding site for Yin Yang (YY)-1 and also demonstrates great homology to the cAMP response element (CRE) which bind the Ca2+ - dependent transcription factor, CRE binding protein (CREB). The fact that Ca2+ can induce transcription of the LDLR gene may, at least in part, explain the association between the -175g→t variant and elevated diastolic blood pressure.

Cholesterol is important for various processes, such as apoptosis, maintenance of cellular membranes and immune function. The -59 c→t mutation in repeat 2 of the LDLR gene abolishes binding of the sterol regulatory element binding protein(SREBP) to the SRE-1 site. SREBP is proteolytically activated during apoptosis by two caspases (CPP32 and Mch3) to

induce cholesterol levels. Our results imply that the -59C/T mutation, in repeat 2 of the LDLR gene promoter, may inhibit apoptosis under normal immunological conditions.

Atherosclerosis can be considered an immunological disease, since various humoral and cellular immune processes can be detected throughout the course of the disease. The finding that certain lipoproteins can protect against infection by binding and lysing of pathogens, or competing with pathogens for cellular receptors, prompted the investigation into the potential role of variation in the LDLR gene promoter in immune function. A significant difference in allelic distribution was detected between asymptomatic HIV-infected subjects and fast progressors for the -124 c→t variant (P=0.006), shown to increase (~160%) transcriptional activity of the LDLR gene. Of relevance to this particular study is the fact that human herpesvirus (HHV) 6 can transactivate CD4 promoters through a partial CRE site. It has been shown that the CREB and YY1 can regulate viral and cellular promoters, and these transcription factors can potentially bind to the LDLR promoter at the FP2 site.

The mutation enrichment in the LDLR gene promoter seen in the South African Black and Coloured population groups can possibly provide insight into the pathogenesis of various diseases. This could also potentially, provide novel targets for treatment, since manipulation of cholesterol levels may affect the pathogenesis of various diseases.

OPSOMMING

Die volledige DNA volgorde bepaling van die mensgenoom is voltooi vroeg in die nuwe millennium. Slegs ongeveer 30 000 gene is geïdentifiseer, heelwat minder as wat in die verlede voorspel is, wat verantwoordelik is vir die genetiese diversiteit in die mens. Hierdie ontdekking het gelei tot 'n verandering in die benadering van navorsing ten opsigte van siektes, aangesien een geen 'n rol by verskeie siektes kan speel. Hierdie gewaarwording blyk veral waar te wees vir die lae digtheids lipoproteïen reseptor (LDLR) geen. Verskeie stimuli, buiten sterole, kan transkripie van die LDLR geen inisieer.

Verskeie siektes soos hipertensie is algemeen in die ontwikkelende wêreld, en dra by tot die hoë mortaliteit syfers. Die bevinding dat 'n promoter variant in die LDLR geen (-175g→t) geassosieer is met verhoogde diastoliese bloeddruk, kan moontlik verhoogde lipiedvlakke in hipertensiewe individue verklaar. Studies het aangetoon dat die verlaging van cholesterol, veral LDL-cholesterol, die voorkoms van hipertensie kan verlaag. Die –175 g→t variant is geleë in 'n *cis*-regulerende element wat na bewering 'n bindingsetel vir die Yin Yang (YY)-1 transkripsie faktor bevat asook sterk homologie met die cAMP respons element (CRE) toon, wat bind aan die Ca^{2+}- afhanklike transkripie faktor, CRE bindings proteïene (CREB). Die feit dat Ca^{2+} transkripsie van die LDLR geen kan inisieer, kan dalk tot 'n mate, 'n verklaring bied vir die assosiasie tussen die –175 (g→t) variant en verhoogde diastoliese bloeddruk.

Cholesterol is noodsaaklik vir verskeie prosesse soos apoptose, die instandhouding van selmembrane sowel as immuun funksies. Die –59 c→t mutasie in die sterol regulerende element 1 (SRE-1) van die LDLR geen vernietig binding van die sterol regulerende element bindingsproteïen (SREBP) aan SRE-1. SREBP word proteolities geaktiveer tydens apoptose

deur twee kaspases (CPP32 en Mch3) om cholesterolvlakke te induseer. Ons resultate impliseer dat die –59C/T mutasie, in herhaling-2 van die LDLR-geen promoter, apoptose kan inhibeer onder normale immunologiese toestande.

Aterosklerose kan beskou word as 'n immunologiese siekte, aangesien verskeie humorale en sellulêre immuun prosesse deur die verloop van die siekte waargeneem kan word. Die feit dat lipoproteïene beskermend kan wees teen infeksies, deur binding en lisering van virusse of kompeteer met patogene vir sellulêre reseptore, het aanleiding gegee tot 'n ondersoek na die potensiële rol van variasies in die promoter area van die LDLR geen in immuun funksie. Betekenisvolle verskille in alleel verspreiding vir die -124c→t variant (P=0.006) is waargeneem tussen asimptomatiese MIV-geïnfekteerde pasïente en individue met vinnige siekte progressie. *In vitro* studies het voorheen getoon dat die -124c→t 'n verhoging in LDLR geen transkripsie (160%) tot gevolg het. Dit is noemenswaardig dat 'n vroeë studie getoon het dat die menslike herpesvirus-6 (MHV6) transaktivering van die CD4 promoters deur 'n gedeeltelike CRE bindingsetel kan bewerkstellig. Beide CREB en YY1 kan virus en sellulêre promotors reguleer, en hierdie transkripsie faktore toon bindingshomologie met die FP2 element van die LDLR promotor

Die mutasie verryking van die LDLR geen promoter soos waargeneem in Suid Afrikaanse Swart en Kleurling populasies, kan moontlik lig werp op die patogenese van verskeie siektetoestande. Hierdie bevindinge kan potensieël nuwe teikens vir behandeling identifiseer, aangesien manipulasie van cholesterolvlakke 'n effek mag hê op die patogenese van verskeie siektes.

This thesis is dedicated to my parents, Frans and Martha Scholtz, for supporting me in all my endeavours even when they weren't comfortable with it. Their unconditional support has made every late night worth the effort.

All nature is but art unknown to thee;
All chance, direction that thou cannot see;
All discord, harmony not understood;
All partial evil, universal good;
And, spite of pride, in erring reason's spite,
One truth is clear; 'Whatever is, is right.'

Alexander Pope, An Essay on Man, 1289

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ABBREVIATIONS

µg	microgram
µl	microlitre
µM	micromoles per litre
ACE	angiotensin-converting enzyme
ACEH	acid cholesterol esters hydrolyse
AML	acute myelogenous leukaemia
ANOVA	analysis of variance
Apo	apolipoproteins
apo B	apolipoprotein B-100
APS	ammonium persiflage
ARP	Apolipoprotein regulatory element
BMI	body mass index
bp	base pair/s
CCB	calcium channel blockers
C/EBP	CCAAT/enhancer binding protein
cDNA	complementary deoxyribonucleic acid
CE	cholesterol esters
CHD	coronary heart disease
CHX	cyclohexamide
CML	chronic myeloid leukaemia
CRE	cAMP response element
CREB	cAMP response element binding protein
CVD	cardiovascular disease
Δ	deletion
dH_2O	deionized, distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ER	estrogen-receptor
ERK	extracellular signal-regulated kinase
FDB	familial defective apolipoprotein B-100

FH	familial hypercholesterolaemia
FP	footprinting
FSH	follicle stimulating hormone
HbloodM	high blood pressure medication
HDL	high density lipoprotein
HEX-SSCP	heteroduplex-single strand conformation polymorphism
HGF-1	hepatocyte growth factor-1
HIV	human immunodeficiency virus
HMG CoA	3-hydroxy-3- methylglutaryl coenzyme A
HNF	hepatocyte nuclear factor
HRE	hormone response element
HSV-1	human herpes simplex virus 1
IE	immediately early
IL-1	increased interleukin 1
kb	kilobase
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
Lp(a)	lipoprotein(a)
MAPK	mitogen activated protein kinase
MI	myocardial infarction
MAPK	mitogen-activated kinases
ml	millilitre
mM	millimoles per litre
mmol/l	millimoles per litre
NCEH	neutral cholesterol esters hydrolase
OM	Oncostatin M
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PKC	protein kinase C
pmol	picomole
RNA	ribonucleic acid
SIRE	sterol independent regulatory element
SREBP	sterol regulatory element binding protein
SSCP	single-strand conformation polymorphism

TACO	tryptophane aspartate-containing coat protein
TBP	TATA-binding protein
TC	total cholesterol
TEMED	N,N,N',N'-tetramethylethylenediamine
TG	triglyceride
TNF∝	tumor necrosis factor alpha
VLDL	very low density lipopotein
VNTR	variable number of tandem repeats
YY1	Yin Yang 1

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CHAPTER 1

1 CHOLESTEROL

Cholesterol is one of the most important substances in the human body and is essential for various cellular processes, including maintaining the integrity of the cell membrane (Brown and Goldstein, 1999), immune function (Feingold and Grunfeld, 1997) and apoptosis (Härtel, 1998). Cholesterol is transported in the form of lipoproteins, of which low density lipoprotein (LDL) is the major cholesterol carrying lipoprotein in human plasma. Various other lipoproteins such as high density lipoprotein (HDL), very low density lipoprotein (VLDL) and lipoprotein (a) [Lp(a)] have been identified. Cholesterol homeostasis is obtained through receptor-mediated endocytosis (exogenous) and / or the cholesterol biosynthetic pathway (endogenous). Maintenance of cholesterol levels is extremely important since too little or too much can have detrimental effects. Low cholesterol levels have been associated with chronic respiratory and gastrointestinal diseases as well as cancer (Muldoon et al, 1997), while high cholesterol levels are strongly associated with an increased risk for heart disease (Ross et al, 1999). In the human body, cholesterol homeostasis is maintained through a feedback regulatory system, which is modulated through membrane-bound transcription factors called sterol regulatory element binding proteins (SREBPs) (Brown and Goldstein, 1997).

1.1 THE ROLE OF CHOLESTEROL IN CELL MEMBRANE INTEGRITY

A fine balance between the cholesterol levels and amount of unsaturated and saturated fatty acids in phospholipids maintains the integrity of cell membranes (Brown and Goldstein, 1999). Devaux (1991) indicated that cholesterol is essential for modulating fluidity and phase transitions in the plasma membranes of animal cells. It has recently been shown that cholesterol together with sphingomyelin forms membrane rafts of caveolae that are sites

where signalling molecules are concentrated (Simons and Ikonen, 1997; Anderson, 1998). For optimal efficacy cholesterol homeostasis needs to be maintained at all costs. This is achieved by sterol regulatory element binding proteins (SREBP's) which are proteolytically cleaved from the membrane in order to be functionally active (Brown and Goldstein, 1997).

1.2 ROLE OF CHOLESTEROL IN INFECTION AND IMMUNE FUNCTION

Recent studies have indicated that cholesterol and the lipoprotein system play an important role in viral transport (Phalen an Kielen, 1991) and innate immunity (Feingold et al, 1997). Various viruses (like alpha viruses) enter the cell through receptor-mediated endocytosis, while cholesterol forms an essential part of infection for some viruses (Phalen and Kielian, 1991; Bernardes et al, 1998; Agnello et al, 1999; Gatfield and Pieters, 2000; Coppens et al, 2000). Interestingly, Hsu and colleagues (1995) demonstrated that the human herpes simplex virus 1 (HSV-1) infection of arterial smooth muscle cells may alter cholesterol trafficking, leading to accumulation of cholesterol esters (CE). They furthermore showed that HSV-1 infection (a) increased LDL binding and uptake, LDL receptor mRNA steady state and gene transcription; (b) increased CE synthesis and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity but reduces CE hydrolysis and cholesterol efflux; (c) decreased both lysosomal and cytoplasmic CE hydrolytic [acid CE hydrolyse (ACEH) and neutral CE hydrolase (NCEH)] activities, where the latter enzyme is PKA-sensitive; and (d) reduced PKA activity after infection. Gatfield and Pieters (2000) indicated that cholesterol is essential for the cellular uptake of mycobacteria and also for the phagosomal association of TACO (tryptophane aspartate-containing coat protein), which prevents degradation of mycobacteria by lysosomes. It has also been reported that multiple viruses use the LDL

receptor for cellular entry, such as Rous sarcoma virus A (Bates et al, 1993), human rhinovirus (Hofer et al, 1994) and hepatitis C virus (Agnello et al, 1999).

Infection and inflammation induce a wide array of protective metabolic changes (the acute phase response) which is mediated by cytokines, primarily at the level of gene transcription. Various studies have shown that lipoproteins bind, neutralise and compete with certain pathogens (Owens et al, 1990; Xu et al, 1993; Hofer et al, 1994). It has been demonstrated that certain viruses compete with LDL for LDL receptor entry into the cell (Hofer et al, 1994), thus elevated LDL levels in certain species may compete with viruses and may play a protective role. Apolipoproteins (apo), like apo A-I, have been shown to neutralise several viruses, including the human immunodeficiency virus (HIV) by inhibiting virus-induced cell fusion (Owens et al, 1990). LDL has also been shown to bind certain viruses, after which the LDL is oxidised, attracting macrophages, which may kill the organism (Xu et al, 1993). Human HDL has the ability to lyse certain pathogens and it has been shown that by increasing serum HDL (by over-expressing Apo A-I) after endotoxin treatment, survival is improved (Levine et al, 1993).

1.3 THE ROLE OF CHOLESTEROL IN APOPTOSIS

Apoptosis is a physiologically regulated process essential in the development and homeostasis of metazoan animals (Gerschenson and Rotello, 1992; Barinaga, 1994; Steller, 1995). Apoptosis is characterised by the formation of large membrane proturbances, also known as "zeiosis" (Stacey et al, 1985) and cell shrinkage (Cohen et al, 1992). The most typical feature at the molecular level is chromatin condensation and DNA degradation (Hotz et al, 1994). The apoptotic bodies shed during apoptosis are phagocytised by macrophages (Savill, 1997).

Apoptosis differs from the pathological process of necrosis, which can be triggered by various processes including hyperthermia, hypoxia, ischaemia, complement mediated metabolic poisons and direct cell trauma (Schwartzman and Cidlowski, 1993) (Figure 1).

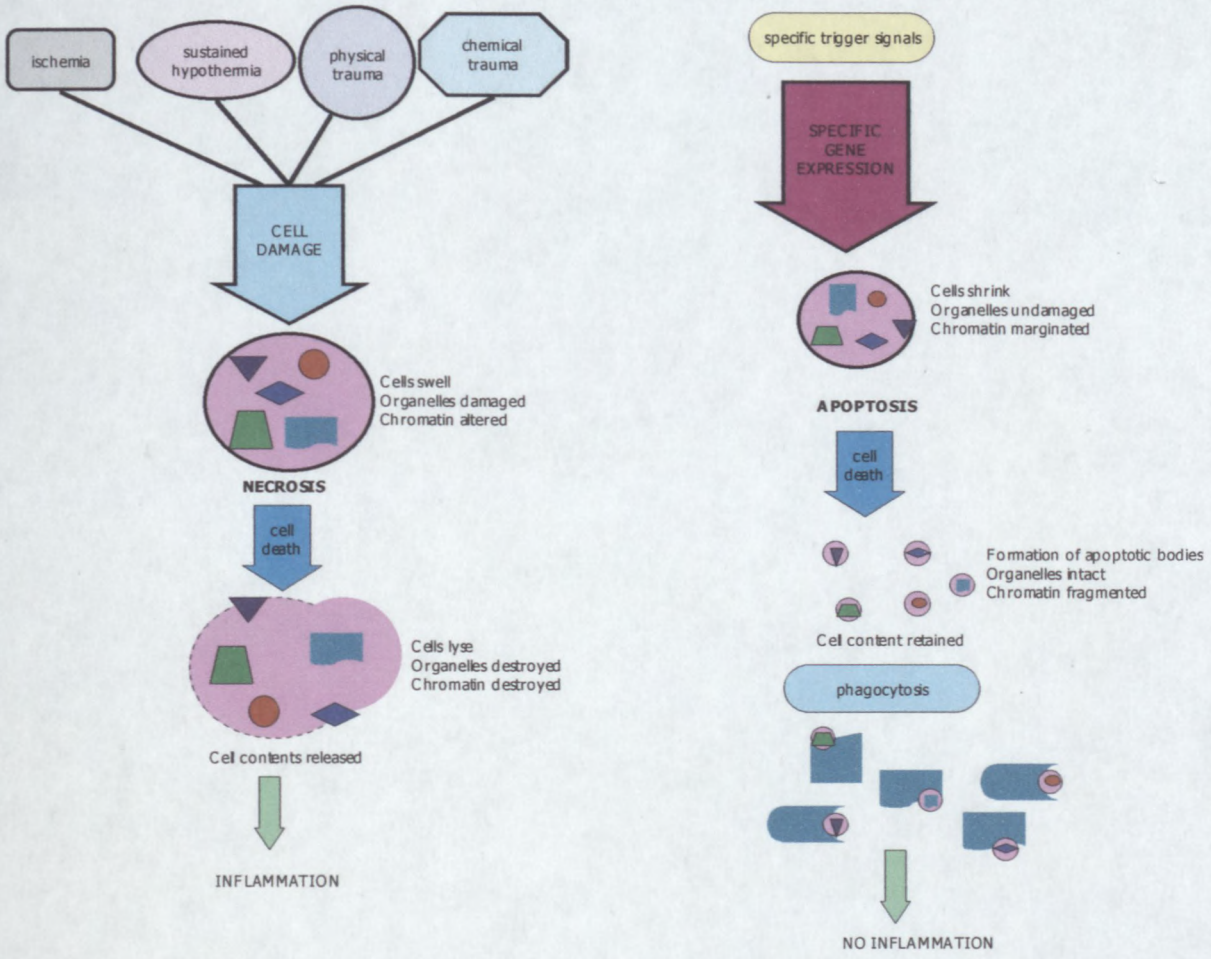

Figure 1: Schematic presentation of the differences between the process of necrosis vs. apoptosis.

In the cell death cascade, where multiple proteins bring about apoptosis, a family of 10 cysteine proteases, called caspases, form an integral part in initiating this process (Voss and Cotton, 1998; Alnemri et al, 1996).

Cholesterol forms an essential part of the cell cycle and its role in apoptosis is well established. Cholesterol homeostasis, as previously mentioned, is under strict control of a feedback regulatory system modulated by SREBPs (Goldstein et al, 1995). SREBPs regulate

transcription of genes encoding the LDL receptor and multiple enzymes involved in the cholesterol and fatty acid biosynthetic pathways (Brown and Goldstein, 1998). SREBPs are proteolytically activated in a two-step cleavage process dependent on cellular cholesterol concentrations (Brown and Goldstein, 1997).

During apoptosis, SREBP's are proteolytically cleaved, irrespective of sterol status, by two members of the caspase family (CPP32 and Mch3), which share 54% homology, but differs in pH (Wang et al, 1996; Pai et al, 1996). The mevalonate pathway (responsible for endogenous cholesterol synthesis) provides isoprenoids for the cell cycle and farnesyl for $p21^{ras}$ activity (Goldstein and Brown, 1990). Interestingly, inhibition of this pathway triggers apoptosis in a wide variety of cells (Padayatty et al, 1997; Clutterbuck et al, 1998; Choi et al, 1999). It thus seems unlikely that the activation of SREBP during apoptosis is to provide isoprenoids and farnesyl, but rather for the provision of cholesterol to maintain plasma membrane integrity (Härtel et al, 1998).

2 REGULATORY REGIONS

Regulation of transcription is an essential prerequisite for homeostasis and considering the complex nature of the eukaryotic genome, unmitigated initiation of genes can be detrimental. The initial predictions formed with regard to the molecular structure and genomic organisation of eukaryotic genomes were obtained from investigating prokaryotes (Ringe, 1992). Although great similarities exist between eukaryotic and prokaryotic genes, expression and regulation of eukaryotic genes demands a more complex approach. A basic rule in eukaryotic systems is that, in general, if a cell does not require a gene product, that gene would not be transcribed (Ringe, 1992). The expression of genes are dependent, in part, on the

combination of both cellular and environmental triggers and the underlying interaction between the signals (Pugh, 2000). The regulatory domains of genes are called promoters, and can encompass several hundred to several thousand base pairs of DNA (Arnone et al, 1997).

2.1 Basic structure

Transcription of genes is dependent on various enzymes, of which the RNA polymerases are the most important for the transcription of the different types of RNA in cells. Given the intricate organs and specialised cells of eukaryotes three different polymerases exist, each making a different kind of RNA (messenger RNA, ribosomal RNA, transfer RNA) (Ringe, 1992). Each RNA polymerase recognises a different promoter type, of which RNA polymerase II promoters are the most well-known.

Promoters recognised by RNA polymerase II are very similar to prokaryotic promoters and contains various binding sites for gene-specific regulatory proteins as well as a core, composing of a TATA box (an A-T rich sequence) and / or initiator elements close to the transcription initiation site (Ringe, 1992; Pugh, 2000). Although the level of transcription relies heavily on the transcriptional activators triggered at certain times, the general transcription machinery assembles over the core promoter and transcription is initiated at the initiator. Binding of TATA-binding proteins (TBP) to the TATA box, seems to be essential for the activation and / or deactivation of transcription (Pugh, 2000). After the binding of various enhancers and the formation of the TBP complex, RNA polymerase (pol) II is recruited to initiate transcription. Once homeostasis is restored, inhibitors are activated to compete with the RNA pol II to deactivate transcription (Pugh, 2000).

Within promoter regions there can be dozens of regulatory elements of various kinds which act as binding sites for distinct transcription factors (Arnone et al, 1997). Interestingly, the presence of a particular regulatory element in a promoter does not reveal much about its influence on the expression of a given gene. There appears to be little logic in the organisation of regulatory elements and even less in the way they interact to regulate gene expression. The same regulatory element can activate transcription in one promoter while it represses expression in another. To decipher how various regulatory elements within a promoter work, extensive experimental analyses are needed, which is not always predictable. There are apparently many ways to switch a gene on or off or to modulate transcription of a given gene depending on the stimulus and the cell type.

A detailed description of the promoter regions of the LDLR and apolipoprotein (apoB) genes is given below, since the present study focuses mainly on the possible role of LDL or LDL-cholesterol in diseases that represent major health problems in the local population. Mutations in these genes may underlie the familial hypercholesterolaemia (FH) phenotype, a condition that has been a focus of research in South Africa ever since it was recognised as a major cause of cardiovascular disease (CVD) in the Afrikaner population of European ancestry. The high prevalence of FH in this population, shown to be due to a founder effect (Kotze et al, 1991), is in striking contrast to the apparently low prevalence of this disease in Africans. The detection several different polymorphisms in the promoter region of the LDLR gene in populations of African origin, whilst apparently absent in Caucasians (Appendix A-C), raised the possibility that some of these alleles may interact with other mutations in the LDLR gene, thereby modifying clinical expression of FH.

2.2 LDLR gene promoter

Low density lipoprotein (LDL) (the most important cholesterol-carrying lipoprotein in human plasma) and the LDL receptor (LDLR) play a pivotal role in the clearance of LDL cholesterol from circulation in the whole body through receptor-mediated endocytosis (Brown and Goldstein, 1986). The LDLR gene is located on the short arm of chromosome 19 (p13.1-p13.3) (Yamamoto et al, 1984; Südhof et al, 1987) and mutations in this gene underlie familial hypercholesterolaemia, a condition characterised by elevated cholesterol levels (Goldstein et al, 1995). Regulation of the LDLR gene is mediated by sterols through a negative feedback mechanism; transcription is initiated when cellular sterol levels are low and down regulated when sufficient sterols are present.

Figure 2 illustrates the essential regulatory region of the LDLR gene that encompasses three direct imperfect repeats (16 bp in length) and two TATA-like sequences (7bp in length), located within 200bp upstream of the transcription initiation site (Goldstein et al, 1995). Repeats 1 and 3 bind Sp1, a transcription factor essential for basal transcription of the LDLR gene (Südhof et al, 1987; Dawson et al, 1988). Repeat 2, designated the sterol regulatory element (SRE-1), is essential for high levels of transcription in the absence of sterols, while sterol-mediated repression is also achieved through this region (Smith et al, 1990; Briggs et al, 1993; Koivisto et al, 1994). Interestingly, two additional *cis*-acting elements (designated footprinting 1 (FP1) and footprinting 2 (FP2), respectively) have also been identified upstream of the three repeats and is thought to be essential for maximal induction of transcription (Mehta et al, 1996). Dhawan and colleagues (1997) proposed a possible interaction between the FP1 and SRE-1 sites. *In vivo* evidence for this possible effect was

observed in an individual (with normal cholesterol levels) reported to have a LDLR promoter variant in both these elements (Scholtz et al, 1999). Variant -59 (C/T) significantly decreased LDLR transcription, while variant -124 (C/T) markedly increased LDLR gene expression *in vitro*.

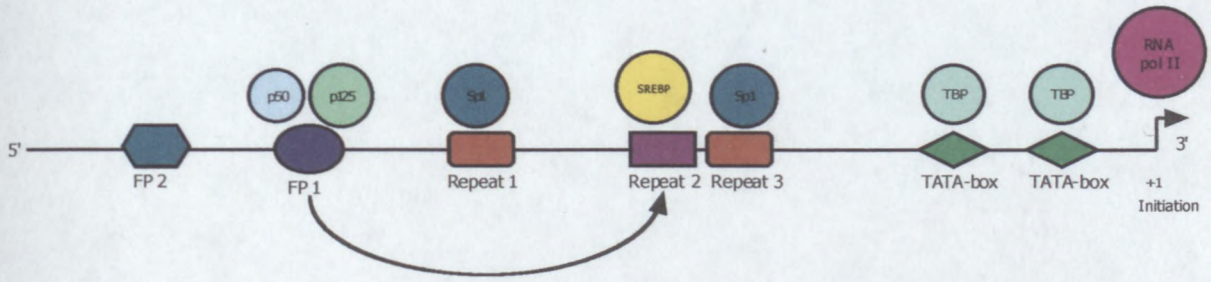

Figure 2: A graphic presentation of the basic cis-acting elements and transcription factors of LDLR gene promoter active in sterol-mediated regulation. Repeat 1 and 3 bind Sp1, while repeat 2 contains the sterol regulatory element 1 (SRE-1) which bind sterol SRE-binding proteins (SREBPS). Footprinting 1 and 2 (FP1 and FP2) are newly defined elements. The arrow connecting FP1 and SRE-1 is indicative of the suggested interaction between the two elements.

Although the mode of interaction between the SRE-1 and FP1 sites is still undefined, Dhawan and colleagues (1997) demonstrated, using protein-binding assays, that two unknown nuclear proteins bind to the FP1 site. They furthermore speculated that these proteins, which they designated p50 and p125 (due to their respective sizes), could be part of a family of transcription factors which recognise and bind identical DNA sequences, or that p50 could be the proteolytically active form of p125. This report further demonstrated that p125 appears to be relevant to LDLR gene transcription since mutations at the crucial sequences in FP1 (positions -135/-136) abolished binding of this protein as well as FP1 induced transcription of the gene.

