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Studies on human sterol 27-hydroxylase with emphasis on its mechanism of regulation and metabolic consequences of a deficient enzyme.

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ABSTRACT

Sterol 27-hydroxylase (CYP27A1) is a mitochondrial cytochrome P-450 enzyme present in most animal cells. In hepatocytes, CYP27A1 has an important role in connection with biosynthesis of bile acids from cholesterol. In other cells the enzyme is responsible for a mechanism by which cholesterol can be converted into more polar products that can easily be eliminated from the cells. The above properties are consistent with an antiatherogenic effect of CYP27A1, which is also illustrated by the fact that patients lacking the enzyme develop premature atherosclerosis and xanthomas in tendons and in the brain (Cerebrotendinous Xanthomatosis).

Upregulation of CYP27A1 would represent a new strategy to treat atherosclerosis, and thus it was regarded to be important to expand the knowledge about the mechanism of regulation of the enzyme in man. In the first part of this thesis the following findings were made, all of which are consistent with an antiatherogenic effect of CYP27A1:

- There was a marked induction of CYP27A1 during differentiation of human monocytes into macrophages, with increased levels of mRNA and production of 27-oxygenated products of cholesterol. This induction has the potential to counteract the effects of induction of scavenger receptors.

- The cytokine TGFβ1 was found to have a stimulatory effect on transcription and enzymatic activity of CYP27A1 in monocyte-derived macrophages. This effect was dependent upon responsive elements in the proximal region of the CYP27A1 gene. The possibility is discussed that part of the antiatherogenic effect of TGFβ1 may be mediated by its effects on CYP27A1.
- Patients with Pulmonary Alveolar Proteinosis have a local accumulation of cholesterol in the lungs, known to contain particularly high levels of CYP27A1. This accumulation was associated with increased levels of 27-oxygenated steroids in the circulation. This increase may reflect a CYP27A1-mediated defence towards a local accumulation of cholesterol.

In the second part of the thesis a novel unique case of CTX is described, with bilateral Achilles tendon xanthomas as the only visible CTX manifestation. The patient was found to be a heterozygote with one CYP27A1 allele containing a previously not described mutation in exon 8: a two-base substitution (C478A and C479A) possibly disrupting the heme-binding domain of the enzyme. Expression of this mutant in HEK293 cells led to a defective enzyme. Despite extensive sequencing efforts, no other mutation was found in the CYP27A1 gene. Reduced levels of 27-hydroxycholesterol in the plasma of other family

members suggest that another genetic defect is present, and that this defect together with the mutation in exon 8 is causing CTX in this patient.

The formation of cholesterol and cholestanol containing xanthomas in the brain of CTX patients is difficult to explain in view of the fact that neither cholesterol nor its 5α-saturated analogue cholestanol are likely to pass the blood-brain barrier. Using a model system of the blood-brain barrier, it was shown that bile acid precursor 7α-hydroxy-4-cholesten-3-one, which is accumulated in the circulation of CTX patients to levels more than 100-fold of normal, is passing cultured brain endothelial cells at a rate about 100-fold higher than that of cholesterol and cholestanol. Cultured human astrocytes, microglial cells and neurogenic cells were able to convert 7α-hydroxy-4-cholesten-3-one into cholestanol. This reaction did also occur in human moncyte-derived macrophages. It is suggested that most of the cholestanol present in brain xanthomas of CTX patients accumulate as a consequence of a flux of 7α-hydroxy-4-cholesten-3-one over the blood-brain barrier. The possibility is discussed that the accumulation of cholesterol is secondary to the accumulation of cholestanol.

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III. 3. Meaney S, Bonfield T L, Hansson M, Babiker A, Kavuru MS.
Serum cholestenoic acid as a potential marker of pulmonary cholesterol homeostasis: Elevated levels in patients with Pulmonary Alveolar Proteinosis.
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J. Int. Med., accept. dec **2006**; Id. 321022-78431

V. 5. Panzenboeck U, Andersson U, Hansson M, Sattler W, Meaney S, Björkhem I.

On the mechanism of cerebral accumulation of cholestanol in patients with Cerebrotendinous Xanthomatosis.