Although the importance of the LDLR gene in cholesterol metabolism as well as sterol-mediated regulation of the gene is well described, recent reports indicated that this only skimmed the surface of the LDLR gene in the overall homeostatic picture. The LDLR gene has been shown to play an important role in the inflammatory response, in such a manner that

it can be considered a primary response or immediate early response gene (Makar et al, 1994; Dhawan et al, 1999). Interestingly, Cuthbert and Lipsky (1990) showed that among the cellular processes initiated upon mitogen activation, an increase in LDLR gene expression could also be observed, irrespective of ambient sterols. This finding therefore implies a role for stimuli, other than sterols, which is important for cellular activation in regulation of the LDLR gene. Various reports have provided evidence that the LDLR gene can also be induced in a sterol independent fashion (Makar et al, 1994, 1998, 2000; Liu et al, 2000).

2.2.1. Cytokines

The finding that various stimuli can induce transcription of the LDLR gene, both independent and dependent on sterol levels, has made it important to determine which essential regulatory elements are important for gene transcription. Liu and colleagues (2000) identified a sterol independent regulatory element (designated SIRE) in the LDLR gene, involved in cytokine (oncostatin M (OM)) induced transcription. This region spanning position -17 to -1 overlapped with the previously described TATA-like element and contained an activator CCAAT / enhancer binding protein (C/EBP) element (-17 to -9) and a cAMP response element (CRE) (-8 to -1). Of interest is the fact that the proximal Sp1 binding site is dispensable, but the binding of a strong transcriptional activator in a location proximal to the SIRE is necessary for this element to function as an independent *cis*-acting element to activate LDLR gene transcription. Another cytokine, tumor necrosis factor alpha (TNFα), have been reported to induce transcription of the LDLR gene (Harada et al, 1990). Hamanaka et al (1992) also showed that apart from induction of LDLR gene transcription, TNFα also increased interleukin 1 (IL-1), another cytokine which increases LDLR gene transcription. Although the mechanism responsible for TNFα-mediated induction of the gene is still unclear, the increase in Sp1 gene expression and enhanced binding of Sp1 to the binding sites,

might confer a possible modus operandi for gene activation (Hamanaka et al, 1992). Various other cytokines including interleukin 1β (IL-1β) (Stopeck et al, 1993) and interleukin 6 (IL-6) (Ruan et al, 1998) have been reported to similarly induce transcription of the LDLR gene, each through different mechanisms. TNFα and IL-1β seem to be sterol dependent (Stopeck et al, 1993) while OM and IL-6 stimulation appear to be sterol independent (Liu et al, 1997; Gierens et al, 2000). Gierens and colleagues (2000) furthermore demonstrated that the mechanism by which IL-6 stimulate LDLR gene transcription is through the activation of nuclear factors binding to the SRE-1 and the Sp1 site in repeat 2 and repeat 3, respectively. The mechanism by which IL-1β induce transcription of the LDLR gene is through activation of extracellular signal-regulated kinase (ERK), a subfamily of the mitogen activated protein kinase (MAPK) cascade (Kumar et al, 1998). Most of these cytokines have mitogenic activities and their effect on LDLR gene transcription might be due to their mitogenic action (Hamanaka et al, 1992).

2.2.2. Hormones and growth factors

Apart from cytokines, stimulation of the LDLR gene by hormones and growth factors has also been reported. The hormone-sensitive region of the LDLR gene has been located between positions –69 to –36, which contains the SRE-1 and Sp1 regulatory sites (Streicher et al, 1996). This report demonstrated that insulin and insulin-like growth factor 1 (IGF-1) induce transcription of the LDLR gene via the SRE-1 site through SREBP-1 / –2 binding. Interestingly, platelet-derived growth factor (PDGF) also activated transcription of the LDLR gene through this region (Mazzone et al, 1989), although the molecular mechanism appears to be different. Roth et al (1991) furthermore demonstrated that although PDGF seems to stimulate transcription of the 3-hydroxy-3-methylglutaryl-CoA (HMG-Co) reductase gene through protein kinase C (PKC- a family of 10 closely related isotypes involved in

transmembranous signal transduction), its effect on LDLR gene transcription appears to be independent of PKC. Basheerudin et al (1995) illustrated that PDGF activation enhanced binding of Sp1 to the LDLR gene, independent of new Sp1 protein synthesis.

Other hormones, like estrogens, have important cardio-protective properties through beneficial effect on lipids and lipoprotein metabolism (Walsh et al, 1991; Lobo, 1991; Hong et al, 1992; Samsioe, 1994; Grodstein and Stampfer, 1995). Estrogens have been shown to increase clearance of LDL (Walsh et al, 1991) and lower plasma LDL levels (Walsh et al, 1991; Lobo, 1991; Nabulsi et al, 1993; Samsioe, 1994). Croston et al (1997) recently illustrated that estrogen may upregulate transcription of the LDLR gene in an estrogen-receptor (ER)-dependent manner, even though the molecular mechanism is undefined. Recently, the molecular mode of operation were elucidated by Li et al (2001), who demonstrated that ER-mediated transcription occurs through interaction with Sp1 and mutations inhibiting Sp1 binding may abolish ER-mediated activation. This study further showed that ER enhanced binding of Sp1-repeat 3 complexes. Although SRE-1 was not directly involved in ER activation, it does seem to be important for optimisation of estrogen's effect on the LDLR gene, suggesting interaction between the Sp1-ER complex and the SRE-1 site (Li et al, 2001). Other growth factors and hormones, comprising epidermal growth factor 1 (EGF-1) (Graham and Russel, 1994), hepatocyte growth factor-1 (HGF-1) (Pak et al, 1996) as well as follicle stimulating hormone (FSH) (LaVoie et al, 1999), also affect LDLR gene transcription irrespective of sterol status.

2.2.3. MAPK and protein synthesis inhibitors

Mitogen-activated kinases (MAPK) are important in mediating cellular responses to various extracellular stimuli (Marshall, 1995; Seger et al, 1995; Treisman, 1996; Robinson and Cobb,

1997). The MAPK cascade comprises protein kinases, which are important for cell proliferation and play a role in both endorsing (p46/54^{JNK} and p38^{MAPK}) and contesting (p42/44^{MAPK}) apoptosis (Robinson et al, 1997). Of interest to this study is the fact that these protein kinases may phosphorylate and regulate the activities of several transcription factors, including cyclic AMP responsive element binding proteins (CREB) which regulate transcription of numerous immediately early (IE) response genes (Cano and Mahadevan, 1995; Hazzalin et al, 1998). Certain members of the MAP kinases (p46/54^{JNK} and p38^{MAPK}), and their subfamily members, are strongly activated in response to stress stimuli. Due to its importance in various cellular processes, the MAPK cascade is under tight control by MAPK kinase kinases and MAPK kinases (Marshall, 1995;Waskiewicz and Cooper, 1995). Interestingly, anisomycin, the most potent protein inhibitor, has the ability to specifically activate p46/54^{JNK} and p38^{MAPK}, as well as induce transcription of the LDLR gene through various mechanisms (Dhawan et al, 1999). Since anisomycin has the ability to act as a stimulant for various signal transduction pathways as well as inhibit translation, Dhawan and colleagues (1999) set out to determine whether anisomycin-induced transcription is due to translational arrest. This study illustrated that the effect of anisomycin could not be due to translational arrest because other protein synthesis inhibitors such as cyclohexamide (CHX) and puromycin did not produced these effects, and induction of transcription was also observed at anisomycin levels below that required for efficient inhibition of protein synthesis. It was further demonstrated that a mild activation of the p42/44^{MAPK} cascade could increase transcription of the LDLR gene (Dhawan et al, 1999). It is important to bear in mind that apart from anisomycin numerous extracellular signals like cytokines (IL-1β; TNFα) (Kumar et al, 1998) employ the p42/44^{MAPK} cascade to upregulate the LDLR gene (Dhawan et al, 1999). Of note is the finding that the p38^{MAPK} pathway also seems to affect the transcription of the LDLR gene (Kumar et al, 1998). Kotzka and colleagues (1998) also demonstrated that the

effect of insulin and PDGF on LDLR gene transcription is linked to the MAP kinase cascade. These and other reports indicate a critical role for the MAP kinase cascade in LDLR gene response.

2.2.4. ACE-inhibitors and CCB's

Angiotensin-converting enzyme (ACE) inhibitors and calcium channel blockers (CCB's) also appears to have cardio-protective abilities through its efficacy in lowering blood pressure and upregulation of LDLR gene transcription in a protein kinase C (PKC)-dependent manner (Block et al, 1993). Interestingly, its been shown that CCB's slightly reduce circulating cholesterol concentration in plasma and can therefore correct disturbances of cholesterol metabolism at the cellular level (Etingin and Hajjar, 1990). Similarly, the positive transcriptional effect of ACE-inhibitors can possibly explain its beneficial role in organ protection (Ambrosioni et al, 1987). Even though Block et al (1993) showed that CCB's and ACE-inhibitors increase binding and internalization of extracellular LDL-cholesterol esters, even at therapeutic concentrations (nanomolar), they failed to block transcription of the HMG-CoA reductase gene, excluding a relevant influence on cellular cholesterol biosynthesis. It is also interesting that different CCB's use different signal transduction pathways to upregulate the LDLR gene (Ruan et al, 1999). It was shown that the calmodulin pathway is commonly used for upregulation of the LDLR gene through diltiazem and verapamil, while the tyrosine kinase and PKC signal transduction pathway also seem to be involved in induction by verapamil. Differential effects were also observed between nifedipine and the two CCB's (diltiazem and verapamil) on LDLR gene expression and protein uptake. Nifedipine appeared to have an inhibitory effect on LDLR mRNA production and binding. Ma and colleagues (1986) also provided evidence that the HMG-CoA reductase

inhibitor mevinolin, induced transcription of the LDLR gene thereby lowering circulating plasma cholesterol levels.

2.2.5. YY1

Yin Yang 1 (YY1) is a multifunctional protein which act as an activator, repressor or initiator of transcription of both cellular and viral genes (Shi et al, 1997). YY1 binds to a core CCAT or ACAT motif and shows substantial heterogeneity in the flanking nucleotides. In 1999 Ericsson and colleagues illustrated that YY1 repress transcription of three SREBP-responsive genes, even though they failed to demonstrate the mechanism of repression of YY1 on the LDLR gene. Bennett and co-workers (1999) however, demonstrated that repression of the LDLR gene by YY1 seems to be independent of direct binding by YY1 to the LDLR promoter region. This study indicated that YY1 interacts with Sp1 in solution and the same region utilised for YY1-Sp1 interaction is also needed for interaction between Sp1 and SREBP. Thus, the specific interaction between Sp1 and SREBP, which upregulate transcription of the LDLR gene, is specifically targeted by YY1 for inhibition of transcription. It is of interest that YY1 can similarly interact with cAMP response element binding protein (CREB) to repress transcription in several genes (Zhou et al, 1995).

2.3 Apolipoprotein B gene promoter

Apolipoprotein (Apo) B is an important component of all lipoproteins involved in atherogenesis, including LDL and lipoprotein (a) (Young et al, 1990). The two forms of apoB (apoB-48 and apoB-100) are encoded by the same gene which has been localised to chromosome 2 (Lusis et al, 1985; Law et al, 1985; Knott et al, 1985; Deeb et al, 1986; Glickman et al, 1986). ApoB-100 is synthesised exclusively by the liver where it plays an

important role in the assembly of various lipoproteins, including LDL, while ApoB-48, synthesised by the gut, is necessary for the assembly of chylomicrons (Kane et al, 1980; Havel and Kane, 1989). The only difference between ApoB-100 and ApoB-48 molecules is that the ApoB-48 is produced by a unique RNA process where the apoB protein is truncated (Powell et al, 1987; Chen et al, 1987). Mutations in the ApoB gene can lead to abnormally high or low apoB and LDL cholesterol levels, depending on the mutation type (Young et al, 1990). ApoB is essential for the clearance of LDL cholesterol from plasma as well as receptor-mediated endocytosis. Elevated cholesterol (LDL levels), as seen in familial hypercholesterolaemia, is commonly due to defective receptor mediated uptake of LDL. Since apoB is the ligand mediating LDL binding to the receptor, similar clinical features can be expected if genetic abnormalities are present in the apoB gene. Familial defective apolipoprotein B-100 (FDB), a condition mimicking these clinical features, have been reported and the genetic abnormality underlying this disease, characterised (Vega and Grundy, 1986). The mutation at amino acid 3500, which changes a glutamine residue to an arginine, were consistently found in individuals showing clinical features similar to FH, but without mutations in the LDLR gene (Soria et al, 1989; Innerarity et al, 1990). To date quite a few mutations have been identified which causes hypobetalipoproteinemia, a syndrome characterised by abnormally low apoB and LDL cholesterol levels (summarised by Farese et al, 1992)

It has been shown that the region 5kb upstream and 1.5kb downstream of the apoB gene is sufficient for hepatic expression, while an additional 315 bp intestinal enhancer is necessary for intestinal expression (reviewed in Zannis et al, 2001). Transcription factors essential for regulation of the apoB gene include the CCAAT enhancer binding protein (C/EBP), hepatocyte nuclear factor-3 (HNF-3), hepatocyte nuclear factor-4 (HNF-4) and various other

nuclear factors, which bind to the proximal and intestinal enhancers. Nucleotides -150 to +124 can initiate transcription of the ApoB gene in hepatic and intestinal cells *in vitro*, but not *in vivo* (Kardassis et al, 1990; Kardassis et al, 1991; Brooks et al, 1994). Through *in vitro* mutagenesis, Kardassis and colleagues (1991) demonstrated that the nucleotide region -112 to -95, which binds HNF-3, is essential for transcription. This region also contains a hormone response element (HRE) which binds orphan (ARP-1 and HNF-4) and ligand-dependent nuclear receptors in the -86 to -62 region and C/EBP in the -72 to -54 region. Mutations abolishing DNA-protein binding reduce transcription of the apoB gene significantly (Kardassis et al, 1991). The proximal promoter contains various additional C/EBP binding sites that do not affect transcription to a similar extent (Figure 3). Interestingly, Brooks and co-workers (1991) demonstrated that intron 2 (+621 to +1064) has a 3-5 fold enhancing effect on the strength of the ApoB promoter. This study furthermore demonstrated that the inclusion of the second enhancer region was sufficient for liver-specific but not intestinal-specific expression of the ApoB gene (Brooks et al, 1991). The region -3067 to -2736 contains a silencer element, which have been shown to suppress the ApoB promoter in CaCo2 cells but not HepG2 cells (Paulweber et al, 1993). This region binds C/EBP, ARP-1 and HNF-4. Ladias and Karathanias, (1991) illustrated that ARP-1 and HNF-4 share sequence homology and have shared DNA binding specificity. When these two elements recognise the same binding site, ARP-1 inhibits HNF-4 mediated transcription (Ladias et al, 1992), but when binding to unique sites, ARP-1 has the capacity to enhance the activity of HNF-4 through protein-protein interaction (Ktistaki and Talianidis, 1997; Kardassis et al, 1998). The intestinal enhancer was localised within 315 bp approximately 57 kb upstream of the ApoB gene (Antes et al, 2000). This region contains binding sites for liver-specific transcription factors (C/EBP beta, HNF-3 beta, HNF-4), and can upregulate transcription of a minimal promoter in the absence of these transcription factors (Figure 3).

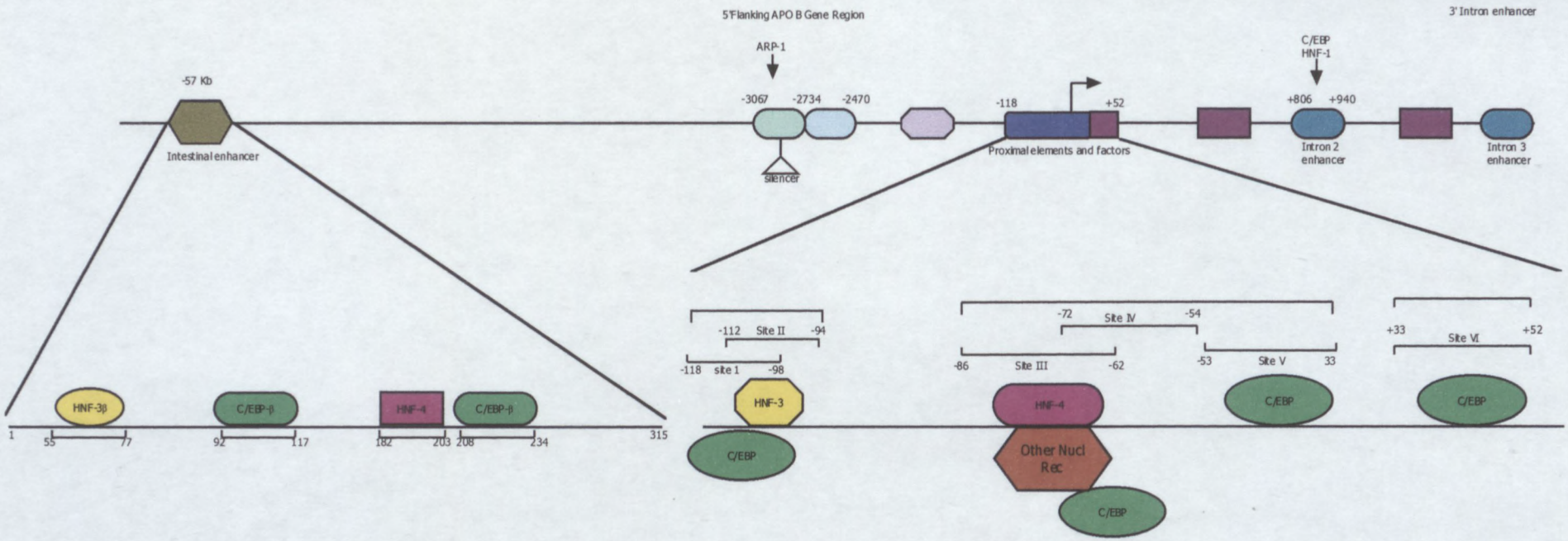

Figure 3: Schematic presentation of the regulatory region of the ApoB gene (adapted from Zannis et al, 2001). HNF,hepatocyte nuclear factor; C/EBP, CCAAT / enhancer binding protein; ARP, apolipoprotein regulatory protein

3. OBJECTIVES

3.1. To investigate the potential role of LDLR promoter polymorphisms in the aetiology of various diseases where lipoprotein metabolism may be involved.

It has recently been suggested that a promoter variant at nucleotide position –175 (g→t) of the LDLR promoter may contribute to the phenotypic expression of familial hypercholesterolemia (FH) in Black patients (Thiart et al, 2000/Appendix B). Since family studies indicated that this variant is not responsible for the FH phenotype on its own, it seems plausible that it may jeopardises the ability of carriers to handle certain metabolic stresses, thereby contributing to disease risk in genetically predisposed subjects.

3.2. To determine the possible role of the –59 c→t mutation in repeat 2 of the LDLR gene in apoptosis

Cholesterol is very important in maintaining cell membrane integrity, which is essential for apoptosis. Scholtz et al (1999/Appendix A) identified a mutation at nucleotide position –59 (C/T) of the LDLR promoter, which markedly reduce transcription of the LDLR gene. This variant is located within the sterol-regulatory element-1 (SRE-1), which is important for sterol-mediated regulation of the LDLR gene (Dawson et al, 1988; Smith et al, 1990). Mutations at position –59 of the LDLR gene promoter have been shown to abolish binding of SREBP to the SRE-1 site (Smith et al, 1990). SREBP, which bind to SRE-1, plays an important role in the regulation of various pro- and anti-apoptotic genes. This prompted the investigation of the effect of the naturally occurring –59 c→t mutation on apoptosis.

3.3. To assess the significance of lipoproteins in host defense

It has recently become apparent that lipoproteins play an important role in innate immunity (Feingold and Grunfeld, 1997). Infection and inflammation induce a wide array of protective metabolic processes, which is mediated by cytokines. The induction by cytokines is primarily at the level of gene transcription. The LDLR gene can be considered an immediate early (IE) or primary response gene and it has been reported that various stumuli, other than sterols, can induce transcription of the gene (Makar et al, 1994; Dhawan et al, 1999). This has prompted the screening of subjects infected with human immunodeficiency virus (HIV) for variation in the LDLR promoter, to determine the possible significance of variation in this gene region in the pathogenesis of infectious disease.

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CHAPTER 2

Association of an African-specific variant (-175g→t) in a *cis*-acting regulatory element of the LDL receptor promoter with diastolic blood pressure

Charlotte L. Scholtz[1], Christiaan F. Hoogendijk[1], Krisela Steyn[2], Carl J. Lombard[3], H Van Jaarsveld[4], Maritha J. Kotze[1]

[1]MRC Cape Heart Group, Division of Human Genetics, Faculty of Health Sciences, University of Stellenbosch, Tygerberg, [2]Centre for Epidemiological Research, Medical Research Council, Tygerberg, South Africa and [3]Biostatistics Unit, Medical Research Council, Tygerberg, [4]Department of Chemical Pathology, University of Free State, Bloemfontein, South Africa.

Abstract

A –175 g→ variant was recently identified in the footprinting 2 (FP2) regulatory element of the low density lipoprotein receptor (LDLR) gene promoter. In an attempt to define the possible phenotypic effect of this variant, mutation analysis was performed on DNA of 414 South African individuals, on whom full clinical, biochemical and physiological parameters were available. In order to minimise confounding effects that may result from population substructures (due to difficulties in selecting matched cases and controls), the study population was recruited from the general Coloured population (Mamre community). Subjects with the

-175g→t variant (51/414) presented with a significantly increased diastolic blood pressure (difference 4.2 mmHG, 95% CI: 1.0 to 7.4 mmHG) compared with those who tested negative ($p<0.01$). Although the polymorphism was not associated with systolic blood pressure, the direction of the estimated effect (difference 3.7 mmHG, 95% CI: -1.7 to 9.0 mmHG) was close to that observed in the diastolic blood pressure regression model. Previous haplotype studies have largely excluded the possibility that these findings may be related to association with another mutation or gene, since the -175t allele occurs on different chromosomal backgrounds. The significant association detected between the apparently African-specific variant -175g→t and diastolic blood pressure therefore suggest that variation in the LDLR promoter may predispose individuals with an African genetic background to hypertension, or may reflect the inability of carriers to handle certain metabolic stresses.

Introduction

African populations in many developing countries are experiencing rapid urbanisation characterised by a double burden of disease in which noncommunicable diseases become more prevalent and infectious diseases remain undefeated (Vorster et al, 1999). While the rates of coronary heart disease (CHD) remain relatively low in the Black population of South Africa, high rates of hypertension, stroke, and obesity are observed in this group (Walker et al, 1993). Since disease risk would be significantly increased in genetically susceptible individuals, genetic studies aimed at disease prevention should be focussed on identification of mutations that may be of relevance in the process of nutritional transition. Genes involved in lipoprotein metabolism, such as those underlying familial hypercholesterolaemia (FH), may represent good candidates for such studies due to the finding that the frequency and spectrum of mutations in the low-density lipoprotein receptor (LDLR) gene contribute significantly to ethnic differences in disease risk (Loubser et al, 1999; Thiart et al, 2000).

Although more than 600 mutations underlying FH have been identified in the coding region of the LDLR gene (Hobbs et al, 1992, Day et al, 1997, http://www.ucl.ac.uk/fh ; Varret et al, 1998, http://www.umd.necker.fr), promoter variants appear to be rare (Top et al, 1992). Recently, several sequence changes have been identified in the promoter region of the LDLR gene in African populations, whilst apparently absent in Caucasians (Scholtz et al, 1999). The most common variant, -175g→t, was over-represented in Black FH patients compared to population-matched controls, even though family studies demonstrated that this mutation alone does not cause the FH phenotype (Thiart et al, 2000). Haplotype studies have excluded the possibility that this finding is due to association of the -175t allele with another mutation in the LDLR gene, since the sequence change occurs on different chromosomal backgrounds (Hoogendijk, 1999; Thiart et al, 2000). It therefore is highly likely that the

-175g→t LDLR promoter variant enhances the FH phenotype in LDLR-deficient patients, particularly since it has been detected *in cis* with two different missense mutations in the LDLR gene (Scholtz et al, 1999; Thiart et al, 2000). It has previously been shown that the combined effect of two LDLR gene mutations occurring on the same chromosome may lead to abnormal receptor function, while the individual mutational effects did not reach statistical significance (Jensen et al, 1997).

In the present study extended analysis of 414 South African individuals of mixed ancestry (Coloured population) were performed, in an attempt to define the possible phenotypic effect of the -175g→t LDLR promoter variant. This recently admixed but genetically distinct population has been defined as an ideal group to investigate the genetics of complex traits such as hypertension, diabetes and obesity (Loubser et al, 1999). Since previous studies have demonstrated an association between polymorphisms (ApaLI, HincII) in the LDLR gene and obesity in essential hypertensives (Zee et al, 1992; 1995), we focussed on these conditions.

Materials and Methods

Subjects

Blood samples were collected with informed consent from 414 individuals (Table 1) of the Coloured population of South Africa, a people of mixed ancestry (KhoiSan, West African Negro, Madagascar, Javanese, Malay and European origin) (Nurse et al, 1985; Loubser et al, 1999). In order to minimise the likelihood of population substructures, all study participants were recruited randomly from the Moravian mission Mamre, located on the western perimeter of the Swartland. For the purpose of this study body mass index (BMI), blood pressure (diastolic and systolic blood pressure), and the diagnosis (Hblood) and use of high blood pressure medication (HbloodM) were denoted. Blood pressure was recorded in all study

participants as previously described (Steyn et al, 1996). The diastolic blood pressure was taken as the point of disappearance of the Korotkoff sound (phase V). Three intermittent readings were taken and recorded. The minimum diastolic blood pressure reading together with the corresponding systolic reading was applied in this study.

DNA Analysis

Genomic DNA was amplified by the polymerase chain reaction (PCR) using previously described primers spanning the -175g→t polymorphism of the LDLR gene (Thiart et al, 2000). Mutation detection was performed using a combined heteroduplex-single-strand conformation polymorphism (HEX-SSCP) technique (Kotze et al, 1995). PCR products demonstrating altered mobility were verified by repeated HEX-SSCP analysis together with a positive control sample for the -175g→t variant and/or direct sequencing on an automated system ABI310.

Statistical Analysis

Linear regression was used to model diastolic and systolic blood pressure on a set of covariates. The covariates included age, sex, body mass index (BMI), high blood pressure medication (HbloodM), LDL particle size and the -175g→t polymorphism as well as significant interactions between them. Since LDL particle size was not determined on all 414 individuals, the linear regression models included only the samples (404) with LDL particle size determinations. P-values <0.05 were regarded as statistically significant.

Results

HEX-SSCP analysis of 414 Coloured individuals from the general South African population (Table 1) demonstrated the presence of the -175g→t LDLR promoter polymorphism in 51 subjects (12.3%). Since the clinical, biochemical and physiological features of four individuals found to be homozygous for the –175g→t variant did not differ significantly from that denoted in the heterozygous group (Table 2), the data of mutation-positive subjects were pooled for further comparative analysis.

Table 2 shows mean values of BMI, blood pressure and lipid determinations in 51 subjects with and 363 subjects without variant -175g→t. Diastolic blood pressure appears to be significantly associated with the promoter variant (p=0.047). Using linear regression, covariates were independently modelled against both diastolic and systolic blood pressure (Table 3). The -175t allele again showed a significant effect (p=0.009) on diastolic blood pressure (4.2 mmHG, 95% CI: 1.0 to 7.4 mmHG) compared to subjects without this variant. Although the polymorphism did not have a significant effect on systolic blood pressure (p=0.18), the direction of the estimated effect (3.7 mmHG, 95% CI: -1.7 to 9.0 mmHG) was close to that observed in the diastolic blood pressure regression model. Several covariates, including age, gender, BMI and the use of high blood pressure medication, showed significant effects on diastolic and systolic blood pressure, independently as well as in combination with each other. LDL particle size modelled against both diastolic and systolic blood pressure did not affect diastolic blood pressure (p=0.5), but significantly affected systolic blood pressure (p=0.01) (K. Steyn et al, unpublished data).

Table 1. Characteristics of the study population

Variable	MALES (n=175)			FEMALES (n=239)		
	Mean	SD	Range	Mean	SD	Range
Age	37.54	16.844	15.0-76.0	39.3	16.968	15.0-80.0
BMI	23.35	5.021	15.2-41.2	27.56	6.996	15.5-51.9
DBP	77.68	15.502	46.0-138.0	73.67	13.03	44.0-116.0
SBP	132.29	24.67	98.0-232.0	126.8	26.165	76.0-242.0
TC	5.18	1.159	2.8-8.9	5.41	1.26	2.8-9.6
Trig	1.32	0.438	0.6-3.6	1.31	0.362	0.6-2.6
HDL	3.29	1.065	0.6-6.8	3.61	1.146	1.2-7.3
LDL	1.2	0.824	0.3-5.4	1.03	0.529	0.3-3.2

BMI=body mass index; DBP=diastolic blood pressure; SBP=systolic blood pressure; TC=total cholesterol;TG=triglycerides; HDL=high density lipoprotein; LDL=low density lipoprotein; SD=standard deviation.

Table 2. Comparative analysis of various parameters in South African subjects with and without the -175g→t polymorphism.

		-175 gg			-175 gt			-175 tt		
Gender	Variable	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Male	Number	153			19			3		
	Age	37.46	17.103	15.0-76.0	36.26	14.383	15.0-64.0	50	18.52	29.0-64.0
	BMI	23.31	4.963	15.2-41.2	23.84	5.811	16.5-36.4	21.85	3.364	18.4-25.2
	DBP	76.99	15.114	46.0-138.0	81.58	17.238	52.0-110.0	88	23.065	66.0-112.0
	SBP	131.9	24.665	98.0-232.0	133.37	21.798	98.0-184.0	145.33	46.49	110.0-198.0
	TC	5.16	1.135	2.8-8.9	5.22	1.399	2.9-8.5	5.73	0.945	5.0-6.8
	TG	1.19	0.773	0.3-4.4	1.22	1.206	0.3-5.4	1.63	0.493	1.3-2.2
	HDL	1.31	0.426	0.6-3.6	1.44	0.535	0.7-2.4	1.13	0.351	0.8-1.5
	LDL	3.3	1.07	0.6-6.8	3.12	1.059	1.3-6.2	3.87	0.97	2.9-4.7
Female	Number	210			28			1		
	Age	39.72	17.32	15.0-80.0	36.46	14.24	18.0-66.0	30		
	BMI	27.6	6.969	15.5-51.9	27.23	7.436	16.0-46.5	28.59		
	DBP	73.53	13.182	44.0-116.0	74.57	12.243	56.0-102.0	78		
	SBP	126.7	26.306	76.0-242.0	127.86	25.9	98.0-190.0	114		
	TC	5.42	1.254	2.8-9.3	5.37	1.336	3.7-9.6	4.4		
	TG	1.03	0.512	0.3-3.2	1	0.653	0.4-2.7	0.6		
	HDL	1.31	0.358	0.6-2.6	1.38	0.392	0.6-2.1	0.9		
	LDL	3.63	1.152	1.2-7.3	3.53	1.137	2.2-6.6	3.2		

Table 2/continue on page 50

Table 2 / continued: Comparative analysis of various parameters in South African subjects with and without the -175g→t polymorphism.

		-175 gg			-175 gt			-175 tt			
Gender	Variable	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	
	Number	363			47			4			P value*
	Age	38.77	17.242	15.0-80.0	36.38	14.141	15.0-66.0	45	18.129	29.0-64.0	
	BMI	25.79	6.548	15.2-51.9	25.86	6.963	16.0-46.5	23.53	4.348	18.4-28.6	0.88
	DBP	**74.99**	**14.113**	**44.0-138.0**	**77.4**	**14.708**	**52.0-110.0**	**85.5**	**19.485**	**66.0-112.0**	**0.0473**
Combined	SBP	128.9	25.72	76.0-242.0	130.09	24.231	98.0-190.0	137.5	41.065	110.0-198.0	0.2918
	TC#	5.31	1.21	2.8-9.3	5.31	1.348	2.9-9.6	5.4	1.02	4.4-6.8	0.6664
	TG#	1.1	0.639	0.3-4.4	1.09	0.912	0.3-5.4	1.38	0.655	0.6-2.2	0.7382
	HDL#	1.31	0.388	0.6-3.6	1.4	0.451	0.6-2.4	1.08	0.31	0.8-1.5	0.2506
	LDL#	3.49	1.129	0.6-7.3	3.37	1.114	1.3-6.6	3.7	0.812	2.9-4.7	0.7499

BMI = body mass index; DBP = diastollic blood pressure; SBP = systolic blood pressure; TC = total cholesterol; TG = triglycerides; HDL = high density lipoprotein; LDL = low density lipoprotein; SD = standard deviation

*Due to the small sample size of the homozygotes (-175 tt) their data were pooled with that of the heterozygotes (-175 gt) and p-values determined on combined groups after correcting for age and gender

#Lipid levels are from Hoogendijk 1999 / Appendix C)

Table 3: Parameter estimates of linear regression of diastolic and systolic blood pressure

Parameter	Diastolic Blood Pressure			Systolic Blood Pressure		
	Estimate	Standard Error	P-value	Estimate	Standard Error	P-value
Intercept	134.5	10.78	0.0001	172.65	18.15	0.0001
Age	-0.34	0.13	0.0116	0.81	0.22	0.0003
Sex (female)	-5.55	1.17	0.0001	-8.35	1.97	0.0001
HBloodM	-59.39	11.30	0.0001	-46.80	19.02	0.0143
-175 g→t†	-4.23	1.63	0.0099	-3.67	2.75	0.1826
BMI	-0.71	0.22	0.0015	-1.68	0.37	0.0001
LDLpart‡	-4.84	7.26	0.5053	-32.14	12.21	0.0088
HBlood	-14.56	2.52	0.0001	-22.95	4.24	0.0001
Age-HbloodM	0.61	0.14	0.0001	-0.17	0.23	0.4682
BMI-HbloodM	0.03	0.13	0.0001	1.87	0.40	0.0001
BMI-LDLpart	0.30	0.25	0.2535	1.16	0.43	0.0068

HBloodM=high blood pressure medication; BMI=body mass index; LDLpart=LDL particle size; HBlood=high blood pressure diagnosis
† -175 variant present; ‡ fairly small to small

Discussion

The main objective of this study was to define the possible phenotypic effect of the -175 g→t variant, shown to occur on different LDLR gene haplotypes (Hoogendijk, 1999; Thiart et al, 2000). This mutation is located in the FP-2 *cis*-acting regulatory element of the LDLR promoter, which contains a putative binding site for the multifunctional transcription factor, F-ACT1 (YY1) (Mehta et al, 1996). Interestingly, this region containing the single base change also shows strong homology (6 out of 8 bp) to the cAMP response element (CRE) which binds CRE-binding proteins (CREB) (Sheng et al, 1991). Since the CREB protein functions as a Ca^{2+}-regulated transcription factor, combined with the fact that LDLR gene transcription is induced by calcium (Makar et al, 1994), may shed some light on the mechanism by which elevated LDL is associated with hypertension.

Since the -175g→t variant is present in African populations while apparently absent in Caucasians, we considered the likelihood that the relatively high frequency and statistically significant association with hypertension in the Coloured study cohort could be the result of population admixture. If there is a relationship between the frequency of hypertension and the degree of admixture, the observed association may not necessarily indicate a relationship between the LDLR gene and hypertension. Such a phenomenon is, however, unlikely, since the Coloured population has prevalence rates of hypertension comparable to that in the South African Black population (both approximately 25%), which does not differ significantly from that (23%) in white males (15% in white females) (Seedat and Seedat, 1982). The -175 t allele was detected at a carrier frequency of 3.8% in the KhoiSan population of South Africa, (Hoogendijk, 1999), which contributed at least 40% to the gene pool of the Coloured population (Loubser et al, 1999). This indicates that the polymorphism was not introduced into the Coloured population as a consequence of recent admixture with the Black population. Although

cholesterol levels in the Coloured population is comparable to the Caucasians (Rossouw et al, 1985), but higher than the Black and Khoisan population (Hoogendijk, 1999 / Appendix C), the lack of significant difference in cholesterol levels between individuals with and without the –175g→t variant, furthermore argued against false association due to population substructures.

Although the precise mechanism is not clear, the significant association detected between the -175 t allele and increased diastolic blood pressure in relation to the known effect of Ca^{2+} on LDLR activity (Makar et al, 1994), suggests that variation in the LDLR promoter might predispose individuals to hypertension. A non-lipid mechanism may be involved, since it has been shown that cholesterol lowering drugs such as pravastatin not only decreases cholesterol levels, but also blood pressure, and that this reduction is independent of changes in plasma cholesterol levels (Glorioso et al, 1999). The finding that cytokines, such as tumour necrosis factor alpha (TNFα) shown to be elevated in hypertensive subjects, (Glenn et al, 2000) may also influence LDLR gene transcription irrespective of sterol status (Ruan et al, 1998), raises the possibility that a complex interaction between external influences affecting LDLR activity may be involved in the development of hypertension (in the African context). The association detected between intragenic LDLR gene polymorphisms and obesity in essential hypertensives, but not in normotensives (Zee et al, 1992; 1995), substantiates this hypothesis. Since glucose, insulin and BMI play a significant role in hypertension, the effect of the –175g→t variant were also investigated using linear regression models for each of these parameters. No statistically significant association could, however, be detected between the –175g→t variant and any of these parameters (fasting glucose (P=0.576), insulin (P=0.364) and BMI (P=0.882)) (data not shown). Further studies are warranted to determine whether variant -175g→t directly causes hypertension, or only jeopardises the ability of genetically susceptible individuals to handle certain metabolic stresses. Preliminary data in the Black population of South Africa have indicated an increased frequency (4/18, 22%) of this promoter polymorphism in hypertensives (H. Van Jaarsvelt and M.J. Kotze, unpublished data)

compared with the normal population where a similar heterozygote frequency (~5%) has been described in different black tribes (Thiart et al, 2000 / Appendix B).

The significant association observed between the apparent African-specific -175g→t LDLR promoter polymorphism and diastolic blood pressure may, at least in part, explain the relatively high prevalence of hypertension in Africans (Steyn et al, 1996). Extended investigation of the consequences of polymorphic variation in the LDLR promoter region may provide clues to understanding the apparently low incidence of coronary heart disease in the Black population, despite the high prevalence of other known risk factors such as hypertension and obesity (Vermaak et al, 1991).

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CHAPTER 3

The significance of variation in the SRE-1 element of the LDLR gene promoter and its possible role in apoptosis

J Devine[1], CL Scholtz[2], MJ Kotze[2], P Bouic[1]

[1]Department of Microbiology, Immunology Unit, Tygerberg Hospital and [2]Division of Human Genetics, Faculty of Health Sciences, University of Stellenbosch, Tygerberg, South Africa.

Abstract

The low-density lipoprotein receptor plays an integral part of cholesterol homeostasis and is under strict control of sterol regulatory binding proteins (SREBPs), which also regulate transcription of various enzymatic pathways. CPP32 and Mch3, which form part of the family of caspases reported to regulate apoptosis, can proteolytically cleave SREBP to its active DNA binding form. A mutation (-59 c→t) identified in the sterol regulatory element (SRE-1) in repeat 2 of the LDLR gene promoter, markedly reduced transcription of the LDLR gene *in vitro*. This mutation co-segregated with the FH phenotype in the South African family, and individuals with this mutation was investigated together with population, age and gender-related controls to determine the significance of this variant on apoptosis. This study also attempted to define the links, if any, between cholesterol and the levels of immunological cells present in these individuals as well as to determine whether any immunological deficits may be present. This study strongly suggests that the -59 c→t mutation may inhibit apoptosis under normal immunological conditions. The CD4 and CD8 T-cells seem to be the most sensitive to the effects of this mutation and possibly also the eosinophils and basophils. We thus conclude that the activation of the SREBP and thereby elevated LDLR expression is essential for apoptosis induction under normal immunological conditions.

Introduction

The importance of cholesterol in apoptosis is well established and it forms an integral part of the cell cycle. Cholesterol is essential for maintaining the integrity of the cellular membrane since, unlike necrosis, the cell membrane stays intact during apoptosis. The concentration of cellular cholesterol is maintained by a finely tuned balance between the *de novo* synthesis (mevalonate pathway) and the uptake of exogenous cholesterol via receptor mediated endocytosis (low-density lipoprotein receptor (LDLR) pathway) (Goldstein and Brown, 1990). Cholesterol regulation is tightly controlled by a family of proteolytically activated membrane-bound transcription factors, called sterol regulatory element binding proteins (SREBP's) (Goldstein and Brown, 1990) through a negative feedback system. SREBP's and other transcription factors are recruited to induce transcription when cellular cholesterol levels are low, while optimum cellular cholesterol levels inhibit the recruitment of transcription factors and transcription is down regulated (Brown and Goldstein, 1986). SREBP's regulate transcription of the LDLR gene (Dawson et al, 1988; Smith et al, 1990), as well as the expression of various enzymes regulating cholesterol homeostasis (Kawabe et al, 1999). These enzymes include HMG-CoA synthase, HMG-CoA reductase, farnesyl pyrophosphate synthase as well as regulators involved in triglyceride synthesis, glycerol-3-phosphate acyltransferase and fatty acid synthesis. Interestingly, two members of the caspase family, CPP32 (Wang et al, 1995,1996) and Mch3 (SCA-2) (Pai et al, 1996) (sharing 54% homology), have both been shown to target SREBP during apoptosis, producing an active SREBP molecule. This activation of SREBP is completely independent of the cellular cholesterol level and occurs even if sterols are supplied to the cell at high levels (Wang et al, 1996). It seems unlikely that the activation of SREBP during apoptosis occur to provide isoprenoids and farnesyl, which are products of the mevalonate pathway. Interestingly it has

been shown that the inhibition of the mevalonate pathway (which is responsible for endogenous cholesterol and the provision of isoprenoids for the cell cycle and farnesyl for p21), induces apoptosis in a wide variety of cells (Clutterbuck et al, 1998; Padayatty et al, 1997; Choi and Jung, 1999). It appears likely that the role of the activation of SREBP is to provide cholesterol to maintain the integrity of the plasma membrane. This suggestion has been supported by reports that the addition of small amounts of cholesterol incorporated into the membrane enhances cell sensitivity to apoptosis (Härtel et al, 1998). Too much cholesterol however, inhibits apoptosis, probably to protect the membrane from lipid peroxidation.

The low-density lipoprotein receptor (LDLR) gene is essential for the recruitment of exogenous cholesterol and contains a sterol regulatory element (SRE-1) in repeat two of its promoter (Brown and Goldstein, 1986; Dawson et al, 1988; Smith et al, 1990). It has been shown that variation in this region of the LDLR gene abolish binding of SREBP and consequently results in down regulation of the LDLR gene (Dawson et al, 1988; Smith et al, 1990). Recently, variations in the LDLR gene promoter have been reported in a South African family of mixed ancestry with familial hypercholesterolaemia (FH) (Scholtz et al, 1999). *In vitro* studies demonstrated that the mutation at position -59 (c→t), located in the SRE-1 element, markedly reduces transcription of the LDLR gene irrespective of sterol status. It should also be noted that this mutation co-segregated with the FH phenotype in the family. Of further note is the fact that two additional variants were detected in this family (-124 C/T and -175 G/T). These two polymorphisms are located in newly defined *cis*-acting regulatory regions, designated footprinting 1 (FP1) and footprinting 2 (FP2) (Mehta et al, 1996), respectively. One individual with both the -59 and -124 variants presented with plasma cholesterol levels within the normal range according to age and gender. This finding might

provide *in vivo* evidence for the proposed interaction between the SRE-1 and FP1 sites as suggested by Dhawan and colleagues (1997)

The aim of this study was to determine the significance of the activation of SREBP (and therefore the LDLR gene) in the cell death cascade, by investigating the levels of apoptosis in lymphocytes isolated from the FH family. This study also attempted to define the links, if any, between cholesterol and the levels of immunological cells present in these individuals as well as to determine whether any immunological deficits may be present.

Materials and Methods

Subjects

The study cohort consisted of 3 individuals with the -59 c→t mutation in the LDLR promoter and a single individual with both the -59 c→t mutation and the -124C/T variant (Scholtz et al, 1999), as well as age, sex and population matched normocholesterolaemic controls. Informed consent was obtained from the study cohort and they were counselled regarding the importance of an HIV test, which was done as part of the study. The determination of the HIV status was important since a positive test could influence the overall results. Various parameters were denoted for each individual, including a fasting lipogram, full blood count with a differential count, IgG, IgM and IgA levels, HIV serology, the percentage and total CD3 (T-cells), CD4 (T-helper cells), CD8 (cytotoxic T-cells), CD 19 (B-cells) and CD16+56 (NK-cells) as well as the levels of apoptosis in CD4, CD8 and CD19 positive cells.

Immunophenotyping

For the purpose of immunophenotyping EDTA blood was drawn from the study subjects and 50µl of the blood was combined with 20µl of the appropriate monoclonal antibodies (supplied

by Becton Dickinson®): CD4 determination: CD3+ FITC, CD4+ PE, CD45+ PerCP, CD8 determination: CD3+ FITC, CD8 PE, CD45+ PerCP, CD19 determination: CD3+ FITC, CD19 PE, CD45+ PerCP and CD16+56 determination: CD3+ FITC, CD16+56 PE and CD45+ PerCP. The blood and monoclonal antibodies were incubated for 20 minutes after which 450µl Facs lysing buffer was added and left for 10 minutes to lyse the red blood cells. Data was acquired by flow cytometry (FacsScan from Becton Dickinson®), with all sample analysed within 1 hour. Analysis was performed using CellQuest software.

Apoptosis determination

For apoptosis determination, 50µl EDTA blood of each individual was used and red blood cells were lysed using 2.5ml Facs Lysing buffer. The samples were incubated for 10 minutes and then centrifuged for 10 minutes at 1800 rpm. Samples were washed with phosphate buffered saline (PBS). Samples were again centrifuged for 10 minutes at 1800 rpm and the PBS discarded. Appropriate monoclonal antibodies were then added (supplied by Becton Dickinson®): CD4 apoptosis: CD3+ FITC, CD4+ PE, CD8 apoptosis: CD3+ FITC, CD8+ PE and CD19 apoptosis: CD3+ FITC, CD19+ PE. The cellular pellet was incubated with the monoclonal antibodies for 20 minutes followed by another PBS wash phase. After the PBS wash, the cell pellet was fixed with 50µl 4% formaldehyde and left for 10 minutes and washed with PBS. After the wash step, 400µl PBS was added together with 100µl Apostain (a DNA intercalating dye produced by Ridge Diagnostics) to the pellet and allowed to incubate until the data was acquired by flow cytometry. All data was acquired within 6 hours of the samples being drawn.

The peripheral blood mononuclear cells were gated according to their scatter properties. The % CD4, % CD8 and the % CD19 was then respectively gated within this gate. The number of

apoptotic cells within these gates was determined according to their fluorescence (far red) and size (forward scatter).

It was noted that within the $CD4^+$ and $CD8^+$ populations there were two distinct sub-populations, which were divided according to their degree of CD4 and CD8 expression. These populations were designated CD4/CD8 Hi and CD4/CD8 Low (Figure 1). The differentiation was made according to the degree of fluorescence and the presence/absence of a homogenous cell population. The determinations were all made in the control individuals and the gates were retained to determine whether the associated patient had any shifts in their degree of fluorescence and the % apoptotic cells present within the gate. CD19 was excluded due to the fact that this population was found to be a single homogenous group and could not be divided into Hi and Low sub-populations.

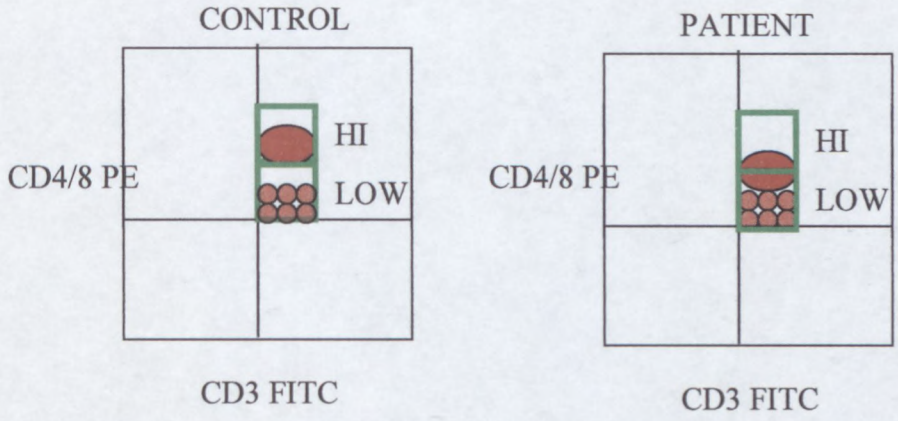

Figure 1: Method used to differentiate $CD4^+$ and $CD8^+$ cells according to the degree of fluorescence and the presence/absence of a homogenous cell population.

Statistical analysis

As the groups in this study were small in nature, two independent statisticians were used to analyse the data in an attempt to provide objectivity and avoid any possible over-interpretation of the results. Statistical analysis focussed on the use of paired analysis, rank analysis and correlation matrices to interpret the data. A value of +4 or -4 represents a significant upward or downward trend respectively when analysing the results with paired analysis. A rank value or 16 suggests a significant trend. A $P<0.05$ was regarded as significant when the data was analysed using correlation matrices.

Results

Immunophenotyping

The only significant difference found between the patient and control groups existed in the difference between the absolute CD3 levels and the %NK levels. The absolute CD3 levels were significantly increased in the patient group with a pair value of +4 and a rank value of 16. The %CD3 levels also showed a trend to being raised in the patient group although this was not significant. The %NK cells were significantly lower in the patient group with a pair

value of -4. The absolute NK levels show a trend to being lower in the patient group although this was also not significant. There exists a trend to an increase in the absolute CD4, CD8 and CD19 levels in the patient group although these trends were not significant (Figure 2).

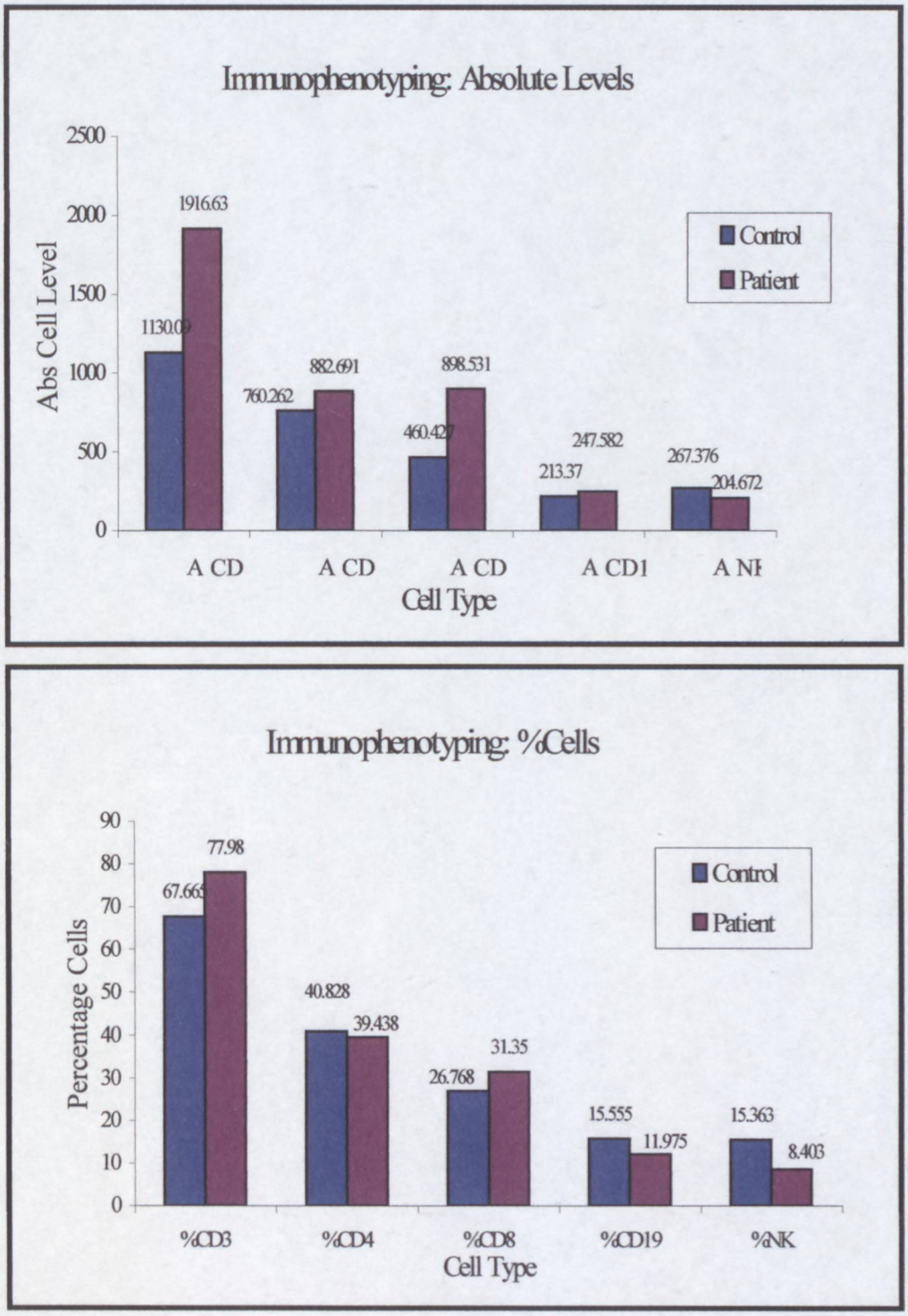

Figure 2: Immunophenotyping of the percentage and absolute cell levels observed in the patient and control groups.

Apoptosis

The apoptosis results are summarised in Table 1. It is noteworthy that apoptotic events predominantly occurred in the low gate of both the CD4 and CD8 groups in the control group. There exist a significant upward trend in the Hi gates and a significant downward trend in the low gates in the patient CD4 and CD8 group. These observations are due to a decrease in the expression of the CD4 and CD8 molecules. Evidence for the latter statement can be found in the reduced levels of fluorescence of the CD4 and CD8 molecules of which the reduction in CD4 expression is significant (Table 1) and the decrease in CD8 expression shows a trend to significance.

Table 1: A summary of the apoptotic events observed in the study cohort

PARAMETER	MEAN C	MEAN P	PAIR	RANK
S CD8H	6.86	28.31	+4	
S CD8L	82.17	68.1	-4	
S CD8T	36.19	22.09	-4	
S CD4H	6.99	51.66	+4	+16
S CD4L	79.42	60.62	-4	
S CD4T	27.89	24.54	-3	
S CD19	6.25	5.22	+2	
FL CD8	468.97	172.97	-3	
FL CD4	359.6	147.34	-4	-16
FL CD19	84.59	61.33	-4	

A significant reduction in the level of total CD8 apoptosis were observed in the patient group with a trend to a significant reduction in the level of total CD4 apoptosis. No significant difference was observed between patient and control groups in the level of CD19 apoptosis, but a significant reduction was detected in the level of CD19 expression in the patient group. With these observations in mind, it would stand to reason that if there exists a reduction in the levels of apoptosis in the patient group then an increase in the number of cells should be visible in the coulter count (Table 2).

Table 2: A summary of the number of cells visualised in the coulter counter.

PARAMETER	MEAN C	MEAN P	PAIR	RANK
%LYMPH	23.8	34.1	+4	
A LYMPH	1.69	2.45	+4	+16
%EOS	3.1	5.32	+4	
A EOS	0.2	0.39	+4	
%BAS	0.45	0.73	+4	+16
WBC	7.375	7.308		

It is important to note in table 2 that a significant increase can be observed in the percentage and absolute lymphocyte count which is in correlation with the inhibition of apoptosis seen in the CD4 and CD8 T-cells. There also exist a significant trend to an increase in the eosinophil counts and the percentage basophils. One would expect the WBC count to be increased in the patient group, however, for all intent of purposes the WBC count is nearly identical between the two groups. This finding possibly suggests that different cell types may show varying sensitivity to the –59c→t mutation and the CD4, CD8 and possibly the eosinophils and basophils are more dependent on the LDLR for the maintenance of their cell cycle.

Lipogram and antibody levels.

No significant difference could be observed between patients and controls regarding the production of antibodies. This result must be seen against the background of a lack of significant difference in the CD19 apoptosis and the CD19 count despite a significant reduction in the degree of expression of the CD19 molecule in the patient group. This suggests that the -59 c→t mutation does not cause any significant disruption of normal B-cell function.

A significant increase was detected in the LDL cholesterol levels in the patient group, consisting of 4 individuals, compared with controls (figure 3). Failure to detect a statistically significant difference in total cholesterol levels between the groups may be due to the presence of both the –59 c→t and –124 c→t mutations in the normocholesterolaemic subject, due to possible allelic interaction (Scholtz et al. 1999).

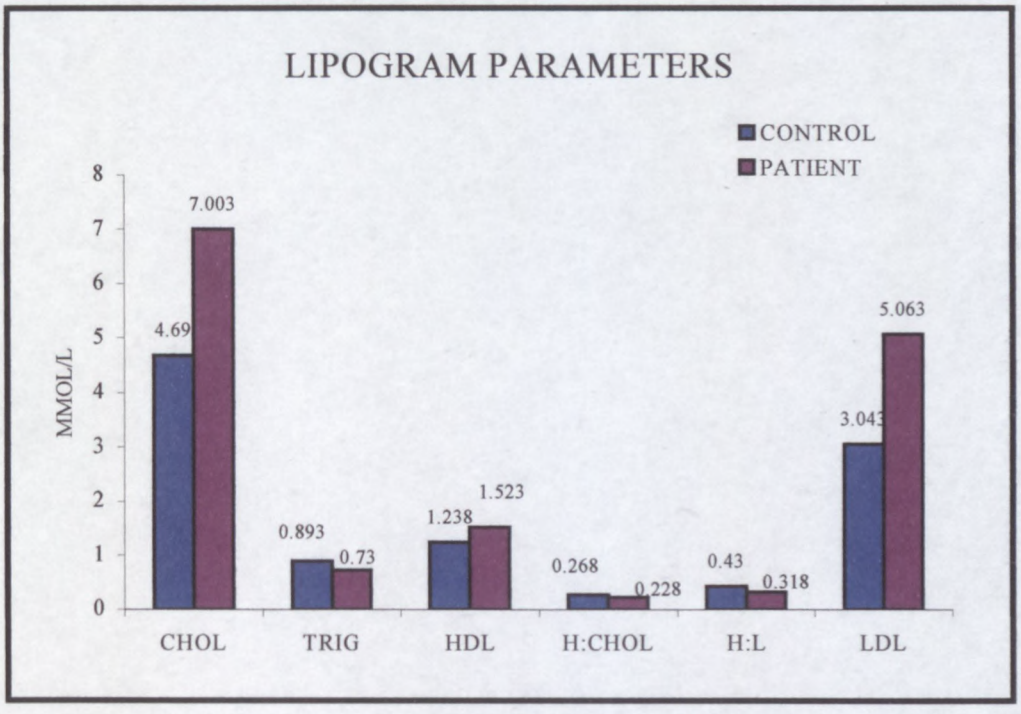

Figure 3: Difference in lipid and lipoprotein levels between patients with the –59 c→ mutation and mutation negative controls.

Discussion

This study demonstrated that the -59 c→t mutation, shown to reduce transcription of the LDLR gene by 60% *in vitro* (Scholtz et al, 1999), have a significant inhibitory effect on apoptosis. A possible mechanism of inhibition by the -59 c→t mutation may be by moderating lipid peroxidation since it may lead to an increase in membrane cholesterol (Härtel et al, 1998). This might play a small role in these patients as there is a trend to an increase in the serum cholesterol concentration. This finding, however, could not explain the trend of apoptosis inhibition in two individuals with normal cholesterol levels.

A second potential mechanism involves interaction with p53 and bcl-2 with transcription factors which regulate SREBP activity and thereby LDLR expression. The bcl-2 gene contains a SRE-like domain within its promoter region and its expression is also increased upon signalling through receptor C_k (Tsujimoto et al, 1986). With the reduction of SREBP binding to SRE-1 of the LDLR gene it may make more SREBP available for binding to apoptosis inhibiting genes such as bcl-2. Furthermore, YY1 is a negative regulator of SREBP activity (Ericcson et al, 1999) in the expression of the LDLR gene as well as p53 activity (Furlong et al, 1996). The inhibitory effect of YY1 on SREBP can be circumvented by increased SREBP activity and therefore LDLR gene expression in cases of sterol depletion (Bennett et al, 1999). The -59 c→t mutation might result in a positive feedback loop so as to increase LDLR gene expression, which will lead to a reduction of YY1 activity. Reduced YY1 activity would lead to increased p53 levels (DNA repair enzyme) (Voss and Cotton, 1998), resulting in increased correction of DNA faults and induction of cell apoptosis.

An interesting result of this study is the shift in the apoptosis Hi/Low groups between patient and control groups, which has been found due to a reduced expression of CD4 and CD8. The

results also demonstrated a reduction in CD19 expression despite the absence of clear Hi/Low apoptotic groups. The reason for this phenomenon is hard to explain. It is well known that cholesterol modulate membrane receptor function (Gimpl et al, 1997) which might explain this result. The absence of an elevated serum cholesterol in two of the patients despite them showing signs of reduced CD4, CD8 and CD19 expression, makes cholesterol mediated alteration of receptor function an unlikely mechanism for this phenomenon. The most likely mechanism rests upon the modulation of the activity of the transcription factors, which regulate CD4, CD8 and CD19 expression. This may involve YY1, which has been implicated as a regulator of a vast number of genes (Shi et al, 1997). Alternatively, the transcription factors c-fos and c-myc, which form part of the receptor C_k transduction pathway (Kaur et al, 1998), might be involved as c-fos has been shown to be particularly important in regulating CD4 and CD8 expression during T-cell maturation in the thymus (Chen et al, 1999). It is also important to note that various cholesterol-independent stimuli regulate the expression of the LDLR gene, most of these stimuli utilise the SRE-1 site for activation. Mitogenic activation of lymphocytes induces the LDLR gene (Makar et al, 1994, Chan et al, 1998), providing possibly a logical explanation for the reduced CD4, CD8 and CD19 expression even though the effect of the -59 mutation in mitogenic activation was not determined in this study.

As previously indicated the expression of LDLR is induced through the interaction of SREBP with other transcription factors including CBP (cAMP response element binding protein (CREB) binding protein) (Meier, 1997). Somatic mutations of CBP is associated with acute myeloid leukaemia (AML) as well as Rubenstein Taybi syndrome, which also predisposes to haematological malignancies (Petrij et al, 1995). Cholesterolgenic mutations associated with AML include the absence of receptor C_k, which results in the dysregulation of the mevalonate pathway, bcl-2, cyclin D and c-fos as well as overexpression of LDLR (Tatidis et al, 1997;

Kaul and Kaur, 1998). All these changes serve to immortalise the cell and secondly to provide it with a high level of cholesterol, which is essential for maintaining cell membrane integrity, as the cells are dividing rapidly. The CBP translocation in AML (8:16) results in the formation of a fusion protein between CBP and MOZ (monocyte zinc finger protein), the latter is believed to have acetyltransferase activity. A second fusion protein described in AML is the fusion between MOZ and TIF2 (a nuclear transactivator). It has been demonstrated that the latter recruit CBP or can mimic its activity. These fusion proteins probably act as "super transcription factors" which immortalise the cell by interacting with the tumour suppressor gene p53 and other cell cycle regulators (Giles et al, 1998).

Although high degrees of LDL degradation have been reported in various cancers such as AML (Ho et al, 1976), no increase in LDLR gene transcription was detected (Rudling et al, 1998). Even though no significant elevation in transcription were reported, it seems likely that the -59 c→t mutation, which seems to reduce SREBP binding (preventing high degree expression of the LDLR gene) (Scholtz et al, 1999), might influence the development of AML in individuals with this mutation. The presence of SREBP is important for the activity of CBP as the latter is a trans-activator and does not bind directly to the DNA to modulate transcription (Ericcson et al, 1998). Therefore, unless the CBP-MOZ/MOZ-TIF2 can bind directly to SRE-1 regions, they will be unable to direct SREBP-dependent up regulation of the LDLR. Theoretically, even if the aforementioned translocations were to occur in patients with the -59 mutation, it is unlikely that the low level of LDLR expression would fulfil the demands of the high cell turnover seen in AML.

In summary, our results strongly suggest that the -59 c→t mutation, in repeat 2 of the LDLR gene promoter, may inhibit apoptosis under normal immunological conditions. The cells

which seem to be the most sensitive to the effects of this mutation are the CD4 and CD8 T-cells and possibly also the eosinophils and basophils as seen by the increase in cell numbers of the latter two populations. The conclusion made from these results suggest that transcriptional activation by SREBP which affects LDLR gene expression, is essential in normal cellular processes for the progression to apoptosis.

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CHAPTER 4

Analysis of the low-density lipoprotein receptor gene promoter region in host susceptibility to HIV infection: A pilot study

CL Scholtz[1], M Zeier[2], G George[3], MJ Kotze[1]

[1]Division of Human Genetics, Faculty of Health Sciences, University of Stellenbosch, Tygerberg, [2]Infectious Diseases Clinic, Tygerberg Hospital, Tygerberg and [3]Department of Medical Biochemistry, Faculty of Medicine, University of Transkei, Transkei, South Africa.

Abstract

Recent data have indicated that lipoproteins might be involved in innate immunity. In this study the likelihood that variation in the promoter region of the low-density lipoprotein receptor (LDLR) gene might play a role in susceptibility to HIV/AIDS has been investigated. DNA samples of 222 HIV-seropositive South African individuals and 173 population-matched controls were subjected to mutation screening. Three LDLR promoter variants, –124 c→t, –136 g→a and –175 g→t were identified in the Black and Coloured populations. In the Black population, a statistically significant difference in allelic frequency for the –124 c→t variant was detected between HIV-seropositive patients with fast disease progression compared with asymptomatic subjects (P=0.006). A marginally significant difference (P=0.07) was also detected between fast progressors and normal/slow progressors, but no statistically significant allelic differences could be detected for the –175 g→t variant. These findings support the hypothesis that variation in the promoter region of the LDLR gene may influence disease progression in HIV infection and propose a possible significant role for the LDLR gene in host defence. It seems possible that not only the rarity of protective mutations in the CC chemokine receptor 5 (CCR5) gene, but also the enrichment of polymorphic variation in the LDLR promoter, may explain the apparently fast disease progression in HIV-seropositive Africans compared with affected Caucasians. Further studies are warranted to confirm these findings in a larger sample and to elucidate the mechanisms underlying the proposed disease association.

Introduction

Comprehending the basic principles of susceptibility and / or resistance to infection in relation to the genetic make-up of the host, that may greatly affect the fate of infectious agents, is extremely important (Marquet et al, 1999). The importance of lipoproteins has repeatedly been highlighted in innate immunity, even though the exact mechanisms remain unclear. Lipoproteins such as apolipoprotein (apo) A-I and high density lipoprotein (HDL) neutralise (Owens et al, 1990) and lyse (Levine et al, 1993) certain pathogens, including the human immuno-deficiency virus (HIV), while LDL has been shown to bind and target parasites for uptake by macrophages (Xu et al, 1993). It has also been demonstrated that some viruses compete for cellular receptors like the LDLR to gain entry into the cell (Hofer et al, 1994). Interestingly, Netea and colleagues (1996) have shown that LDLR deficient mice were protected against certain lethal infections, which further highlighted the importance of LDL in immunity.

LDL is the major cholesterol carrying lipoprotein in human plasma and is transported into the cell through receptor-mediated endocytosis (Goldstein et al, 1995). The finding that mitogenic activation can induce transcription of the LDLR gene, irrespective of sterol status (Cuthbert and Lipsky, 1984; 1990), furthermore suggests that the LDLR gene can be considered an immediate early response gene (Makar et al, 1994, Dhawan et al, 1999). Infection and inflammation induce a wide array of metabolic changes, the acute phase response, which are primarily mediated by cytokines (Mackiewicz et al, 1993). The regulation by cytokines is mostly at the level of gene transcription, as has been demonstrated for the LDLR gene (Stopeck et al, 1993). It is noteworthy that abnormal cholesterol levels have been repeatedly

detected early during infections (Nilsson-Ehle and Nilsson-Ehle, 1990; Grunfeld et al, 1992; Hsu et al, 1995), indicating a possible earlier host response.

Various mutations underlying familial hypercholesterolaemia (FH) have been reported in the coding region of the LDLR gene, while sequence changes in the promoter region appear to be rare (Hobbs et al, 1992; Top et al, 1992; Day et al, 1998; Peeters et al, 1998; http://www.ucl.ac.uk/fh and http://www.umd.necker.fr). The regulatory region of the LDLR gene lie within 200 bp upstream of the transcription initiation site and three direct imperfect repeats are essential for transcription of the gene (Südhof et al, 1987) Repeats 1 and 3 bind Sp1, a transcription factor important for basal transcription, while repeat 3, the sterol regulatory element (SRE-1), bind SREBP (Smith et al, 1990; Briggs et al, 1993). Two additional regulatory elements, designated footprinting 1 (FP1) and footprinting 2 (FP2) elements, were also shown to be important for maximal induction of transcription (Mehta et al, 1996). Dharwan and colleagues (1997) furthermore reported possible interaction between FP1 and SRE-1, while it has been suggested that the FP2 site might contain a putative binding site for YY1 (Mehta et al, 1996). This multifunctional transcription factor may act as both an activator and repressor of various cellular and viral genes (Shi et al, 1996) and may repress transcription of the LDLR gene indirectly (Bennett et al, 1999).

Schulte (2001) has suggested that subtle variations in promoter gene regions might influence the ability of an organism to adapt to a change in the environment. Since the LDLR gene can be considered an immediately early response gene, we screened DNA of HIV-infected and apparently healthy controls for variation in the promoter region to determine possible allelic associations.

Materials and Methods

Subjects

Blood samples were collected with informed consent from 222 HIV-seropositive South African subjects. These included 11 Caucasian, 50 Coloured and 84 Black patients who attended the Tygerberg Hospital Infectious Clinic and 77 Black asymptomatic HIV-infected individuals from the THUSA survey (Table 1). The THUSA samples were collected as part of a cross sectional survey of apparently healthy Black individuals performed in the Northwest Province of South Africa (James et al, 2000). In this study "Coloured" refers to a recently admixed but genetically distinct population of mainly Khoisan and European origin and "Black" to subjects of central African origin (Nurse et al, 1985).

Table 1: HIV infected individuals recruited for the study grouped according to disease progression.

Disease	**HIV-infected individuals**			
Progression	**Caucasian**	**Coloured**	**Black**	**THUSA samples**
Fast	2	4	11	0
Normal	1	12	14	0
Slow	0	5	2	0
Unknown	8	29	57	77
Total	**11**	**50**	**84**	**77**

HIV-positive individuals were enrolled either prospectively at the time of HIV infection or retrospectively. HIV infection was determined serologically by detection of anti-HIV antibodies by standard clinical protocols. Subjects were classified as being slow progressors if

they were infected with HIV for longer than ten years before they progressed to AIDS and as fast progressors if the progression to AIDS was within two years after infection. DNA samples of 40 seronegative Blacks and 76 seronegative Coloureds were included as controls. Due to the difficulty of obtaining control samples screened for HIV, DNA samples of 57 Black individuals recruited from the general South African population were pooled with the HIV seronegative samples to obtain a statistically viable sample size. Allele frequencies for the polymorphisms analysed were similar in these groups for the Black population.

DNA analysis

DNA was extracted from whole blood using the Qiagen extraction kit according to the manufacturer's instructions. Genomic DNA was amplified by the polymerase chain reaction (PCR) using primers 5'GAGGCAGAGAGGACAATGGC3' (forward) and 5'CCACGTCATTTACAGCATTTCAATG3' (reverse). Mutation detection was performed using a combined heteroduplex-single-strand conformation polymorphism (HEX-SSCP) technique (Kotze et al, 1995). Repeated HEX-SSCP analysis and/or direct sequencing on an automated sequencer (ABI 310) were performed to characterise PCR products demonstrating altered mobility.

Statistical analysis

Genotype distribution and allele frequencies were compared between groups using the chi-square and/or Fisher exact tests. P-values <0.05 were regarded as statistically significant.

Results

Mutation status in HIV-infected Black and Coloured individuals is grouped according to disease progression in Table 2. The –124 c→t variant was detected in three Black fast progressors (13.6%) and in one asymptomatic HIV-infected subject (0.6%), but not in any of the other disease progression groups or the Coloured population. It is not possible to comment on disease progression in the individual recruited as part of the THUSA survey, since this study population was not available for follow-up studies due to ethical constraints. In addition to the previously described –124 c→t and –175 g→t promoter variants (Scholtz et al, 1999), a novel g→a base change was detected at nucleotide position –136 in two Black HIV-positive samples but not in either the Black control individuals nor the Coloured population. Although the numbers are small, it is noteworthy that none of the Black or Coloured slow progressors carried any of the LDLR promoter variants. None of the sequence variants identified in the LDLR promoter region were detected in the Caucasian HIV-seropositive patients and therefore these subjects were excluded from further analysis.

The genotype distribution and allele frequencies of the LDLR promoter polymorphisms identified in the study cohort are summarised in Table 3. The –175 g→t variant was detected in seven Black HIV-infected patients of whom two were homozygous. Homozygosity for the –175 g→t variant was not observed in the asymptomatic Black individuals or any of the Coloured individuals, although the frequency of this polymorphism has previously been reported to be highest in the Coloured population of South Africa (Hoogendijk, 1999 / Appendix C). In this study the frequency of variant –175 g→t was higher in the HIV-seropositive Black patients (excluding asymptomatic group) than in the Coloured HIV-seropositive patients or controls. However, possibly due to small numbers analysed, no

statistically significant differences could be detected between the HIV-infected subjects and controls. No significant difference in genotype distribution or allele frequencies could be detected between HIV-infected subjects and population matched controls for either the –124 c→t or the –175 g→t variants in the Black or Coloured populations. To determine whether any differences would be detected when only serologically tested controls were included, comparisons between these individuals and the HIV-seropositive samples were performed. No significant difference could be detected when either pooled controls ($p=0.452$) or seronegative samples were included ($p=1.00$). A significant difference could also not be detected when the THUSA samples were compared with the Western Cape HIV-seropositive patient group for either the –124 c→t or the –175 g→t variants (data not shown).

To determine whether the –124 c→t or the –175 g→t variants could be associated with disease progression, statistical analysis was performed between the various disease progression groups and the asymptomatic (THUSA) HIV-infected subjects (Table 4). Due to the small sample sizes and to exclude possible confounding factors due to Caucasian admixture in the Coloured population, this analysis was only performed in the Black population upon pooling of slow (no mutations detected, Table 2) and normal progressors. A significant difference was detected in allelic frequency for the -124 c→t variant between the fast progressors and the asymptomatic HIV-seropositive group ($p=0.006$). No significant difference could be detected between the pooled normal / slow progressors and the asymptomatic THUSA subjects for either variants. The results remained significant for the -124 c→t when all the HIV-seropositive patients (in an attempt to increase sample size) with known disease progression were compared with the asymptomatic group ($p=0.046$). Comparison between the fast progessors and normal/slow progressors were marginally significant for the -124 c→t variant ($p=0.07$).

Table 2. Allelic distribution of variants detected in the South African Black and Coloured HIV study-population in various stages of disease progression.

LDLR Variant	Allele	Fast progressors		Normal progressor		Slow progressor		Progression unknown		Asymptomatic
		Blacks (n=22)	Coloureds (n=8)	Blacks (n=28)	Coloureds (n=24)	Blacks (n=4)	Coloureds (n=10)	Blacks (n=114)	Coloureds (n=58)	Blacks (n=154)
-124										
	C	19 (86.4)	8 (100)	28 (100)	24 (100)	4 (100)	10 (100)	114 (100)	58 (100)	153 (99.4)
	T	3 (13.6)	0	0	0	0	0	0	0	1 (0.6)
-136										
	G	22 (100)	8 (100)	27 (96.4)	24 (100)	4 (100)	10 (100)	113 (99.1)	58 (100)	154 (100)
	A	0	0	1 (3.6)	0	0	0	1 (0.9)	0	0
-175										
	G	20 (90.9	8 (100)	26 (92.9)	24 (100)	4 (100)	10 (100)	109 (95.6)	56 (96.6)	150 (97.4)
	T	2 (9.1)	0	2 (7.1)	0	0	0	5 (4.4)	2 (3.4)	4 (2.6)

Table 3. Genotype distribution and allele frequencies of the two LDLR promoter variants in the South Africa Black and Coloured HIV study-populations

		BLACKS						COLOUREDS			
		HIV samples (n=84)		Controls (n=97)		THUSA samples (n=77)		HIV samples (n=50)		Controls (n=76)	
LDLR variant		No	%	No	%	No	%	No	%	No	%
124	cc	81	96.4	96	99	76	98.7	50	100	76	100
Genotype	ct	3	3.6	1	1	1	1.3	0	0	0	0
	tt	0	0	0	0	0	0	0	0	0	0
Allele	c	165	98.2	193	99.5	153	99.4	100	100	152	100
	t	3	1.8	1	0.5	1	0.6	0	0	0	0
-175	gg	77	91.7	90	92.8	73	94.8	48	96	70	92
Genotype	gt	5	5.9	7	7.2	4	5.2	2	4	6	8
	tt	2	2.4	0	0	0	0	0	0	0	0
Allele	g	159	94.6	187	96.4	150	97.4	98	98	146	96
	t	9	***5.4***	7	***3.6***	4	2.6	2	***2***	6	***4***

Table 4. Comparison of allele frequencies at various stages of disease progression in Black HIV positive individuals and controls.

LDLR variants	Allele	**Fast**	THUSA samples	P	**Normal / Slow**	THUSA samples	P	**HIV patients**	THUSA samples	P
-124										
	C	19	153	0.006	32	153	NS	51	153	0.046
	T	3	1		0	1		3	1	
-175										
	G	20	150	NS	30	150	NS	50	150	NS
	T	2	4		2	4		4	4	

NS, not significant

Discussion

Increasing data support the concept that, in addition to its well recognised role in lipid transport, the lipoprotein system also plays an important role in innate immunity (Feingold and Grunfeld, 1997). The fact that the LDLR gene can be considered an immediately early or primary response gene, highlights the importance of this gene in immune function (Makar et al, 1994; Dhawan et al, 1999). The identification of various promoter variants in the LDLR gene in HIV-infected individuals, particularly in the Black population where disease progression to AIDS appears to be relatively fast, may imply an important role in susceptibility to infectious diseases. Variants –124 c→t, –136 g→a and –175 g→t were detected in the Black population but not in the Caucasian population, while only the base change at nucleotide position –175 was present in the Coloured population. In a previous study to determine the global distribution of this polymorphism (Hoogendijk, 1999 / Appendix C), it was detected at carrier frequencies of 12.6% in the Coloured population and 4.6% in the Black population. The -175 g→t variant was furthermore absent in more than 700 Caucasians screened and occurred at a carrier frequency of 3.8% in the Khoisan; these populations contributed roughly equal (~40% each) to the gene pool of the Coloured population of South Africa. Thiart et al (2000 / Appendix B) have also reported that the frequency of this polymorphism is similar in different Black tribes (Xhosa, Zulu, Sotho, Pedi) in South Africa.

The novel variant –136 g→a, identified in this study in two HIV-seropositive Black patients only, is located within the *cis*-acting FP1 site of the LDLR gene promoter. Dhawan and colleagues (1997) demonstrated, through site-directed mutagenesis, that variation at position –135 / –136 completely abolishes binding of p125 to the FP1 site and also eliminates SRE-1

mediated induction of transcription. The –124 c→t variant is located directly adjacent to the FP1 site and has previously been shown by *in vitro* studies to increase transcription of the LDLR gene to ~160% of normal (Scholtz et al, 1999). The –175 g→t variant is located in the FP2 site, which contains a putative binding site for YY1 (Shi et al, 1997) as well as a potential CRE site (which binds CRE-binding proteins) (Flamand et al, 1998) spanning the mutated allele. The transcription factors YY1 and CREB are both present and involved in the regulation of cellular and viral genomes. In 1998, Flamand and colleagues demonstrated that the CD4 promoter, which also contains a potential CRE site (6/8 bases homologous), is transactivated by the human herpesvirus (HHV) 6. HHV 6 is proposed to play a cofactorial role in disease progression in AIDS patients since it can infect CD4 and T-cells, hence contributing to the decline in this cell population.

From Table 2 it was clear that the frequency of variant –175 g→t is consistently lower in the Coloured population compared to the Black population within the different progression groups (except in slow progressors who all tested negative), despite the fact that the frequency of this mutation was shown to be significantly higher in Coloureds compared to Blacks in the general population (Hoogendijk, 1999 / Appendix C). As expected in the control population, the highest allele frequency for variant –175 g→t was found in HIV-seronegative blood donors from the Coloured population (Table 3). Although the numbers are small, it is therefore noteworthy that the lowest overall allele frequency for this variant was detected in HIV-seropositive Coloured patients. The allele frequency of 2% detected in this group is even lower than that of the asymptomatic Black HIV-seropositive subjects (2.6%), shown to have the lowest frequency for the rare allele of variant –175 g→t among all the Black patient and control groups analysed. By using intragenic LDLR gene polymorphisms/mutations and a highly informative microsatellite marker upstream of the gene, it has previously been

demonstrated that the –175 g→t variant is associated with different chromosomal backgrounds (Hoogendijk, 1999 / Appendix C; Thiart et al, 2000 / Appendix B), and therefore the only logical explanation for the above-mentioned findings is admixture linkage disequilibrium.

In a recent study of the CC chemokine receptor 5 (CCR5) gene performed in the South African population (Petersen et al, 2001), similar findings were obtained with respect to a novel polymorphism identified in the gene. It was demonstrated that the frequency of the A-allele of the African-specific polymorphism at codon 35 (P35, CCG to CCA) was significantly higher in HIV-seropositive compared with HIV-seronegative Coloureds, while the frequency of this allele was lower in Black HIV-seropositive patients compared with HIV-seronegative Black controls. The difference in allele frequency between Black patients and controls, however, did not reach statistical significance. It is therefore clear that if the significance of the CCR5 gene in HIV/AIDS has not been so well defined in Caucasian populations (Martinson et al, 1997), failure to detect significant differences in allelic frequencies for any of the known or novel mutations in the South African Black population (Petersen et al, 2001) could have led to dismissal of this gene as an important co-factor for HIV infection. These findings highlights the value of the Coloured population as a source of genetic material to identify genes/mutations involved in complex conditions (Loubser et al, 1999), based on the admixture linkage disequilibrium approach (McKeigue, 1998).

Since the likelihood of spurious associations due to population substructures in the Coloured population has to be considered before any conclusions can be drawn from these findings, we are in the process to type the study cohort for other African-specific and Caucasian-specific mutations in an attempt to exclude possible confounding effects. Data obtained with the only

African-specific marker completed to date, a mutation in the HFE gene (IVS 3-48c→g), did not demonstrate a similar reversal of allele frequencies in HIV-seropositive patients and controls in the Black versus the Coloured population (G.S. Pretorius and M.J. Kotze, unpublished results) as reported for certain polymorphisms in the CCR5 and LDLR genes, which argues against population substructures in our study cohort. Detection of the Caucasian-specific 32-bp CCR5 deletion in some of the Coloured subjects, particularly the slow progressors, demonstrated the Caucasian admixture in this population group (Petersen et al, 2001). Apart from the fact that this deletion-mutation has, as expected, not been detected in the South African Black population, none of the other known and novel CCR5 gene mutations identified in this population was detected in a high risk subgroup consisting of HIV-seronegative commercial sex workers (Petersen et al, 2001). Failure to identify any of the LDLR promoter variants in these subjects of Zulu descent appears to substantiate our hypothesis that this gene may be involved in HIV/AIDS susceptibility. It therefore seems possible that not only the presence of protective mutations in the CCR5 gene, but also the absence of polymorphic variants in the LDLR promoter, may be beneficial with respect to HIV/AIDS, particularly since this is the case in Caucasians.

Comparative analysis between HIV-seropositive Black subjects with different stages of disease progression (when known) has indeed demonstrated a statistically significant difference between the fast progressors and the asymptomatic group for the –124 c→t variant ($p=0.006$). A marginally significant difference ($P=0.07$) was also detected between fast progressors and normal/slow progressors. No significant difference could be detected for the -175 g→t variant. Due to small numbers and to exclude possible confounding factors as a consequence of Caucasian admixture in the Coloured population, the aforementioned analyses were only performed in the Black population.

In order to investigate the likelihood that the association with disease progression in HIV-seropositive patients may be related to opportunistic infections, we have also performed mutation screening in 96 Coloured patients with tuberculosis (TB) and 47 control individuals above the age of 20 years, drawn from the same community (Western Cape area where one in every three houses has had at least one case of TB in the last 10 years). No significant difference in allelic frequency could, however, be detected between these groups (E van Helden and CF Hoogendijk, unpublished data). Preliminary data obtained in Black TB patients and controls from the Transkei area also failed to show any association between susceptibility to TB and variation in the LDLR gene promoter. Studies are underway to identify patients infected with both HIV and TB for further comparative analysis, in order to enhance our understanding of the complex interplay between infectious agents and host factors in innate immunity.

Comparative analysis of various biochemical parameters in the THUSA study cohort (including approximately 1000 individuals of whom more than 200 were HIV-infected) have recently demonstrated significantly lower plasma cholesterol levels in the asymptomatic HIV-seropositive subjects compared with the seronegative subjects (HH Vorster et al, unpublished data). The finding that the –124 c→t variant, which significantly increases transcription of the LDLR gene leading to reduced LDL-cholesterol levels (Scholtz et al, 1999), predominates in HIV-seropositive fast progressors therefore supports the notion that low plasma cholesterol levels may be associated with higher risk towards disease progression in HIV/AIDS. If the proposed association with disease progression can be confirmed in a larger/additional study population, it seems plausible that appropriate alterations in lipid status might slow down the progression of the infectious complications of HIV infection.

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CHAPTER 5

Variation in regulatory regions of genes involved in lipoprotein metabolism: Are we missing the obvious?

CL Scholtz

Division of Human Genetics, Faculty of Health Sciences, University of Stellenbosch, Tygerberg

Abstract

Survival of any species is dependent on its ability to swiftly adapt to any changes in their environment. At a molecular level, this could represent variation in their genetic composition, which leads to changes on the cellular level. Modifying gene regulation would therefore contribute significantly to any species' ability to adapt evolutionary to a changing environment. Cholesterol homeostasis is regulated by various genes involved in lipoprotein metabolism, including genes encoding lipoprotein receptors and apolipoproteins. Mutations causing abnormalities in the structure of lipoprotein receptors and / or the tempo of transcription of these genes would therefore, greatly influence any subject's ability to handle environmental change. Cholesterol is essential for various processes and abnormalities in cholesterol homeostasis can have dire consequences. The low-density lipoprotein receptor (LDLR) gene forms part of the acute phase response and are considered an immediately early response gene. Although mutations in the promoter region of the LDLR gene are considered to be rare, several sequence changes have recently been identified in Africans. In this overview of variation detected in the LDLR promoter, the possible role of subtle phenotypic effects caused by regulatory mutations is discussed within the context of the pathogenesis of different diseases.

Introduction

Slight variations in regulatory sequences could provide evolutionary significant changes and greatly impact on environmental adaptation of organisms (Schulte, 2001). Since cholesterol forms an essential part of the cell cycle, it would be logical that alterations in lipid levels could be indicative of environmental change. Various studies have demonstrated that changes in lipid profiles are one of the primary compensatory changes initiated upon infection (Nilsson-Ehle and Nilsson-Ehle, 1990; Hsu et al, 1995). The low-density lipoprotein receptor (LDLR) gene plays an important role in the regulation of cholesterol homeostasis through receptor-mediated endocytosis (Brown and Goldstein, 1986). Various reports have indicated that the LDLR gene can be considered an immediately early (IE) or primary response gene, since transcription is initiated upon mitogenic stimulation (Makar et al, 1994; Dhawan et al, 1999).

In addition to sterols, various other molecules, including cytokines (Stopeck et al, 1993), hormones (Streicher et al, 1996; Croston et al, 1997; LaVoie et al, 1999), growth factors (Mazzone et al, 1989; Graham and RussellL, 1994; Basheerudin et al, 1995; Pak et al, 1996), protein synthesis inhibitors (Dhawan et al, 1999), 3-methyl-3-glutaryl coenzyme A (HMG-CoA) reductase inhibitors (Ma et al, 1986), angiotensin converting enzyme (ACE) inhibitors and Ca2+ channel blockers (CCBs) (Block et al, 1993) as well as mitogen activated protein kinases (MAPK) (Kumar et al, 1998; Kotzka et al, 1998; Dhawan et al, 1999) can induce transcription of the LDLR gene.

Promoter region of the LDLR gene

Transcription of the LDLR gene is under stringent control and all the essential regulatory elements are located within 200 bp upstream of the transcription initiation site (Goldstein et al, 1995). This region contains two TATA-like sequences and three direct imperfect repeats (16bp in length), essential for basal transcription as well as sterol mediated regulation of the LDLR gene. Repeats 1 and 3 (nucleotide positions-103 →-88 and -51→-36, respectively) bind Sp1, a known transcription factor, important for basal transcription of the LDLR gene (Südhof et al, 1987; Dawson et al, 1988). Repeat 2 (position -68→-53) contains the sterol regulatory element (SRE-1) (position -63→-57), which binds sterol regulatory binding proteins (SREBPs) and is also responsible for sterol-mediated regulation of the LDLR gene (Smith et al, 1990; Briggs et al, 1993; Koivisto et al, 1994). Recently, two additional *cis*-acting elements, designated footprinting 1 (FP1) and footprinting 2 (FP2) elements (located between positions -144→-125 and -187→-173, respectively) were identified, and deemed essential for maximal induction of transcription (Mehta et al, 1996). Dhawan and colleagues (1997) identified two nuclear proteins (p50 and p125) which bind to the FP-1 site, and suggested possible interaction between FP-1 and SRE-1.

LDLR promoter mutations

To date, relatively few promoter mutations causing familial hypercholesterolaemia (FH) have been identified in the LDLR gene, which may signify the importance of this region. Although Top et al (1992) failed to identify any promoter mutations in a large cohort of heterozygous FH patients, Hobbs and colleagues (1992) reported the presence of three mutations in the two Sp1 binding sites. These included a 3-bp deletion at nucleotide position -92 and two point mutations, -44 c→t and -42 c→g. This study did not include functional analysis of these variants, and Peeters and colleagues (1998) have subsequently determined through transcient

transfection studies and bandshift assays that mutation -92Δcct identified in a South African Pedi significantly reduces transcription of the LDLR gene and abolishes binding of Sp1 to repeat 1.

Koivisto et al (1994) presented the first detailed report of a disease causing mutation in the LDLR promoter (-43 c→t), which co-segregated with the FH disease phenotype in a Finnish family. *In vitro* assays indicated that this variant, located in repeat 3, significantly decreased LDLR gene transcription and abolishes binding of Sp1. Mutation -45 t→c associated with a mild FH phenotype, was identified in the proximal Sp1 site in a Welsh patient (Sun et al, 1995). Functional studies demonstrated that the -45 t→c variant reduces the binding affinity for Sp1 to this site as well transcription of the LDLR gene *in vitro.*

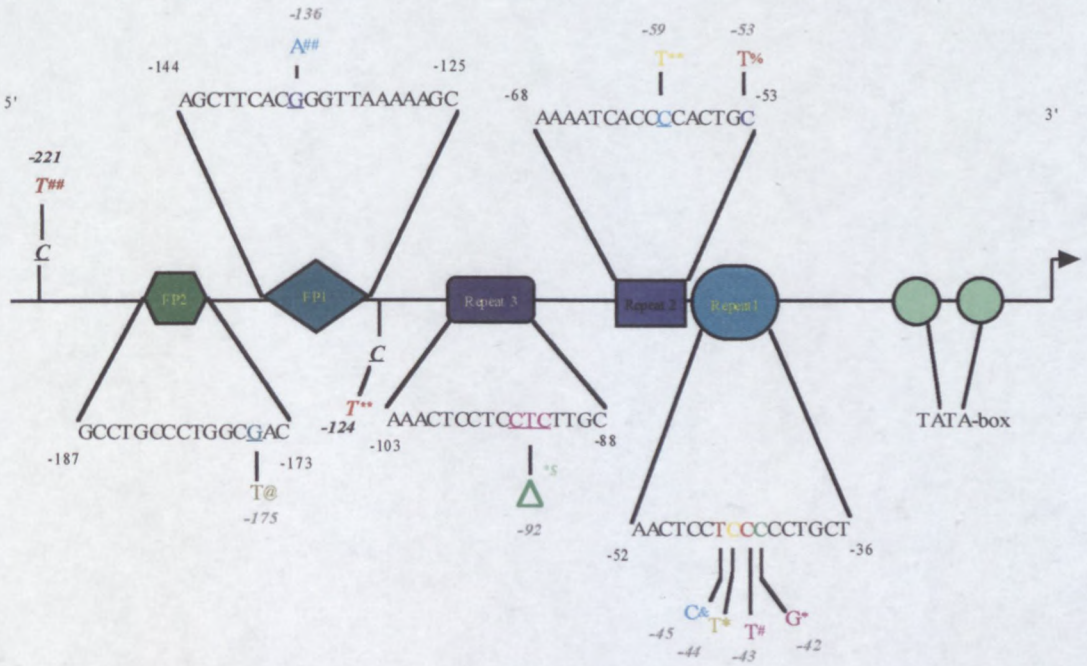
In 1997, Day and colleagues reported the first naturally occurring LDLR promoter mutation in repeat 2 (-53 c→t) of the LDLR gene. This variant, however, is unlikely to have a major effect on transcription of the LDLR gene, since it is located outside the region (SRE-1 site) identified to be critical for protein binding and sterol-mediated transcription (Smith et al, 1990; Briggs et al, 1993).

Further analysis of the LDLR promoter gene region in the South African population revealed an enrichment of promoter variants in the Black and Coloured (a people of mixed ancestry) populations, while apparently absent in Caucasians (Appendix A-C). Scholtz and colleagues (1999) reported the presence of a disease-causing mutation at position -59 (c→t) within repeat 2 of the LDLR gene in a South African Coloured family with FH. This mutation significantly reduced transcription of the LDLR gene and co-segregated with the FH disease phenotype in the family. In addition to this mutation, two other sequence changes, -124 c→t and -175 g→t,

were detected in the same family. The -124 c→t variant is located immediately adjacent to the FP-1 site and significantly increased LDLR transcription (~ 160% compared to control) while the -175 g→t variant, located within the FP-2 site, only slightly (non-significant) reduced transcription of the LDLR gene *in vitro*. The -59 c→t mutation and the -124 c→t polymorphism were detected together in a single individual in this family who presented with plasma cholesterol levels within the normal range. This finding appears to provide the possible *in vivo* evidence for interaction between the SRE-1 and FP1 site, as previously suggested by Dhawan and colleagues (1997).

Figure 1 provides a schematic presentation of the mutations and polymorphisms that have been identified in the LDLR promoter region. The influence on LDLR promoter activity is illustrated in Figure 2, for four of the variants that have been identified in South Africa.

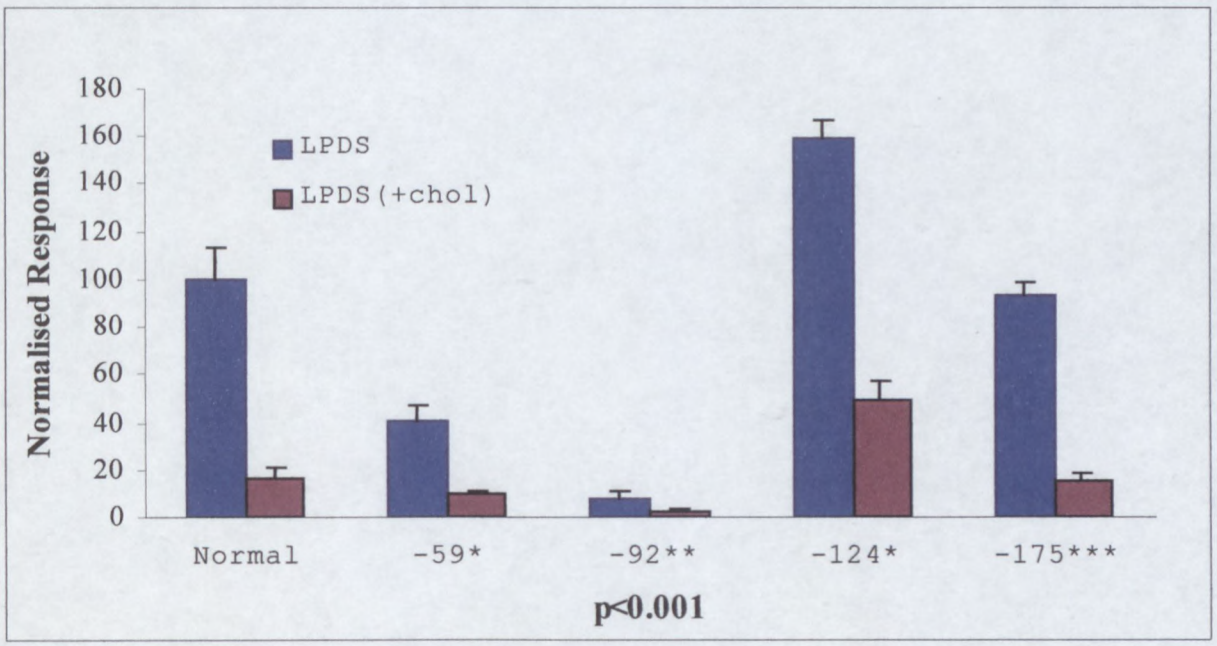
Figure 1: Schematic presentation of naturally occurring mutations in the promoter region of the LDLR receptor gene.



FP2 = footprinting 2 site; FP1=footprinting 1; TATA-box = TATA like sequences; underlined sequences indicate variants in the South African population

* Hobbs et al, 1992; #Koivisto et al, 1994; &Sun et al, 1995; %Day et al, 1997; $ Peeters et al, 1998; ** Scholtz et al, 1999; ## novel variants

Figure 2: Transient expression of luciferase reporter gene constructs containing the LDLR gene promoter. Illustration of the expression patterns of mutations identified in South Africa (-59 c→t; Δ -92 cct; -124 c→t; -175 g→t) compared to a mutation negative LDLR promoter construct. Luciferase gene activity was normalised against β-galactosidase activity.



*Scholtz et al, 1999; ** Peeters et al, 1998; *** Hoogendijk, 1999

Extended mutation analysis of the LDLR promoter region has recently resulted in the identification of two additional mutations, -136 g→a and -221 c→t, in South African subjects. The -136 g→a variant is located within the FP-1 site, where sequence variation was shown to influence binding of p125 to the FP-1 site and abolishes SRE-1 mediated LDLR transcription (Dhawan et al, 1997). The c to t base change at position –221 lies outside the described regulatory region of the LDLR gene (200 bp upstream of the transcription initiation site) and therefore further *in vitro* studies are required to determine the possible effect of this point mutation on transcriptional activity.

LDLR activity and disease pathogenesis

Infection and inflammation induces a wide array of metabolic changes, the so-called acute phase response, which is mediated by cytokines. Numerous reports have indicated that high lipoprotein levels are beneficial in combating infections (Owens et al, 1990; Xu et al, 1993; Hofer et al, 1994). Various cytokines, including oncostatin M (OM), tumour necrosis factor α (TNF α), interleukin 1β (IL-1β) and interleukin 6 (IL-6) have been shown to regulate transcription of the LDLR gene (Stopeck et al, 1993). In disease pathogenesis this would suggest an increase in LDLR gene transcription and increased endocytosis of LDL.

It has been reported that LDLR gene transcription is enhanced upon infection with herpersvirus 1 (HSV-1), leading to increased LDL endocytosis (Hsu et al, 1995). Other reports have indicated that cholesterol is essential for cellular transport as well as survival of certain pathogens (Phalen and Kielian, 1991; Bernardes et al, 1998; Gatfield and Pieters, 2000). Interestingly, it has recently been reported that certain pathogens compete with lipoproteins for cellular receptors (Coppens et al, 2000). Although the exact mechanism of cholesterol recruitment by pathogens is not fully understood, it is reported that viruses exploit transcription factors, (Sp1, YY1, LSF and CREB) important for gene expression (Romerio et al, 1997; Flamand et al, 1998; Rahaus and Wolff, 2000). Of interest to this study is the fact that binding and putative binding sites for these transcription factors are present in the LDLR gene promoter. Since the LDLR gene is considered an immediately early response gene, and the fact that certain pathogens exploit the LDL receptor to gain entry into the cell (Hofer et al, 1994), it appears likely that mutations in the promoter region of the gene will affect transcription and therefore viral entry. The -175 g→t variant, detected in the South African

Black and Coloured populations (Scholtz et al, 1999), are located within a putative YY1 binding site (Shi et al, 1997).

The FP2 site where the –175 g→t variant is located, contains a potential CRE-site which binds CRE-binding protein. Sheng et al. (1991) demonstrated that CREB is a Ca^{2+}-regulated protein and this might explain the finding that Ca^{2+} can increase LDLR gene transcription (Makar et al, 1994). The fact that LDL can increase intracellular Ca^{2+} levels (Sachinidis et al, 1991) might furthermore explain the possible link for the assosiation found between hypertension and the –175 g→t LDLR promoter variant. Various reports have indicated that increased LDL levels are associated with hypertension and decrease in lipid levels may decrease the incidence of hypertension (Ekelund, 1988; Flesch et al, 1994).

Atherosclerosis is a multifactorial disease which has been suggested to commence early in childhood, manifesting later in life (Stary, 1989). It is also one of the most prevalent and well-studied diseases in the Western world and is predicted to become the leading cause of death in developing countries by the year 2020. Interestingly, Wick et al (1995) (and other researchers) have provided evidence that atherosclerosis can be considered an immunologically mediated disease, an aspect that has been greatly neglected in classical atherosclerosis research. Evidence exists that humoral and cellular immune responses are involved at all stages of atherosclerotic development (Hansson et al, 1989; Libby and Hansson, 1991).

Abnormalities in cellular cholesterol homeostasis have been widely reported in the initiation and progression of various cancers, including chronic myeloid leukemia (CML) (Goel et al, 1996) and acute myelogenous leukemia (AML) (Rudling et al, 1998). Vitols and colleagues (1984) demonstrated that LDLR activity was increased in different

leukemia patients and also reported that hypocholesterolemia was frequently found in leukemia. In 1996, Goel and colleagues described a particular 47kDa protein (with affinity for SRE sequences) which was present in lymphocytes of normal individuals but not in CML patients. This finding suggests that the enhanced degradation of LDL observed in leukemia patients could be due to SRE-1 enhanced expression of the LDLR gene. When the normal cells proliferates, LDL receptor numbers are increased through increased LDLR gene expression (Kruth et al, 1979). Ho et al (1978) and Vitols et al (1984) demonstrated a significant increase in LDL degradation and receptor activity in mononuclear cells of leukemic patients compared to normal subjects, but the exact mechanism underlying this phenomenon is still unclear. Vitols et al (1990) have demonstrated that LDL could possibly be used as a carrier to target lipophilic cytotoxic drugs to specific leukemic cells *in vivo*. In 1996, Rudling and Collins reported that LDLR and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase mRNA levels were reduced in renal cell carcinoma. This finding prompted close scrutiny of other cancer cells and showed that the increased LDL degradation observed in cancer patients, appears to be independent of increased LDLR gene expression (Rudling et al, 1998).

In conclusion, it is evident that the importance of cholesterol is not always considered when defining the pathogenesis of disease. It has been reported that naturally low or clinically lowered cholesterol levels are associated with higher mortality rates due to increased suicide risk, violence-, and injury-related deaths not caused by malignancies (Forrette et al, 1989). With this in mind, Rauchhaus et al (2000) have suggested an alternative endotoxin-lipoprotein theory and stressed the need for a paradigm shift in the approach to the treatment of atherosclerosis. This suggested paradigm shift, however, does not eliminate the role of traditional risk factors, such as elevated cholesterol in the pathogenesis of

atherosclerosis, but only emphasise the importance of alternative approaches to managing heart disease and in concomitantly elevated cholesterol levels. The fact that various substances, apart from sterols, regulate LDLR gene transcription could be beneficial for the treatment regime of various diseases.

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CONCLUSION

The regulation of eukaryotic gene expression is complex and poorly understood and it is clear that, even from the present perspective, evolution has yielded a wide variety of mechanisms to control genomic activity. By studying the survival adaptation of the fish population (Fundulus heteroclitis) off the East Coast of North America, Schulte (2001) demonstrated that variations in regulatory sequences could provide evolutionary significant changes, which can greatly impact an environmental adaptation.

The role of lipoproteins (especially low-density lipoprotein) has been well established in cholesterol homeostasis and various diseases, particularly atherosclerosis. Insight gained during this study, however, implies that even though we have acquired immense knowledge on the functionality of LDL and cholesterol in the global homeostatic picture, this does not even begin to emphasise the importance of these substances in cellular survival.

The LDLR gene can be considered an early response gene (Makar et al, 1994; Dhawan et al, 1999), and its promoter region is highly conserved (few regulatory variants present). Even though some of the variants identified in the gene may only slightly affect transcription of the LDLR gene, it seems possible that these variants might hamper the ability of individuals to handle certain metabolic stresses, underlying some noncommunicable diseases. The association detected between the -175 g→t variant and diastolic blood pressure appears to support this hypothesis. We postulate that the effect of Ca2+ on LDLR gene expression and the subsequent increase in intracellular Ca2+ levels by LDL, might at least underline this association. Of further interest is the fact that this region contains a putative binding site for

YY1 and shows strong homology to a cAMP response element (CRE-1). It is noteworthy that both these factors have been implicated in the regulation of both cellular and viral genes.

Classical atherosclerotic research has failed to consider the role of humoral and cellular immune complexes involved in atherosclerosis. The presence of immune responses can be detected throughout atherosclerosis (Wick et al, 1995) and suggests that it can be considered an immunological disease. The endotoxin-lipoprotein hypothesis (Rauchhaus et al, 2000), which suggests an alternative approach to managing and treating atherosclerosis, could revolutionise the way we see atherosclerosis, thereby providing new targets for drug development.

Upon mitogenic stimulation, immediately early genes are expressed to adapt to the cellular changes. This process, and the fact that certain pathogens compete for the LDL receptor to gain cellular entry, emphasises the importance of the LDLR gene regulation in innate immunity.

The significant increase in clearance of LDL in various cancer cells emphasises the importance of cholesterol in proliferation, even though the precise mechanism is still unclear. Even though the LDLR and HMG-CoA genes are down regulated in renal cell carcinoma, this does not necessarily mean that these genes imply non-significance in the aetiology of cancer. It has been suggested that LDL could be used as a carrier to target specific lipophilic cytotoxic drugs to leukemic cells (Vitols et al, 1990).

The observed effect of the -59 c→t mutation on apoptosis suggests that this variant might inhibit apoptosis under conditions of normal immunological conditions. The cells which seem

to be the most sensitive to the effects of this mutation are the CD4 and CD8 T-cells and possibly also the eosinophils and basophils as seen by the increase in cell numbers of the latter two populations.

This study highlighted the importance of regulatory gene regions in the pathogenesis of disease. Since the sequencing of the human genome was completed a radical paradigm shift is needed in the approach to disease pathogenesis, since approximately 30 000 genes (instead of the initial 80-100 000 genes predicted) have been identified to be responsible for human diversity. This would therefore, necessitate further investigation into non-structural variations and protein-protein interaction in defining modern diseases. The LDLR gene represents as an excellent example of a single gene that may be involved in various conditions such as cancer and risk for heart disease. It thus seems plausible that alterations in serum lipid levels might affect progression of various diseases. This might be especially important in the African context, since variation in promoter regions may play an important role in adaptation to a more Westernised lifestyle/environment.

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APPENDIX A

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Mutation –59c→t in repeat 2 of the LDL receptor promoter: reduction in transcriptional activity and possible allelic interaction in a South African family with familial hypercholesterolaemia

Charlotte L. Scholtz[1,2,+], Armand V. Peeters[2,+], Christiaan F. Hoogendijk[1,2], Rochelle Thiart[1,2], J.N.P. de Villiers[1,2], Renate Hillermann[1,2], Jingwen Liu[3], A. David Marais[1,4] and Maritha J. Kotze[1,2,§]

[1]MRC Cape Heart Group, [2]Division of Human Genetics, Faculty of Medicine, University of Stellenbosch, PO Box 19063, Tygerberg 7505, South Africa, [3]Palo Alto VA Health Care System, Palo Alto, CA, USA and [4]Department of Medicine, University of Cape Town, Cape Town, South Africa

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The low-density lipoprotein receptor (LDLR) plays a major role in cholesterol homeostasis. Mutations in the regulatory region of the LDLR gene, although rare, have been shown to alter transcriptional activity of the gene and can cause familial hypercholesterolaemia (FH). In this study, a transition (c→t) was identified at nucleotide position –59 within repeat 2 of the LDLR promoter in a South African FH patient of mixed ancestry. By screening 17 family members of the index case for this promoter mutation, two additional single base changes (–124c→t and –175g→t) were identified, located at recently described *cis*-acting regulatory sequences of the LDLR promoter. Both the –59c→t and the –124c→t transitions were identified in the normocholesterolaemic son of the index patient. Reporter plasmids containing the normal and mutant promoter fragments were constructed by directional cloning. Transcription studies using a luciferase reporter system demonstrated that the –59c→t mutation significantly reduces promoter activity in both the presence and absence of sterols (~40% of normal activity), while the –124c→t variant increases transcription (~160%) of the LDLR gene. The intra-familial phenotypic variability observed amongst individuals with the –59c→t mutation can probably be ascribed to allelic interaction, suggesting that variation in the LDLR promoter region may contribute significantly to the phenotypic expression of FH-related mutations in populations where these mutations prevail.

INTRODUCTION

Familial hypercholesterolaemia (FH) is an autosomal dominant disorder affecting the regulation of cholesterol homeostasis. Clinical and biochemical features of FH include xanthomata, premature coronary heart disease (CHD) and elevated plasma cholesterol (1). Most FH-related mutations identified to date are located in the coding region of the low-density lipoprotein receptor (LDLR) gene, while mutations in the promoter region appear to be rare (2–5).

The essential regulatory elements of the LDLR gene lie within ~200 bp upstream of the transcription initiation site, and three imperfect direct repeats are largely responsible for promoter activity (6). Repeats 1 and 3 bind Sp1, a *trans*-acting transcription factor, which promotes transcription of the LDLR gene in the presence and absence of sterols (6,7). The 10 bp core sequence of repeat 2, designated the sterol regulatory element (SRE-1), is essential for high levels of transcription of the LDLR gene (8–10). In the case of sterol depletion, SRE-1 interacts with the essential transcription binding proteins (SREBP) to induce transcription of the LDLR gene (11–13), whilst responsible for sterol-mediated repression of the gene when cellular cholesterol levels are high (8,9). Mutations in the core of repeat 2 have been shown to reduce transcription significantly only in the absence of sterols (8,9). Two additional *cis*-acting regulatory elements have recently been identified and are designated footprinting 1 (FP1) and footprinting 2 (FP2) elements (14). FP1 and FP2, spanning nucleotide intervals –145 to –126 and –187 to –175, respectively, are deemed essential for maximal induction of transcription. In 1997, Dhawan *et al.* (15) suggested that FP1-induced transcription might be through interaction with SRE-1. A variety of substances, such as cytokines, growth factors and hormones, has also been reported to influence regulation of transcription of the LDLR gene (16,17).

In this study, we identified single base changes at nucleotide positions –59(c→t), –124(c→t) and –175(g→t) of the LDLR gene promoter in a South African family of mixed ancestry. Of these, only the –59c→t mutation located in repeat 2 was associated with the FH phenotype. Together with the sequence

+These authors contributed equally to this work
§To whom correspondence should be addressed. Tel: +27 21 9389441; Fax: +27 21 9317810; Email: mjk@gerga.sun.ac.za

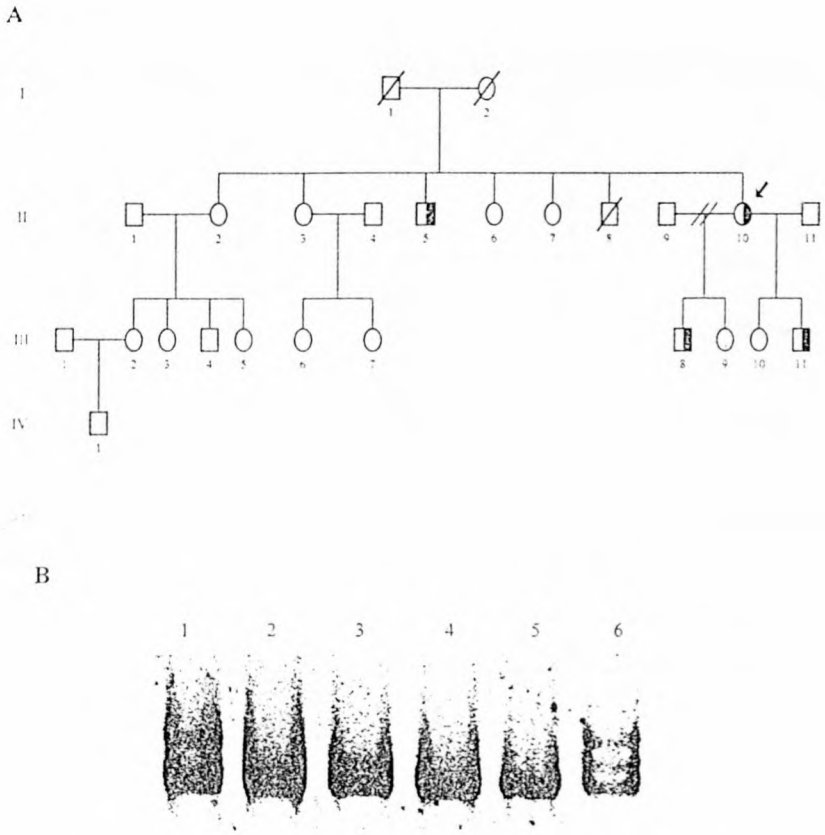

Figure 1. Mutation analysis in the index family. (A) Structure of the family. Family members with the apparently disease-related mutation –59c→t, including the normocholesterolaemic son (III-8) of the index patient (identified by an arrow), are indicated by half-filled symbols. (B) Heteroduplex analysis of the LDLR promoter region. DNA of a control individual with the –124c→t variant was loaded in lane 1. The index patient (lane 2) and one of her sons (lane 3) were heterozygous for mutation –59c→t, while the eldest son (lane 6) was heterozygous for both –59c→t and –124c→t. The promoter mutations were absent in the two daughters (lanes 4 and 5).

changes described here, nine different defects/polymorphisms have now been reported in the LDLR promoter region (3,4,10,18,19).

RESULTS

A base change (c→t) at nucleotide position –59 was identified by combined heteroduplex and single-strand conformation polymorphism (HEX–SSCP) analysis (20) in repeat 2 of the LDLR gene promoter in a South African FH patient of Afro-Euro-Malay origin. Subsequent screening for the mutation in 17 additional relatives revealed the presence of mutation –59c→t in the brother (II-5) and two sons (III-8, III-11) of the index case (Fig. 1A). Individuals II-5 and III-11 presented with total cholesterol (TC) levels above the 80th percentile for age and gender (21), while individual III-8 demonstrated an apparently normal lipid profile (Table 1). Interestingly, further mutation analysis in this individual (III-8) revealed an additional single base change (c→t) at nucleotide position –124 (Fig. 1B, lane 6). Furthermore, we detected a base change (g→t) at nucleotide position –175 in the samples of two normocholesterolaemic nieces (III-2, III-5) of the index patient. DNA sequence analysis of the three promoter mutations detected in the family is illustrated in Figure 2. The lipid profiles of the mutation-negative daughters of the index patient were within the normal range, while her mutation-negative siblings had moderately raised TC concentrations (below the 70th percentile) for age and gender according to Rossouw *et al.* (21).

DNA screening of an additional 151 FH heterozygotes from the same population for the presence of promoter variants led to the identification of a single patient with the –175g→t base change. Subsequent DNA screening of this individual for mutations in the coding region of the LDLR gene revealed a G→A base change (E237K) in exon 5 (R. Thiart and H. Nissen, unpublished data). In contrast, no disease-causing mutation could be identified in the coding region of the FH index patient with the –59c→t mutation. We also failed to identify an LDLR gene mutation in the hypercholesterolaemic sister (TC > 7 mmol/l and normal triglycerides) of the index case (II-7), who tested negative for mutation –59c→t. This individual was selected for extensive analysis of the LDLR gene (together with the index case) to screen for another genetic factor underlying the hypercholesterolaemia in the family. Mutations –124c→t (previously detected at a low frequency in Africans) and –59c→t were absent in 60 healthy blood donors of mixed ancestry included in this study, while the promoter variant at nucleotide position –175 was detected at a heterozygote frequency of 13% (8/60) in the control population.

Table 1. Characteristics of the index case and several relatives

Subjects	Gender	Age (years)	TC (mmol/l)	TG (mmol/l)	HDLC (mmol/l)	LDLC (mmol/l)	Mutation	Apo E genotype
II-2	F	71	6.5	2.7	0.9	4.4	–	3/3
II-3	F	68	7.7	3.0	0.9	5.4	–	3/4
II-5	M	63	9.2	1.7	1.2	7.2	–59c→t	3/3
II-6	F	60	5.9	1.3	1.5	3.8	–	3/3
II-7	F	57	7.3	1.0	1.4	5.4	–	3/4
II-10[a]	F	54	9.5	0.9	1.3	7.8	–59c→t	3/3
II-11	M	60	3.0	0.9	0.9	1.7	–	3/4
III-2	F	44	4.0	0.8	1.1	2.5	–175g→t	3/3
III-3	F	31	3.7	1.0	1.2	2.0	–	3/3
III-4	M	38	5.2	2.3	1.0	3.1	–	3/3
III-5	F	42	4.5	1.0	1.0	3.0	–175g→t	3/3
III-6	F	37	6.1	1.2	0.8	4.7	–	3/3
III-7	F	42	7.2	0.9	0.7	6.1	–	4/4
III-8	M	36	4.9	0.5	2.1	2.6	–59c→t/–124c→t	3/3
III-9	F	30	5.3	0.5	0.8	4.3	–	2/3
III-10	F	23	4.5	0.5	1.3	3.0	–	3/3
III-11	M	15	5.1	0.7	1.6	3.2	–59c→t	3/3
IV-1	M	23	4.4	0.9	1.0	3.0	–	3/3

Subject numbering according to the pedigree in Figure 1A.
[a]Index case.

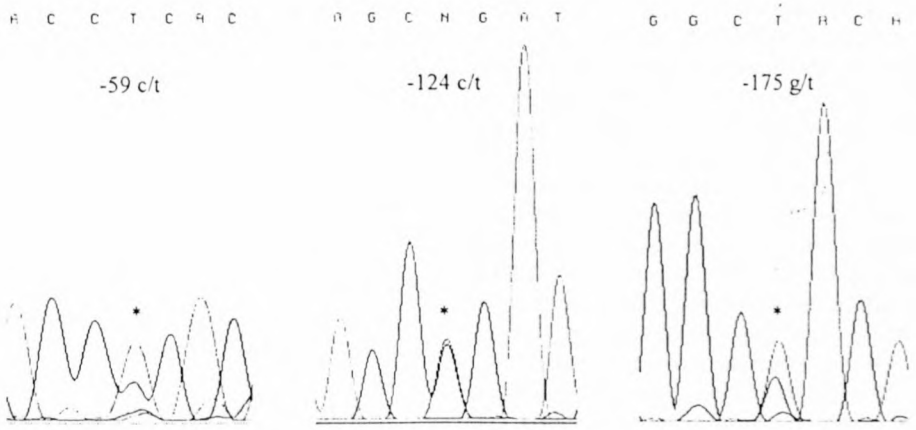

Figure 2. DNA sequence analysis in members of the index family with mutations –59c→t, –124c→t and –175g→t. Each base change is depicted in the sequence by an asterisk (*).

In order to determine the possible allelic effects of the sequence changes identified at nucleotide positions –59 and –124 in individual III-8, PCR-amplified products encompassing nucleotide interval –244 to +55 of the wild-type and mutated promoters were cloned into a luciferase reporter vector and transiently transfected into human hepatoma (HepG2) cells. High levels of transcription were observed for the wild-type promoter, while the promoterless vector (pGL3 Basic) showed virtually no effect in the HepG2 cells. In comparison, the –59c→t transition significantly reduced transcription of the LDLR gene promoter to ~40% of normal activity, while the –124c→t base change increased promoter activity to ~160% of normal in sterol depleted cells ($P < 0.05$). In HepG2 cells supplemented with sterol-containing medium, transcription of the LDLR gene decreased to ~16%, while transcription of the mutant promoters was reduced to 10% (–59c→t) and 50% (–124c→t), respectively (Fig. 3).

Apolipoprotein (apo) E genotyping was performed in an attempt to determine whether mutation –59c→t exhibits only a mild effect on LDLR function that is exacerbated by the E4 allele of this polymorphism (22). All individuals with mutations in the LDLR promoter region were found to be homozygous for the neutral E3 allele of the apo E polymorphism (Table 1). Notably, the presence of the cholesterol-raising E4 allele was detected in all

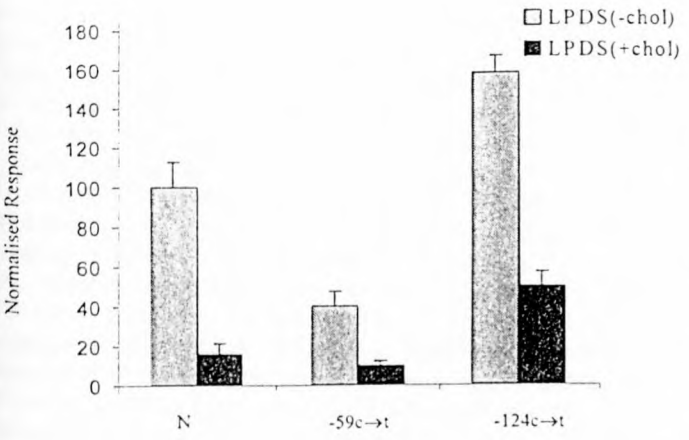

Figure 3. Analysis of wild-type (N) and mutated (–59c→t, –124c→t) LDLR promoter activity under transient transfection conditions. Luciferase gene activities were normalized against β-galactosidase activity.

three family members (II-3, II-7, III-7) with TC levels raised 7 mmol/l in the absence of mutation –59c→t.

DISCUSSION

The base change (c→t) identified at nucleotide position –59 in the index family represents the first report of a naturally occurring mutation in the 10 bp core sequence of repeat 2 in the LDLR promoter. *In vitro* results demonstrated that this mutation dramatically diminishes transcription of the LDLR gene in both the absence and presence of sterols. The transfection assays were performed in HepG2 cells, where a reduction of transcriptional activity to ~40% of normal in sterol-deficient cells was observed. It has been shown previously that the nucleotide interval –65 to –56, the core sequence of repeat 2, is essential for high levels of transcription, as well as sterol-mediated repression of the gene (8,9). Interestingly, transversions (c→g/a) incorporated at nucleotide position –59 (8,9) virtually abolished (to ~10% of normal activity) the induction of transcription in the absence of sterols, but did not reduce transcription of the LDLR gene in the presence of sterols as observed with the –59c→t mutation identified in the South African patient. This observation may be explained by the fact that the naturally occurring mutation represents a transition, reflecting the possible significance of the specific base content at a given position within repeat 2. Since a mutation at nucleotide position –59 results in non-binding of nuclear proteins (9), and hypermethylation of guanine –59 is positively associated with activation of LDLR gene transcription in skin fibroblasts (23), we conclude that the –59c→t mutation is the most likely cause of the FH phenotype in the index patient. Further evidence in favour of a causative role of this mutation includes the failure to identify an additional disease-causing mutation in the coding region of the LDLR gene in the index patient, its apparent absence in healthy control individuals and the fact that this site has remained conserved throughout evolution. The –53c→a mutation reported previously in repeat 2 (4) (I.N.M. Day, personal communication) is located at a position unlikely to have a major effect on sterol-mediated regulation of the LDLR gene (8,9).

The –59c→t transition was detected in three family members of the index case. In keeping with the above-mentioned data, her brother (II-5) and youngest son (III-11) with (only) mutation –59c→t, presented with TC levels above the 80th percentile for age and gender. In a follow-up study of lipid determinations (data not shown) performed after a 10 month period in nine (II-5, II-7, II-10, II-11, III-2, III-4, III-8, III-11, IV-1) relatives, TC concentration in the 15-year-old son (III-11) of the index patient with mutation –59c→t was only moderately raised (4.6 mmol/l; >60th percentile), which can probably be ascribed to altered expression of FH-related mutations during childhood (24). The follow-up TC determinations also confirmed the FH status of the index case's brother (II-5; TC 8.4 mmol/l), the non-FH status of her sister (II-7; 6.4 mmol/l), and the normal lipid profile of her eldest son (III-8; 4.9 mmol/l) carrying both mutations –59c→t and –124c→t. Plasma cholesterol levels remained normal in subjects II-11, III-2, III-4 and IV-1 without mutation –59c→t, which is consistent with the finding that this mutation co-segregates with the FH phenotype in family members without the sequence variant at nucleotide position –124(c→t).

Although it was not possible to study the influence of the numerous environmental (e.g. diet) and genetic factors that may contribute to the abnormal lipid profile in the index family, possible allelic effects imposed by the common apo E polymorphism were excluded in those subjects with mutations in the LDLR promoter region. The presence of the apo E4 allele, previously shown to be associated with raised plasma cholesterol concentrations (22), may nevertheless explain the raised TC levels in mutation-negative hypercholesterolaemics II-3, –7, III-7 (TC > 7 mmol/l).

The normal plasma cholesterol levels detected in the mutation-positive son (III-8) of the index patient can probably be ascribed to allelic interaction between the mutations at nucleotide positions –59 and –124, since the transfection results revealed a statistically significant increase in promoter activity for the –124c→t construct alone, to ~160% of normal in sterol depleted cells. The –124c→t variant is located adjacent to the FP1 *cis*-acting regulatory element (position –126 to –144), previously implicated in maximal induction of the human LDLR gene transcription in response to low cellular cholesterol levels. Demonstration in this study that the variant (c→t) at nucleotide position –124 increases LDLR transcriptional activity indicates that the boundary of the FP1 enhancer sequence should probably be extended to include nucleotide position –124. It is noteworthy that this variant was absent in the FH patients analysed. This observation may be due to chance, but as illustrated in the index family, it is highly unlikely that an individual with this apparently favourable variant as well as a disease-causing LDLR gene mutation would present with elevated plasma cholesterol levels; except maybe when the two sequence changes occur on the same chromosome. Interestingly, DNA screening of >1000 individuals from eight different ethnic groups demonstrated the presence of variant –124c→t at a low frequency (1–3%) in populations with an African genetic element, while apparently absent in Caucasians (25).

Identification of variant –175g→t in normocholesterolaemic individuals, as well as in a single proband (who died recently at

the age of 50 years of a heart attack) with an FH-related mutation in the coding region of the LDLR gene, indicates that this base change does not cause the FH phenotype. However, as suggested by our preliminary data obtained in the South African Black population (26), it is possible that the presence of the –175g→t variant imposes susceptibility or an increased risk for the development of symptomatic FH in patients with other disease-related mutations. In a study of the coding region of the LDLR gene in South African FH patients of mixed ancestry, we demonstrated recently that Caucasian admixture has contributed significantly to the disease phenotype in this indigenous population (27). Since the –175g→t polymorphism has not been detected in Caucasians, we postulate that the significantly lower frequency (1/151) of this variant in FH patients of mixed ancestry compared with controls from the same population (~13%), is probably a reflection of the genetic profile at the LDLR gene locus in FH patients as a consequence of admixture linkage disequilibrium. Interestingly, variant –175g→t is located within a recently defined FP2 *cis*-acting regulatory element, and disrupts a putative binding site for the multifunctional transcription factor F-ACT1 (YY1) (14). However, whether the presence of the rare –175t allele increases CHD risk in FH patients of mixed ancestry was not the focus of this study, and therefore further data are not included in this report.

This study highlights the role of the SREBP in the regulation of LDLR gene transcription and suggests that the –59c→t mutation, leading to reduced transcriptional activity both in the presence and absence of sterols, is the causative FH mutation in the index family. In contrast to clinical FH homozygotes who present with severely elevated plasma cholesterol levels due to the presence of two mutant LDLR alleles, we have identified an individual with two point mutations in the promoter region of the gene, whose cholesterol concentration is within the normal range. Further studies are warranted to determine whether the normocholesterolaemic status in this 'compound heterozygote' may provide the sought-after *in vivo* evidence for interaction between FP1 and SRE-1, as postulated previously by Dhawan *et al.* (15).

MATERIALS AND METHODS

Details of the index patient and study subjects

The index case is a 54-year-old South African woman of mixed ancestry (San, Khoi, African Negro, Madagascar, Javanese and Western European origin), clinically diagnosed with FH. She presented at age 44 with angina, xanthomata and arcus corneae, smoked ten cigarettes per day and had no documented family history of premature CHD. At this age, her pre-treatment TC concentration was 7.3 mmol/l, TG 0.6 mmol/l, high-density lipoprotein cholesterol (HDLC) 1.6 mmol/l and low-density lipoprotein cholesterol (LDLC) 5.5 mmol/l. For inclusion in this study, her plasma lipid levels were measured again together with that of 17 additional family members, using standard techniques (Table 1).

Follow-up mutation screening was performed in 151 unrelated FH heterozygotes of mixed ancestry attending the Groote Schuur Hospital lipid clinic. The selection of the samples was based on previously described criteria for a diagnosis of FH (24), including TC > 7 mmol/l, the presence of tendon xanthomas and/or premature CHD in the index patient or first-degree relatives. DNA of 60 healthy blood donors from the same population was also included. All blood samples were obtained with informed consent and the study protocol was approved by the appropriate Institutional Ethics Review Committee.

DNA analysis

Genomic DNA extracted from whole blood was amplified by PCR on an OmniGene Thermal Cycler (Hybaid, UK) using LDLR promoter primers 5′-GAGGCAGAGAGGACAAT-GGC-3′ (forward) and 5′-CCACGTCATTTACAGCATT-TCAATG-3′ (reverse). PCR products were denatured and fragments resolved on a low cross-linked polyacrylamide gel for combined HEX-SSCP analysis (20) of the LDLR gene. Fragments demonstrating altered mobility were sequenced manually and variation confirmed on an automated sequencer, ABI373 (Perkin Elmer, Foster City, CA). Determination of apo E genotypes was performed using oligonucleotide primers F4 and F6 (28), restriction enzyme digestion with *Hha*I and gel electrophoresis (29).

Reporter vector constructs and transient transfection assays

Construction of reporter plasmids containing the normal and mutant promoter fragments and transient transfection assays were performed as described previously (18). The calcium phosphate method was used to transfect HepG2 cells with the plasmid DNA. Triplicate wells were assayed for each transfection condition and at least three independent transfection assays were performed for each construct. Luciferase activity was normalized against β-galactosidase activity to correct for transfection efficiency. The *in vitro* results obtained with the –59t and –124t transcripts described in this study have been verified by Peeters (30) in CHO cells using Transfectam (Promega, Madison, WI) as the transfection reagent.

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APPENDIX B

Predominance of a 6 bp deletion in exon 2 of the LDL receptor gene in Africans with familial hypercholesterolaemia

Rochelle Thiart, Charlotte L Scholtz, Joseph Vergotine, Christiaan F Hoogendijk, J Nico P de Villiers, Henrik Nissen, Klaus Brusgaard, Dairena Gaffney, Michael S Hoffs, W J Hayward Vermaak, Maritha J Kotze

Abstract

In South Africa, the high prevalence of familial hypercholesterolaemia (FH) among Afrikaners, Jews, and Indians as a result of founder genes is in striking contrast to its reported virtual absence in the black population in general. In this study, the molecular basis of primary hypercholesterolaemia was studied in 16 Africans diagnosed with FH. DNA analysis using three screening methods resulted in the identification of seven different mutations in the coding region of the low density lipoprotein (*LDLR*) gene in 10 of the patients analysed. These included a 6 bp deletion (GCGATG) accounting for 28% of defective alleles, and six point mutations (D151H, R232W, R385Q, E387K, P678L, and R793Q) detected in single families. The Sotho patient with missense mutation R232W was also heterozygous for a de novo splicing defect 313+1G→A. Several silent mutations/polymorphisms were detected in the *LDLR* and apolipoprotein B genes, including a base change (g→t) at nucleotide position −175 in the FP2 *LDLR* regulatory element. This promoter variant was detected at a significantly higher ($p<0.05$) frequency in FH patients compared to controls and occurred in cis with mutation E387K in one family. Analysis of four intragenic *LDLR* gene polymorphisms showed that the same chromosomal background was identified at this locus in the four FH patients with the 6 bp deletion. Detection of the 6 bp deletion in Xhosa, Pedi, and Tswana FH patients suggests that it is an ancient mutation predating tribal separation approximately 3000 years ago.

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Keywords: apolipoprotein B; hypercholesterolaemia; low density lipoprotein receptor; mutation

MRC Cape Heart Group, Division of Human Genetics, Faculty of Medicine, University of Stellenbosch, Tygerberg, South Africa
R Thiart
C L Scholtz
J Vergotine
C F Hoogendijk
J N P de Villiers
M J Kotze

Department of Clinical Chemistry, Odense University Hospital, Odense, Denmark
H Nissen
K Brusgaard

Department of Pathological Biochemistry, Glasgow Royal Infirmary University NHS Trust, Glasgow, UK
D Gaffney
M S Hoffs

Institute of Chemical Pathology, University of Pretoria, South Africa
W J H Vermaak

Correspondence to:
Dr Kotze, Division of Human Genetics, Faculty of Medicine, University of Stellenbosch, Francie van Zijl Drive, Clinical Building Room 2126, PO Box 19063, Tygerberg 7505, South Africa, mjk@gerga.sun.ac.za

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Autosomal dominant hypercholesterolaemia (ADH) is most commonly caused by mutations in the low density lipoprotein receptor (*LDLR*) gene causing familial hypercholesterolaemia (FH), or in the apolipoprotein B (*ApoB*) gene causing familial defective apo B (FDB).[1 2] These biochemical defects result in the precipitation of excess cholesterol and clinical characteristics include tendon xanthomata and premature coronary heart disease (CHD). The estimated incidence of both FH and FDB is approximately 1 in 500 in most white populations.

In the Afrikaner population of South Africa, the prevalence of FH has been increased to approximately 1 in 70, as a consequence of a founder effect following the introduction of at least three defective *LDLR* gene alleles by European settlers.[3–5] This is in striking contrast to the apparently low prevalence of FH in the black population, reported to have migrated from central Africa to the south in three main groups, the Ngunis (Xhosa, Tembu, Swazi, and Zulu) along the east coast, the Sothos (South Sotho, North Sotho/Pedi, West Sotho/Tswana) who settled further west on the Transvaal highveld, and the Vendas living in the northern Transvaal area.[6 7] We suspect that FH is not frequently recognised in Africans because of altered clinical expression and not because of a lower mutation prevalence compared to most other populations. Previous studies have indicated that the mutational mechanisms giving rise to germline mutations are largely a function of the local DNA sequence environment.[8–10]

Since the situation in South Africa is ideal for studies of underlying lipid related genetic differences among population groups,[11] we attempted to identify black hypercholesterolaemics to determine the spectrum of mutations in the promoter and coding region of the *LDLR* gene and in exon 26 of the *ApoB* gene. FDB has not previously been studied in the South African black population, but was found to be rare in other South African populations, most likely because of a "negative" founder effect that diluted the frequency of the common *ApoB* 3500 mutation in the immigrants relative to their parent populations.[12]

Subjects and methods

SUBJECTS

Blood samples were collected from 56 black patients attending lipid clinics in South Africa, after obtaining informed consent and ethical approval from the regional Review Committees. Details on clinical features and ethnicity were provided by the referring clinicians. Sixteen patients with a diagnosis of "classical" or "probable" FH, including two FH homozygotes, were selected for extensive mutation analysis for the coding and promoter region of the *LDLR* gene and exon 26 of the *ApoB* gene. Blood samples were also obtained from 38 of their family

Table 1 Characteristics of African probands analysed for LDLR and apo B gene mutations

Index	*Ancestry*	*Sex*	*Age*	*TC* (mmol/l)‡	*TG*	*HDL*	*LDL*	*Clinical*	*LDLR gene sequence changes*	*Apo B gene sequence changes*	*Relatives tested*
CM	Xhosa	F	52	8.5	2.7	1.5	5.8	CHD	R793Q		0
MX	Xhosa	M	50	10.8	2.0	0.9	9.0	Arc, Xan	−175g→t		0
AN	Swazi	F	58	10.1	0.9	1.3	8.4	Arc, Xan, CHD	D151H		0
AS	Swazi	M	49	8.0	1.0	2.1	5.4	Arc, CHD	P678L		0
AM*	Swazi/Zulu	F	56	8.3	1.5	1.9	5.7	Arc			11
CK†	Zulu	M	26	13.8	0.8	1.3	12.1	Arc, Xan	−175g→t		1
SH	Sotho	M	33	12.7	2.2	1.2	10.5	Arc, Xan	313+1G→A; R232W		4
RK	Sotho	M	58	10.7	2.3	1.0	8.6	CHD	R385Q		0
KN†	Pedi	F	32	14.9	0.8	1.4	13.1	Arc, Xan	6-bp del, 6-bp del		0
EF	Pedi	F	56	13.1	1.1	1.2	11.4	Arc, PVD, CHD	E387K; −175g→t; C347C	T3552T; T3540T	10
LP	Pedi	F	61	9.4	0.8	0.9	8.1	Arc, Xan	6-bp del		3
CN*	Pedi	F	57	7.4	2.6	0.9	5.3	Arc, PVD			6
RM*	Pedi/Tswana	F	54	10.8	0.4	1.3	9.3	Arc, ?Xan			3
LM*	Tswana	F	56	6.1	1.8	1.8	3.5	Arc, CHD	−175g→t	T3552T	0
RL	Tswana	F	30	9.3	0.8	1.7	7.2	Arc, Xan	6-bp del		0
CS	Tswana	F	47	7.9	0.7	1.7	5.9	Arc	6-bp del		0

The majority of mutations summarised in this table were included in a recent mutation update.[22]
Reference plasma cholesterol concentrations in the general black population are given in ref 24.
TC, total cholesterol; TG, triglycerides, HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; CHD, coronary heart disease; PVD, peripheral vascular disease; Arc, arcus cornealis; Xan, xanthomata.
*Probable FH.
†Clinical FH homozygotes.
‡Pretreatment concentrations, except for proband LM for whom pretreatment levels were not available.

members (table 1). Classical FH (12 probands) was defined as the occurrence of pretreatment total cholesterol (TC) >7 mmol/l, with the presence of tendon xanthomata or premature CHD or both in the index case or a first degree relative. Probable FH (four probands) was defined by the same pretreatment cholesterol level and primary hypercholesterolaemia or premature CHD or both in the family (table 1). DNA samples of the 40 lipid clinic patients without the FH phenotype, but who had hyperlipidaemia or normal lipid profiles in the presence of vascular disease, were included for analysis of specific regions of the *LDLR* gene. Ninety six people drawn from the same population (19 Pedis, 21 Sothos, 27 Xhosas, and 29 Zulus) were sampled as controls. TC, high density lipoprotein cholesterol (HDLC), and triglyceride (TG) determinations and extraction of genomic DNA were performed using standard methods.[13] Plasma LDL cholesterol (LDLC) concentrations were calculated with the Friedewald formula (LDLC=TC−(HDL+TG/2.18)).[14]

MUTATION DETECTION

Heteroduplex single strand conformation polymorphism (HEX-SSCP) analysis was performed in South Africa[15] and denaturing gradient gel electrophoresis (DGGE) in Denmark[16] and Scotland[17] to screen polymerase chain reaction (PCR) amplified genomic DNA for mutations in the *LDLR* and *ApoB* genes. For HEX-SSCP analysis, the exon specific primers described by Jensen *et al*[18] were used, while the promoter region of the *LDLR* gene was amplified using primers 5'-GAGGCAGAGAGGACAATGGC-3' and 5'-CCACGTCATTTACAGCATTTCAATG-3'. Base changes in the promoter region were numbered according to Hobbs *et al*,[19] after adding an additional A within the AAAA stretch preceding repeat 1, which is missing from the published sequence.[20] PCR products showing aberrant electrophoresis patterns were sequenced on both strands with a PCR Product Sequencing kit (Amersham) or an automated sequencer ABI 373A or both.

HAPLOTYPE ANALYSIS

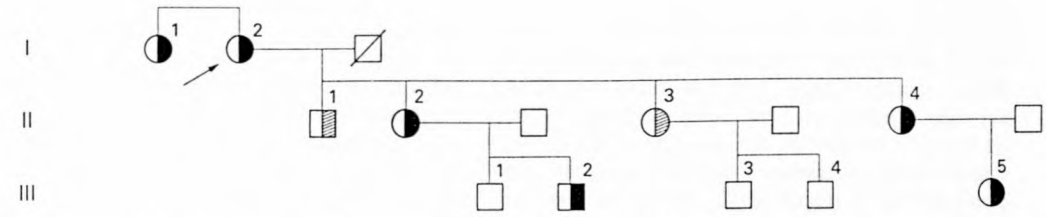
Haplotype analysis using four *LDLR* gene polymorphisms was performed according to Theart *et al*.[21] Microsatellite markers VWA31, F1A1, and TH01 (Profiler kit, Applied Biosystems) were used to test for biological consistency in two families.

STATISTICAL ANALYSIS

Allele frequencies were determined by allele counting. Testing for significance of heterogeneity in mutation frequencies among patient and control groups was based on the chi-square and Fisher's exact tests.

Results

Extensive DNA screening of the *LDLR* gene in 16 black FH patients, using both the DGGE and HEX-SSCP screening methods, showed six missense mutations in individual families and a 6 bp deletion in four probands (table 1).[22] The deletion (FH Cape Town-1), previously described in a Xhosa FH homozygote,[23] and missense mutations D151H and R385Q have not (yet) been reported in other populations. Haplotype *Sma*I+/*Stu*I+/*Ava*II− was associated with the deletion in all three FH heterozygotes and a homoallelic FH homozygote. Screening of the coding region in DNA of the four FH patients heterozygous for a base change (g→t) at nucleotide position −175 of the *LDLR* gene promoter resulted in the detection of a recycling deficient mutation E387K[19] in the DNA of subject EF. Interestingly, this Pedi proband was found to be extremely heterogeneous at the DNA level, since a silent C to T base change was furthermore detected at nucleotide position 1104 in exon 8, in addition to two silent mutations in the *ApoB* gene. The G to C change in the third base of codon 3540 (T3540T) and the T to C change in the third base of codon 3552 (T3552T) in the *ApoB* gene have previously been reported in



Age (y)	62	57	38	35	15	13	31	12	7	26	5
TC (mmol/l)	9.3	13.1	5.8	10.5	2.8	6.1	7.4	3.5	5.2	8.6	7.3
LDLC (mmol/l)	5.9	11.4	2.8	8.8	1.8	4.8	5.2	1.9	4.0	7.2	6.2
HDLC (mmol/l)	3.0	1.2	2.4	1.1	0.8	0.8	1.4	1.3	0.9	1.0	0.8
TG (mmol/l)	0.9	1.1	1.3	1.5	0.4	1.1	1.7	1.6	0.6	0.8	0.7
Xanthomas	–	–	–	–	–	–	–	–	–	+	–
CVD/PVD	–	PVD	–	–	–	–	–	–	–	–	–
E387K	+–	+–	––	+–	––	+–	––	––	––	+–	+–
–175G/T	+–	+–	+–	++	+–	+–	––	––	––	+–	+–
*Sma*I	++	++	++	++	++	++	++	++	++	++	++
*Stu*I	++	++	++	++	++	++	+–	++	++	+–	++
*Ava*II	––	––	––	––	––	––	––	––	––	––	––
*Nco*I	++	++	++	++	++	++	++	++	++	++	++

Figure 1 Pedigree of proband EF (arrow) clinically diagnosed with heterozygous FH. Clinical, biochemical, and genetic data are provided for people for whom DNA samples were available. Those with raised plasma cholesterol levels are indicated by dark (mutation positive for E387K) and shaded symbols. The presence (+) or absence (–) of LDLR gene mutations and recognition sites for SmaI, StuI, AvaII, and NcoI are indicated.

Nigerian and African-American subjects, respectively.[17] One of the daughters of proband EF (II.3 in fig 1) carried two copies of the silent *ApoB* mutation at codon 3540. RFLP analysis indicated that haplotype *Sma*I+/*Stu*I+/AvaII–/*Nco*I+ cosegregated with the −175t allele in the family (fig 1). This chromosomal background was also identified in two of the other probands with the sequence substitution at −175 in the *LDLR* promoter region, while haplotype *Sma*I–/*Stu*I+/*Ava*II+/*Nco*I+ was associated with the t allele in the Tswana proband (LM), who also carried the T3552T variant in the *ApoB* gene.

In order to determine whether the two mutations identified in each of probands EF and SH occur in cis or in trans on their respective chromosomes, blood samples were obtained from additional family members for segregation analysis. Pedigree analysis in the family of EF showed that mutation E387K and the −175g→t variant occur on the same chromosome (fig 1). All the family members who inherited the 387K/−175t haplotype (I.1, I.2, II.2, II.4, III.2, and III.5) had abnormally high TC and LDLC levels. II.2, with a clinical diagnosis of heterozygous FH, was homozygous for the t allele at nucleotide position −175. This implies that her deceased father (husband of the index case) also carried the −175g→t promoter variant, but in the absence of mutation E387K. Her normocholesterolaemic son (III.1), as well as her brother, inherited this paternal chromosome, the latter presenting with a moderately raised TC value. The proband's son (II.1) and one of her daughters (II.3) (confirmed by marker studies using highly informative microsatellites) had moderately raised plasma cholesterol concentrations in the absence of either the promoter variant or the exonic mutation, indicating that another unknown factor contributes to the abnormal lipid profile observed in this family. TC concentrations were found to be very low in the general black population (approximately 3 mmol/l) compared with other South African groups.[24 25]

DNA screening of the 53 year old father of proband CK, diagnosed with homozygous FH, showed homozygosity for the t allele at nucleotide position −175. His TC and LDLC levels were 6.11 mmol/l and 4.29 mmol/l, respectively, which is comparable to that of a FH heterozygote. Plasma TG and HDLC concentrations were 1.49 mmol/l and 1.14 mmol/l, respectively, and the only clinical feature indicative of hyperlipidaemia in this obligate FH heterozygote was corneal arcus.

HEX-SSCP analysis indicated that the splicing defect identified in exon 3 represents a de novo event in the family of SH, since it was not present in any of his close relatives analysed. Familial relationship was illustrated by transmission of the exon 5 mutation (R232W) from the father (72 years, TC 4.1 mmol/l), and was further substantiated by marker studies using three highly informative microsatellites (data not shown). Mutation R232W was absent in the normocholesterolaemic brother (30 years, TC 3.5 mmol/l) and sister (42 years, TC 3.3 mmol/l) of the proband. Their mother, aged 62 years, presented with a TC level of 2.9 mmol/l. It was therefore not possible to determine whether the splice mutation occurred in cis in the proband on the paternal chromosome bearing mutation R232W, or in trans on the normal maternal chromosome.

Subsequent DNA screening of 96 controls from the general black population comprising 56 Ngunis (27 Xhosas, 29 Zulus) and 40 Sothos (19 Pedis, 21 Sothos) resulted in the identification of six subjects (four Ngunis (one Xhosa, three Zulus) and two Sothos (one Pedi, one Sotho)) heterozygous (6%) for the −175t allele. Although the number of patients analysed is small, the frequency of this allele appeared to be higher within each tribal group (2/6 Ngunis and 2/10 Sothos with FH)

compared to the controls (4/56 Ngunis and 2/40 Sothos). An overall statistically significant difference ($p<0.05$) was observed between the presence of the rare t allele in the general black population (0.03) compared to its frequency of 0.13 in the patients diagnosed with classical or probable FH ($\chi^2=5.916$, 1 df, $p=0.0149$). We furthermore detected five carriers of the −175g→t polymorphism among 40 lipid clinic patients without the FH phenotype (13%), showing an intermediate allele frequency of 0.06. This was not significantly different from the frequencies observed in the FH ($\chi^2=1.326$, 1 df, $p=0.249$) or control ($\chi^2=1.474$, 1 df, $p=0.224$) groups. Variant −175g→t was also detected in 1/47 DNA samples of controls from the Venda tribe studied by Ehrenborg *et al*,[25] which was absent in more than 300 whites screened.[26]

Discussion

Numerous low density lipoprotein receptor (*LDLR*) gene mutations (>600) have been identified in FH patients, but genetic data on black African populations are rare.[19 22 23 27] A striking finding is that a 6 bp deletion predominates in a small number (5/18) of FH patients (this study)[19 23 28] identified in the South African black population, where this lipid disorder is thought to be rare. This deletion in exon 2 removes an aspartic acid and a glycine from the first cysteine rich ligand binding repeat of *LDLR*, and impairs its transport but not lipoprotein binding in fibroblasts.[23] Frequent detection of a deleterious mutation can be the result of consanguinity, recurrent mutational events, genetic drift, founder gene effect, multiple introduction of the mutation into a population, or heterozygote advantage.

The 6 bp deletion identified originally in a homoallelic Xhosa FH homozygote,[23] and now also in a homozygous Pedi and three FH heterozygotes (Pedi and two Tswanas) on the same haplotype, have not (yet) been reported in other populations. These findings largely exclude the likelihood of a recurrent mutational event because of slipped mispairing or multiple entries of the deletion mutation into the black population. Detection of the deletion in different tribes suggests that it originated in Africa approximately 3000 years ago before tribal separation.[29] Although FH patients with the deletion may therefore be distantly related, family ties cannot at present explain its relatively high prevalence among black FH patients. The apparently low prevalence of FH in South African blacks and the large population size furthermore argue against a founder effect. It is, however, possible that the deletion mutation was propagated and inherited within a small group of people who later evolved separately into different African tribes. Another plausible explanation is that this deleterious deletion mutation may be associated with a selective advantage in Africa. Already in 1990 Hobbs *et al*,[30] noted that the presence of several founder mutations in different South African population groups[4 31] may be indicative of a Darwinian selection that favours the heterozygous state in this region of the world. Since the most likely selective agent in Africa would be infectious diseases, the finding that *LDLR* deficient mice are protected against lethal endotoxaemia and severe gram negative infections[32] supports the likelihood of such an evolutionary selection mechanism conferring a survival advantage. In addition to binding and inactivating endotoxin, lipoproteins also bind certain viruses and inhibit their infectivity.[33]

Although the family data presented in this study show that the −175g→t polymorphism residing in a cis acting element in the LDLR promoter[34] does not cause the FH phenotype in affected subjects, further studies are warranted to investigate the likelihood that this variant may influence disease expression. The possibility that the significantly higher frequency of the −175g→t promoter polymorphism in South African black FH patients compared to controls ($p<0.05$) is caused by linkage disequilibrium with another downstream mutation causing the FH phenotype was excluded by haplotype studies showing that the rare t allele was associated with different *LDLR* haplotypes. This allele furthermore cosegregated with missense mutation E387K in one family. These different chromosomal backgrounds may be the result of recombination events, reflecting the age of the −175g→t variant. Compared to whites, blacks are considered older in evolutionary terms[35] and can therefore be expected to have accumulated variation over longer times. It is possible that the −175g→t polymorphism did not spread to other parts of the world, thereby explaining its apparent absence in whites (this study).[18 36] The African origin of the −175g→t variant was confirmed by detection of the rare t allele at a low frequency in control DNA samples obtained from Nigerians and African-Americans.[26] African-Americans originated mostly from the western African coast and arrived in North America between the 16th and 19th centuries.

One Sotho proband was heterozygous for a known splicing defect in intron 3 (313+1G→A) and for the R232W mutation in exon 5. In all the patients with mutation 313+1G→A studied to date, the splicing defect is associated with a clinical picture of severe hypercholesterolaemia and early CHD.[37 38] Patient SH had a TC concentration of 13 mmol/l, but it is uncertain whether this high level is solely because of the 313+1G→A mutation or whether there is an additional effect of the downstream R232W mutation. Family studies could not rule out the possibility of a double mutation, but showed that the splicing defect is the consequence of a de novo mutation. None of the family members of SH were hypercholesterolaemic, including his 72 year old father (LDLC 1.9 mmol/l), who was heterozygous for mutation R232W. This finding indicates that R232W does not affect LDLR function or, alternatively, that clinical expression of this missense mutation is altered by other genetic/environmental factors.

Although the missense mutations identified have not been characterised further, they are likely contributors to the FH phenotype in our

patient sample, since all the codon changes involve conserved amino acids and were not detected in the normal population. Screening for mutations causing FDB[16 17 39] resulted in the identification of two silent mutations, T3540T and T3552T (data not shown), previously described in a Nigerian and African-American subject, respectively.[17] Failure to identify disease related mutations in all the patients studied may be because of limitations imposed by the screening techniques used, clinical misdiagnosis of FH, or mutations in other genes causing the ADH phenotype.[40 41]

Both the Zulu and Pedi patients clinically diagnosed with homozygous FH presented with relatively low pretreatment TC levels (<15 mmol/l) for this severe condition[1] and neither have yet suffered from CHD. The relatively mild expression of homozygous FH in these subjects largely precludes an estimation of the prevalence of heterozygous FH in the South African black population based on the prevalence of homozygous FH. Raised plasma cholesterol levels causing FH in a family frequently remain undetected until the occurrence of coronary events or clinical signs indicative of FH is observed in one or more family members. This may particularly be the case in the South African black population, as hypercholesterolaemics with lipid profiles compatible with the diagnosis of heterozygous FH frequently lack xanthomata characteristic of this condition (this study).[42] None of the FH heterozygotes with the relatively severe 6 bp deletion in exon 2[23] presented with CHD. These findings provide evidence that FH is probably underdiagnosed in the South African black population, most likely as a consequence of altered expression of FH related mutations. This may be the result of interaction with other genetic and environmental factors, including a prudent diet.[11] Data provided by us and others[43–45] therefore suggest that clinical/biochemical criteria for the diagnosis of FH need to be different by country/population and that DNA methods may assist in making a definitive disease diagnosis.

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APPENDIX C

Inter-ethnic variation and allelic effects of the -175g→t variant in the FP2 *cis*-acting regulatory element of the LDL receptor gene

Christiaan F. Hoogendijk,[1] Charlotte L. Scholtz,[1] Simon M. Pimstone,[2] Ewa Ehrenborg,[2] Johannes J.P. Kastelein,[3] Joep C. Defesche,[3] Rochelle Thiart,[1] Lana du Plessis,[1] J. Nico P. de Villiers,[1] Rhena Delport,[4] David C. Rubinsztein,[5] Leslie J Raffel,[6] Clarence. E. Grim,[7] Sonia Mediene-Benchekor,[8] Philippe Amouyel,[9] Thierry Brousseau,[9] Michael R. Hayden[2] and Maritha J. Kotze[1]

[1]MRC Cape Heart Group, Division of Human Genetics, Faculty of Health Sciences, University of Stellenbosch, Tygerberg, South Africa, [2]Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada, [3]Lipid Research Group, Academic Medical Centre, Amsterdam, The Netherlands, [4]Department of Chemical Pathology, Faculty of Medicine, University of Pretoria, Pretoria, South Africa, [5]Department of Medical Genetics, Addenbrooke's NHS Trust, Cambridge, United Kingdom, [6]Cedars-Sinai Medical Center, Los Angeles, California, USA, [7]Medical College of Wisconsin, Milwaukee, USA, [8]Laboratoire de Biologie Moléculaire et Génétique, Institut des Sciences de la Nature, Université d'Oran es-Sénia, Oran, Algeria and [9]Unité 508 INSERM, Institut Pasteur de Lille, Lille, France.

Address for correspondence and reprints: Dr. MJ Kotze, Division of Human Genetics, Faculty of Health Sciences, University of Stellenbosch, P.O. Box 19063, Tygerberg, South Africa. Phone: 27 21 9389441; Fax; 27 21 9317810; E-mail: mjk@gerga.sun.ac.za.

Abstract

DNA samples of 2303 individuals obtained from nine different population groups were screened for a polymorphic variant (g→t) at nucleotide position -175 of the low-density lipoprotein receptor (LDLR) promoter. This variant, residing in a *cis*-acting regulatory element, predominates in Black South Africans with familial hypercholesterolaemia (FH), most likely due to enhancement of the clinical expression of FH when the -175t allele occurs in association with another disease-related mutation. The objectives of this study were 1) to determine the global distribution of the promoter variant, 2) to determine whether the -175g→t polymorphism affects transcriptional activity of the LDLR gene, and 3) to use this information to assess the likelihood of negative selection against the rare -175t allele during human evolution. The –175g→t variant detected at carrier frequencies of 3-10% in different African population groups was absent in the Caucasian and Asian (Chinese) individuals studied. In contrast to previous findings in Black South African FH patients, the -175t allele occurred at a significantly lower frequency in hypercholesterolaemics from the recently admixed Coloured population of South Africa compared with population-matched controls ($P<0.0001$). Haplotype and mutation analysis excluded the likelihood that this finding is due to association with another FH-related mutation in the patient group, although reversal of the positive association with FH observed in the Black population may, at least in part, be due to admixture linkage disequilibrium. Finally, transient transfection studies in HepG2 cells demonstrated that the -175t allele is associated with a non-significant decrease (~7%) of LDLR transcription in the absence of sterols. The data presented in this study suggest that the -175g→t polymorphism may have subtle effects that become clinically important within certain genetic and/or environmental contexts and highlight the potential consequences of admixture between populations with different disease risks.

Introduction

Elucidation of the role of polymorphic variants in susceptibility to common diseases represents a major challenge in human genetics. Certain commonly occurring polymorphisms may contribute significantly to genetic risk of cardiovascular disease (CVD), while less frequent but highly penetrant mutations may represent the primary cause of disease subtypes such as familial hypercholesterolaemia (FH). FH is inherited in an autosomal co-dominant fashion and is estimated to occur at a frequency of 1/500, although a significantly increased (~1/70) disease prevalence has been reported in the Afrikaner population of South Africa due to the presence of three common founder gene mutations in the low-density lipoprotein receptor (LDLR) gene (Kotze et al. 1991). To date, more than 700 FH-related mutations (http://www.ucl.ac.uk/fh, http://www.umd.necker.fr) have been identified in the LDLR gene, whilst single-nucleotide polymorphisms (SNPs) that alter the amino acid sequence of the protein appear to be rare (Hobbs et al. 1992). This may be due to evolutionary selection against deleterious alleles as suggested by Cargill et al. (1999), although the effect of individual polymorphic variants may be relatively mild and therefore difficult to detect due to potential confounding factors that may differ between populations. By studying more than 500 SNPs in 106 genes, these authors demonstrated that polymorphisms involving amino acid sequence changes are found at a lower rate and with lower allele frequencies than silent substitutions that are less likely to influence disease. This finding relates well to the early prediction (Kotze et al. 1989c) that the non-synonymous StuI polymorphism identified in the LDLR gene (Kotze et al. 1986), and occurring at a relatively low frequency (~0.08) in various populations (Kotze et al. 1989a; http://www.umd.necker.fr), may have a mild phenotypic effect. Gudnason et al. (1995) have subsequently shown that the rare allele of

this mutation (A370W) is indeed associated with elevated plasma cholesterol levels in the general population.

Although the promoter regions of most genes remains poorly characterised, the 5'-untranslated region of the LDLR gene has been studied extensively. The first comprehensive mutation analysis of the LDLR promoter region (Top et al. 1992) suggested that variation in this area does not play a significant role in the aetiology of FH. However, several sequence changes that may affect transcriptional activity of the LDL receptor have subsequently been reported by us (Peeters et al. 1998; Scholtz et al. 1999; Thiart et al. 2000) and others (Hobbs et al. 1992; Koivisto et al. 1994; Sun et al. 1995; Jensen et al. 1996; Day et al. 1997b). Frequent detection of polymorphic variants (allele frequency >1%) in the LDLR promoter region in subjects of African origin (Scholtz et al. 1999) raised the possibility that the phenotypic expression of FH mutations might be influenced by variation in the LDLR promoter region in populations where these mutations prevail (Thiart et al. 2000). This notion was substantiated by the detection of two LDLR promoter mutations with opposite effects on transcriptional activity, in a subject with normal plasma cholesterol levels who was part of a South African FH family (Scholtz et al. 1999). Mutation -59c→t was shown to reduce promoter activity (~40% of normal activity) and co-segregated with the FH phenotype, while the -124c→t polymorphism increased transcriptional activity (~160%). Another point mutation (g→t) detected at nucleotide position -175 in association with different FH-related mutations (Scholtz et al. 1999; Thiart et al. 2000), most likely contributes to or "unmasks" expression of the disease phenotype / mutation in affected South African patients. Segregation analysis in a Black FH family with mutation E387K (Thiart et al. 2000) and a South African Coloured family with mutation E237K (Scholtz et al. 1999), demonstrated that the -175g→t variant

occurs in *cis* with both these mutations in the respective families. These studies demonstrated that the promoter polymorphism at nucleotide position -175 does not cause the disease phenotype in the absence of another FH mutation, since only those subjects with sequence changes in both the promoter and coding regions presented with elevated plasma cholesterol levels.

In this study extended analysis of the -175g→t variant was performed in nine different population groups, in order to determine the global distribution of this polymorphism and its possible allelic effect.

Subjects and Methods

Study Population

DNA samples of 2303 unrelated subjects from ethnically diverse population groups have been screened for the -175g→t polymorphism (Table 1).

Caucasians. The 742 Caucasian subjects were from five distinct groups: 357 white South Africans of European descent, 200 Belgians, 145 French, 20 Australians and 20 New Zealanders. Except for 327 of the white South Africans included as controls, and the 145 French participants, all the other subjects were hyperlipidaemics attending lipid clinics in the different countries.

Asians. The 133 unrelated control individuals of Chinese (Cantonese) ancestry were recruited from six different Chinese family physician practices in Vancouver. These subjects were previously included in a similar study of a promoter variant in the lipoprotein lipase (LPL) gene (Ehrenborg et al. 1997).

Algerians. The 123 unrelated Algerian male subjects were randomly selected from a representative list of households living in Oran region, from the Algerian National census provided by the Algerian national demographic and statistic department.

Nigerian Blacks. The 27 Sub-Saharan Africans from Nigeria were referred to the East Anglian Regional Genetics Service Laboratory in Cambridge, UK, for the diagnosis of sickle-cell anaemia (Rubinsztein et al. 1994).

Khoisan. The 103 Khoisan people were from the area of Schmidtsdrift in the North-West Cape. These individuals belonged to the Vaskela tribe or the Barakwena or Negroid type.

Coloureds. A total of 775 subjects (300 hypercholesterolaemics and 475 controls) from the Coloured population of South Africa, a people of mixed ancestry (Khoisan, West African Negro, Madagascar, Javanese, Malay and European origin). Sixty-one of the control individuals were unrelated healthy blood donors recruited via the Western Province Blood Transfusion services. The other 414 control individuals came from the Moravian mission Mamre, located on the western perimeter of the Swartland. The hyperlipidaemics attended lipid clinics in the Western Cape Province and included 200 patients with a diagnosis of classical or probable FH (Loubser et al. 1999) and 100 non-FH hyperlipidaemics.

South African Blacks. The 211 Black South Africans were from three tribes originating mainly from central Africa: 51 Ngunis (27 Xhosa, 24 Zulu), 66 Sothos (34 South Sotho, 32 North Sotho/Pedi) and 94 Vendas. The 94 control subjects from the Venda tribe living in rural areas of South Africa were previously included in a similar study of a promoter variant in the LPL gene (Ehrenborg et al. 1997). Fifty-four individuals from the different tribes were dyslipidaemic patients (including 14 FH patients) attending lipid clinics in the Southern and Northern regions of South Africa.

American Blacks. The 30 African-Americans included 6 FH patients and 24 non-hyperlipidaemic control individuals recruited as part of a family-based, cardiovascular genetic epidemiology study.

Caribbean Blacks. Twenty-six of the 105 subjects of the Caribbean Black population living in the Netherlands were hypercholesterolaemic patients from Curacao attending a lipid clinic in Amsterdam. The remainder of the subjects, 79 in total, were control individuals from Antilles, Surinam, and Hindustani origin.

Biochemical Analysis

Plasma levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were determined in 706 of the unrelated South African control subjects as previously described (Kotze et al. 1987; Ehrenborg et al. 1997). LDL cholesterol was calculated according to the Friedewald formula [LDLC = TC – (HDLC + TG/2.18)] (Friedewald et al. 1972).

Analysis of genomic DNA

DNA was extracted from whole blood using standard procedures (Miller et al. 1988). Polymerase chain reaction (PCR) amplification of genomic DNA and analysis of the -175g→t variant were performed as previously described (Thiart et al. 2000). Analysis of the tetra-nucleotide repeat marker D19S394 was performed as previously described (Day et al. 1997a), using primers C_{11} (5'-AGACTACAGTGAGCTGTGG-3') and C_{12} (5'-GTGTTCCTAACTACCAGGC-3').

Cloning and transient transfection assays

The wild type (-175g) and mutated (-175t) promoter fragments were inserted within the *Hind*III and *Bgl*II cloning sites of the promoterless luciferase reporter vector pGL3 (Promega), essentially as described previously (Peeters et al. 1998). For cloning purposes, a *Bgl*II site was incorporated in the 3' region of the forward PCR primer (5'-CCA-ATTTGAGGGGGCGTCAGATCTTCACC-3') and a *Hind*III site in the 3' region of the reverse PCR primer (5'-GGGTTTCAAGCTTGGAC-ACAGCAGGTCGTG-3'), that were used together to amplify the fragments containing the different alleles. Plasmid DNA for transfection experiments was prepared using a Promega Plasmid Kit (Promega Corporation, Madison, WI). Human hepatoma (HepG2) cells were cultured in EMEM medium supplemented with 10% foetal calf serum, 100 units/ml of penicillin G, and 100 μg/ml streptomycin. Transfection assays in HepG2 cells were performed using the calcium phosphate co-precipitation method as previously described (Peeters et al. 1998). Absolute luciferase activity was normalised against β-galactosidase activity to correct for transfection efficiency. Triplicate wells were assayed for each transfection condition and at least three independent transfection assays were performed for each luciferase construct.

Statistical analysis

Significance of frequency distribution of the -175t allele between and within populations was determined using chi-square analysis and Fisher's exact two-tail probability when appropriate. Group differences in biochemical parameters were determined using an analysis of variance. P-values <0.05 were regarded as statistical significant.

Results

Frequency of the -175t allele in patients and controls

Allele frequencies of the -175g→t variant were determined in the different population groups indicated in Table 1. The -175t allele was not detected in any of the Caucasian or Chinese individuals, but was present in all the populations of African origin. In the general South African population, the highest carrier frequency (12.6%) was detected in the Coloured population. In this particular subpopulation, a lower frequency of the -175t allele was observed in hyperlipidaemics compared with controls ($p<0.0001$). This finding is in contrast to the results obtained in Black patients attending lipid clinics in South Africa for whom, as previously reported by Thiart et al. (2000), the frequency of the mutated allele was significantly higher compared with controls drawn from the same population ($p<0.01$). Frequent detection of the -175g→t variant in the Khoisan population, who has contributed significantly to the gene pool of the Coloured population, largely excludes the possibility that the rare allele has been introduced into the Coloured population strictly as a consequence of Black admixture. Finally, the -175t allele was absent in hypercholesterolaemic American (a relatively small number of patients and controls were available for analysis) and Caribbean Blacks included in the study, whilst detected at frequencies of 12.5% and 3.8%, respectively, in normolipidaemics subjects from these populations.

Analysis of microsatellite marker D19S394

Genotyping using D19S394 (approximately 250-kb upstream of the LDLR gene) was performed to determine whether the significant differences in allele frequencies observed between patient and control groups in the Coloured population, and the trend observed in

the Caribbean and American Blacks, could be ascribed to allelic association. As previously demonstrated following mutation screening and/or analysis of four intragenic LDLR gene polymorphisms in non-Caucasian FH patients (Scholtz et al. 1999; Thiart et al. 2000), the -175t allele was observed on various chromosomal backgrounds in both the patient and control populations. The results obtained in subjects from the Coloured population are shown in Figure 1.

Phenotypic effects of the -175t allele

Lipid profiles were assessed in 705 individuals from the general South African population (Table 2). No statistically significant differences were found in individuals with or without the -175t allele in the Coloured, South African Black or Khoisan populations, after correcting for age and gender. In the four Coloured individuals, aged 29 to 64 years, found to be homozygous for the -175g→t variant, TC levels ranged from 4.4 to 6.8 mmo/l, and LDLC from 2.9 to 4.7 mmol/l. Plasma cholesterol levels were consistently higher in the Coloured population compared with the Black and Khoisan populations, a phenomenon that can probably be ascribed to Caucasoid admixture as indicated earlier by direct LDLR mutation screening in the Coloured population (Loubser et al. 1999).

Transient transfection assays

To further ascertain a possible phenotypic effect, the activity of the wild-type and mutated LDLR promoter fragments was analysed using transient transfection assays. Since hepatocytes constitute the main cell type synthesising cholesterol, the human hepatocyte cell line HepG2 was used. Using as a reference the activity of the construct containing nucleotide -175g, it was found that the -175t allele decreased transcription (~7%) of the LDLR gene in the absence of sterols (Figure 2), but these differences did not reach statistical

significance. When cells were grown in medium supplemented with sterols instead of lipoprotein-deficient serum, luciferase activity was reduced to approximately 90-95% of normal. The promoterless vector pGL3 Basic demonstrated virtually no effect in the HepG2 cells (data not shown).

Discussion

Failure to identify the -175 g→t LDLR promoter variant in the Caucasian or Asian populations indicates that this is an African-specific polymorphism, possibly due to development of this variant after the migration of these groups from Africa. Detection of the polymorphism in the Khoisan population as well as in different Black tribes in South Africa, however, suggests that it originated before the protonegriform Africans split into two major branches, the Khoisan and the Negro. Based on archaeological evidence, the separation probably began more than 6000 years ago (Tobias 1974). Non-African populations have experienced bottlenecks in population size during which less common alleles such as -175t could have been lost through genetic drift or selection (von Haeseler et al. 1996). After testing certain theoretical predictions, Cargill et al. (1999) suggested that the minor allele of a functional polymorphism in current populations may in a significant proportion of cases be the oldest, and the extent of linkage disequilibrium around this allele would be expected to be quite small.

Among the Black populations with some degree of Caucasoid admixture, viz. South African Coloured, American Black and the Caribbean Black populations, the frequency of the t-allele was consistently higher in controls than in patients attending lipid clinics. In the South African population, the highest allele frequency was detected in the Coloured

population, and the t-allele was significantly more common in the control group (0.07) than in patients (0.01) ($p<0.0001$). None of the American and Caribbean Black FH patients/hyperlipidaemics carried the -175t allele. While this may simply reflect the relatively small number of hyperlipidaemic patients studied, it is also possible that the -175g→t variant does not contribute to the development of dyslipidaemia in these populations. Failure to detect the LDLR promoter variant in these groups may also be a consequence of admixture linkage disequilibrium and/or selection acting against the mutated allele during human evolution. The selection hypothesis, as proposed by Cargill et al. (1999), is supported by the finding that the rare -175t allele appears to be associated with the development of dyslipidaemia in the South African Black population (Thiart et al. 2000), since the frequency was significantly higher in patients attending lipid clinics (0.09) than in the control population (0.02).

In an attempt to explain the aforementioned findings we considered the following explanations with respect to the -175g→t variant: 1) association/linkage disequilibrium with another disease-related mutation(s); 2) contribution to phenotypic variability via interaction with other lipid-related mutation(s); 3) negative selection in Westernised populations as a consequence of "incompatibility" with a non-African genetic background; 4) undetected in lipid clinic patients of recently admixtured populations due to the predominance of other major genetic risk factors which are prevalent in European parent populations; 5) apparent mutation enrichment in the promoter region of the LDLR gene in Africans as a consequence of selective advantage. The latter possibility has not been investigated further, but it is noteworthy that evidence is accumulating for potential mechanisms by which changes in lipid metabolism and serum lipoproteins may be beneficial in host defence (Hardardottir et al. 1995; Netea et al. 1995). It is therefore

possible that alleles considered to be detrimental in relation to disease susceptibility may be maintained as a consequence of improved survival to reproductive age following infectious disease challenge. The multiple founder-related LDLR gene mutations identified in South Africa (Leitersdorf et al. 1989; Kotze et al. 1989b; Meiner et al. 1991) led to a hypothesis by Hobbs and colleagues as early as 1990, that this apparent mutation enrichment may be caused by a Darwinian selection that favours the heterozygous state in Africa (Hobbs et al. 1990). Although we have recently demonstrated (Durst et al. 2001) that the founder effect explains the high frequency of the common Lithuanian mutation (G197del) in the Jewish FH population (80% in South African Jews with FH) (Meiner et al. 1991), positive selection due a selective advantage or genetic factors modifying the response of a mutation's carriers to historical or local environmental constraints remains a possibility in the African context.

Haplotype analysis with four intragenic (*Sma*I, *Stu*I, *Ava*II, *Nco*I) LDLR gene polymorphisms in the South African population (Thiart et al. 2000), and genotyping of a highly informative microsatellite marker D19S394, demonstrated that association with a specific disease-related mutation downstream in the LDLR gene is an unlikely explanation for the differences detected in allele frequencies of the -175g→t polymorphism among hyperlipidaemic and control populations. Specific polymorphisms/haplotypes were not predominantly associated with the mutant allele in hyperlipidaemics or patients with FH, compared with individuals from the respective control populations. Since marker D19D394 is located at a physical distance of 250 kb from the LDLR gene, there is an expected 1/400 chance of recombination between D19S394 and the LDLR locus in a single generation (Day et al. 1997a), while cross-over events would be less likely with respect to polymorphisms and mutations within the LDLR gene.

A possible explanation for the significantly lower frequency of the -175g→t variant in South African Coloured patients attending lipid clinics compared with controls, may be related to the genetic make-up of this "selected" hyperlipidaemic group. It has recently been shown that Caucasian admixture contributes significantly to the FH phenotype in the Coloured population of South Africa (Loubser et al. 1999). Since the -175g→t variant appears to be absent in Caucasians, it is possible that the significantly lower frequency of the mutated allele detected in Coloured hyperlipidaemics compared with controls from the same population, is a reflection of the genetic origin of the LDLR gene locus in the hyperlipidaemic subjects. Afrikaners of European descent are at high risk for FH due to a founder effect in South Africa (Kotze et al. 1991), and African populations such as the Khoisan presumably are at low risk. Since these two populations contributed roughly equally to the gene pool of the present-day Coloured population (Loubser et al. 1999), the risk of FH would therefore be proportional to the number of LDLR copies that are of European descent. Any LDLR gene copy that carries the -175t allele must be of African descent, and therefore this allele would be expected to be inversely associated with FH.

Family studies previously performed in the South African Black population suggested that the -175g→t variant does not directly cause the FH phenotype in affected individuals, but that it may contribute to phenotypic variability via interaction with other lipid-related mutations, such as the allelic association with E387K reported by Thiart et al. (2000). Identification of a new mutation, E237K, in association with the -175t allele (Scholtz et al. 1999) in one of the 200 Coloured FH patients analysed, appears to be in accordance with these findings. Although it is not possible to exclude the likelihood that the -175t allele occurred together with mutation E237K in the FH family by chance, it is noteworthy that the proband died at the age of 50

years of a heart attack, perhaps suggesting that the -175g→t variant may impose a health threat with Westernisation. If the mutated allele is largely incompatible with a non-African genetic background and/or Western lifestyle one would anticipate a decrease in the frequency or absence of the allele as observed in populations of European descent.

Recently, an association has been detected between the -175g→t variant and diastolic blood pressure in the Coloured population of South Africa (C.L. Scholtz et al., manuscript in preparation). Since the study participants included in this study have been recruited from the general population for comparisons between subjects with and without variant –175g→t, the likelihood of population substructures leading to spurious associations was largely excluded. Further studies are, however, warranted to determine whether variant -175g→t directly causes hypertension *per se* or perhaps jeopardises the ability of genetically susceptible individuals to handle certain metabolic stresses, which secondarily result in changes in blood pressure. In light of the results obtained in FH families with this promoter variant (Scholtz et al. 1999; Thiart et al. 2000), failure to demonstrate an association with plasma cholesterol levels in three South African population groups studied may suggest that the 175g→t polymorphism is context-dependent.

Since the -175g→t variant resides in a FP2 cis-acting regulatory element and may disrupt a putative binding site for the multifunctional transcription factor YY1 (Mehta et al. 1996), we investigated the effect of the -175g→t variant *in vitro*. In accordance with the family data and lipid comparisons presented, the cloning and transient transfection studies revealed a non-significant decrease of approximately 7% in transcriptional activity for the mutated allele. However, since the *cis*-acting elements responsible for non-sterol

regulation of the LDLR gene by intracellular calcium and molecules including hormones, cytokines and growth factors have not yet been defined (Makar et al. 1994), further studies are warranted to investigate the possibility that variant -175g→t is involved in this form of gene regulation. Differential expression may be expected in association with different environmental contexts, seeing that the same mutation in the LPL gene has been associated with both increased and decreased transcriptional activity depending on the type of cell line used (Ehrenborg et al. 1997).

In conclusion, this study has provided further evidence that promoter regions may harbour DNA polymorphisms which, instead of being phenotypically and clinically neutral, may exert some influence on the transcriptional activity of the downstream gene. Such comparatively minor effects on transcription may not always be immediately apparent but could still assume clinical significance in combination with other sequence changes, whether it be another polymorphism or a disease-causing mutation in the same/other gene. The trends observed in this study may be a reflection of the serious consequences that may arise from a sequence variant in the regulatory region of the LDLR gene being expressed within a new genetic and environmental framework. Although mild phenotypic effects are unlikely to influence reproductive fitness, further studies are warranted to assess the likelihood that the apparent mutation enrichment in the LDLR gene promoter in populations of African descent may impose a major health threat with Westernisation.

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FIGURE LEGENDS

Figure 1. Analysis of microsatellite marker locus D19S394 in patients and controls of mixed ancestry with the -175g→t variant on a 6% polyacrylamide denaturing gel. Lanes 1-6: PCR-amplified genomic DNA of control individuals. Lanes 7-10: PCR-amplified genomic DNA of hypercholesterolaemic subjects. Fragment sizes corresponding to a M13 sequence marker are indicated in base pairs.

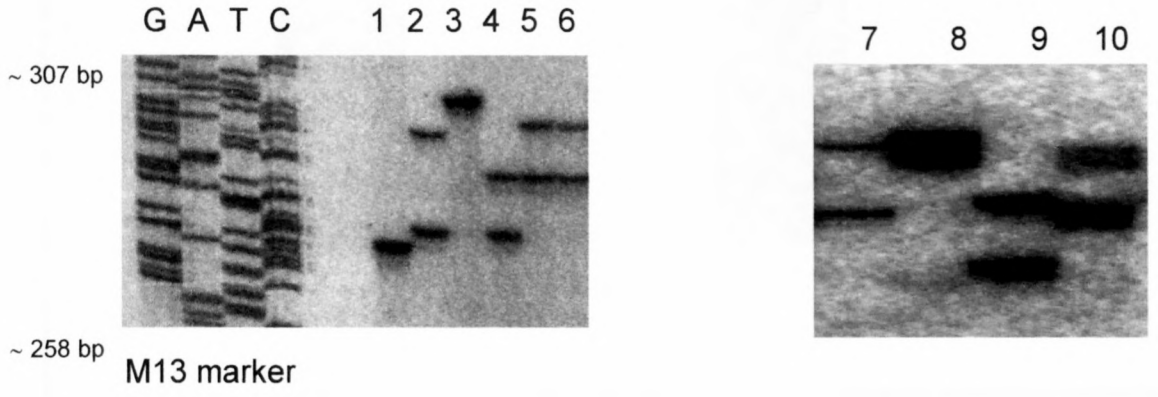

Figure 2. Analysis of wild type and mutated LDLR promoter activity under transient transfection conditions.

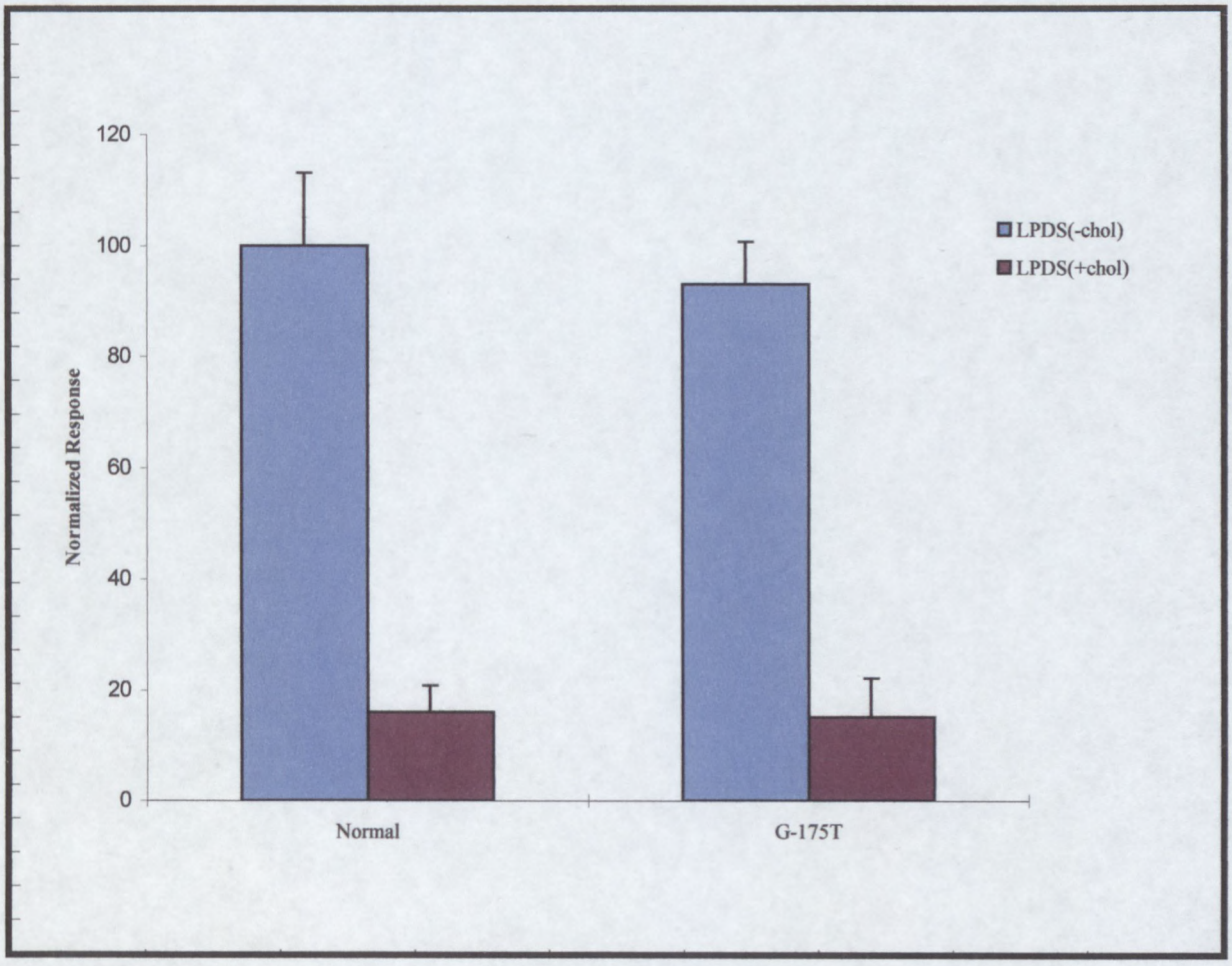

Table 1. Comparison of genotype distribution and allele frequencies of the -175g→t variant in different population groups

Population	n	Frequencies Alleles		Genotypes			Carrier %	P value
		g	t	gg	gt	tt		
Caucasians	742	1.0	0.0	742	0	0	0.0	
Chinese	133	1.0	0.0	133	0	0	0.0	
Algerians	123	0.98	0.02	119	4	0	3.1	
Nigerian Blacks	27	0.98	0.02	26	1	0	3.6	
Khoisan	103	0.98	0.02	99	4	0	3.8	
Coloureds	775						8.4	<0.0001
Patients	300	0.99	0.01	296	4	0	1.3	
Controls*	475	0.93	0.07	415	56	4	12.6	
SA Blacks	265						7.3	<0.01
Patients	54	0.91	0.09	44	10	0	16.8	
Controls	211	0.98	0.02	201	10	0	4.6	
American Blacks	30						18	0.302
Patients	6	1.0	0.0	6	0	0	0.0	
Controls	24	0.88	0.13	18	6	0	21.9	
Caribbean Blacks	105						5.6	0.332
Patients	26	1.0	0.0	26	0	0	0.0	
Controls	79	0.96	0.04	73	6	0	7.3	

P-values refer to both allele and genotype distribution
Patient groups represent individuals attending lipid clinics; the majority has the FH phenotype
*Data from Coloured individuals of the Mamre community and healthy blood donors were pooled, since the genotype distribution and allele frequencies were similar in these two subgroups (data not shown).

Table 2. Lipid and lipoprotein concentrations in the general South African population according to genotype combinations at position -175 in the LDLR gene promoter

		Coloured population Mean (SD)	Black population Mean (SD)	Khoisan Mean (SD)
gg	Number	363	194	86
	Sex (M/F)	153/210	146/48	21/65
	Age (years)	38.77 (17.26)	34.32 (14.32)	35.66 (15.26)
	TC (mmol/l)	5.31 (1.21)	3.36 (0.83)	3.84 (0.69)
	HDLC (mmol/l)	1.31 (0.39)	1.05 (0.62)	
	TG (mmol/l)	1.10 (0.64)	1.17 (0.43)	
	LDLC (mmol/l)	3.50 (1.13)	1.70 (0.85)	
gt	Number	47	9	4
	Sex (M/F)	19/28	9/0	1/3
	Age(years)	36.38 (14.14)	30.0 (8.07)	21.50 (15.86)
	TC (mmol/l)	5.31 (1.35)	2.83 (0.68)	3.64 (0.92)
	HDLC (mmol/l)	1.4 (0.45)	1.17 (0.32)	
	TG (mmol/l)	1.09 (0.91)	0.85 (0.24)	
	LDLC (mmol/l)	3.37 (1.11)	1.27 (0.29)	
tt	Number	4		
	Sex (M/F)	3/1		
	Age(years)	45.0 (18.13)		
	TC (mmol/l)	5.40 (1.02)		
	HDLC (mmol/l)	1.08 (0.31)		
	TG (mmol/l)	1.38 (0.66)		
	LDLC (mmol/l)	3.70 (0.81)		

TC, total cholesterol; HDLC, high-density lipoprotein cholesterol; TG, triglycerides; LDLC, low-density lipoprotein cholesterol; M/F, male/female; SD, standard deviation


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