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CONTENTS

LIST OF ABBREVIATIONS

27-OH	27-hydroxycholesterol
24-OH	24-hydroxycholesterol
ABCA1	ATP Binding Cassette transporter A1
ABCG1	ATP Binding Cassette transporter G1
ACAT1	Acyl-CoA: cholesterol Acyltransferase 1
AD	Alzheimer's disease
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
C4	7α-hydroxy-4-cholesten-3-one
CETP	Cholesteryl Ester Transfer Protein
Cholestenoic acid	3β-hydroxy-Δ5-cholestenoic acid
CSF	Cerebrospinal fluid
CTX	Cerebrotendinous Xanthomatosis
CYP7A1	Cholesterol 7α-hydroxylase
CYP7B1	Oxysterol 7α-hydroxylase
CYP27A1	Sterol 27-hydroxylase
CYP46A1	Cholesterol 24-hydroxylase
GC-MS	Gas chromatography-mass spectrometry
GH	Growth Hormone
GM-CSF	Granulocyte Macrophage - Colony Stimulating Factor
HDL	High Density Lipoprotein
HMG-CoA	3-Hydroxy-3-Metylglutaryl-CoA
IL-6	Interleukin 6
IL-10	Interleukin 10
LCAT	Lecithin: cholesterol acyltransferase
LDL	Low Density Lipoprotein
LXR	Liver X Receptor
M-CSF	Macrophage - Colony Stimulating Factor
mRNA	messenger RNA
NADPH	Nicotinamide Adenin Dinucleotide Phosphate (reduced)
oxLDL	oxidized Low Density Lipoprotein
PAP	Pulmonary Alveolar Proteinosis
StAR	Steroidogenic acute regulatory protein
T3	Triiodothyronine
TGFβ1	Transforming Growth Factor β1

Thesis summary – Main Section

Introduction

Cholesterol

Cholesterol is a hydrophobic 27-carbon steroid with many important functions in the body. It is a vital constituent of plasma membranes of all cells in the body helping to maintain fluidity and structure. It also serves as substrate for the production of steroid hormones (progesterone, estrogen, testosterone, glucocorticoids and mineralocorticoids), vitamin D3 and bile acids. Approximately one half to two thirds of total body cholesterol is *de novo* synthesised in the body, in particular in the liver and in the intestine. The remaining part is supplied in the diet.

Cholesterol synthesis

Cholesterol synthesis starts in the cytosol with the condensation of two acetyl-CoA units to form acetoacetyl-CoA, which then reacts with another acetyl-CoA unit to produce 3-hydroxy-3-metylglutaryl-CoA (HMG-CoA). The next conversion is catalysed by the rate-limiting enzyme in the sequence, HMG-CoA reductase, present on the outer surface of the ER, resulting in mevalonate. Further condensation reactions produce squalene, a 30-carbon compound that is

converted into lanosterol, that is finally converted into cholesterol in several steps.

Regulation of cholesterol synthesis.

The rate-limiting enzyme for cholesterol synthesis, HMG-CoA reductase, is highly regulated by negative feedback inhibition by mevalonate and cholesterol both at the mRNA and the protein level (for a review see [1]). Insulin stimulates and glucagon inhibits HMG-CoA reductase [2, 3]. The statin class of cholesterol lowering drugs effectively inhibits this enzyme as well [4]. To detect and control the levels of free intracellular cholesterol the cell utilizes two coupled proteins, SCAP and SREBP. When SCAP senses a depletion in intracellular cholesterol it escorts the SCAP-SREBP-complex from the ER membrane to the Golgi. Two proteases present in the Golgi successively cleave the SREBP protein to release the amino-terminal part of SREBP that translocates to the nucleus and initiate transcription of genes involved in lipid homeostasis. Specifically, SREBP-1c is mainly responsible for the activation of genes involved in fatty acid synthesis while SREPB-2 is mainly involved in regulation of cholesterol synthesis [5]. When cholesterol is abundant membrane proteins called insig-1 or insig-2 are able to bind SCAP and retain the SCAP-SREBP complex in the ER, thereby inhibiting cholesterol synthesis. Insig proteins may also reduce cholesterol synthesis by degradation of the HMG CoA reductase protein through a ubiquitin dependent mechanism [6].

The two main mechanisms for cholesterol removal from the body are conversion of cholesterol into bile acids, and excretion of unchanged cholesterol into the bile. Both cholesterol and bile acids are recycled in an enterohepatic circulation.

About 5-10% of the bile acids and about half of the cholesterol is lost during each cycle, and thus the cholesterol must be replaced by dietary cholesterol or by *de novo* synthesis.

Low density lipoprotein particles rich in cholesterol in the circulation may be subject to oxidative modification. These oxidised LDL-particles may be taken up by macrophages located to the subendothelial space of the blood vessel walls, expressing scavenger receptors. Since these receptors do not down-regulate in response to cholesterol saturation like the LDL-receptor, the process of un-inhibited cholesterol accumulation may lead to atherosclerosis development.

A high cholesterol level is associated not only with cardiovascular morbidity and mortality, but also with Alzheimer's disease (AD) [7, 8]. It is recommended that the fasting plasma cholesterol concentration should be less than 5.0 mmol/L [9].

Bile acids

Bile is produced by the hepatocytes in the liver and consists of cholesterol, bile salts and phospholipids in a balanced composition to keep cholesterol in solution. If this balance is disturbed cholesterol may crystallize and form gallstones. The bile secreted into the lumen of the small intestine in association with a meal is able to emulsify dietary lipids and make them susceptible to degradation by pancreatic lipase.

Bile acid synthesis.

Bile acids are produced in the hepatocytes via two enzymatic routes, the classical (neutral) or the alternative (acidic, since most of the early intermediates are acids) pathways [10]. The classical pathway is initiated by 7α-hydroxylation of cholesterol in the ER by the cytochrome P450 cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme of this sequence. Cholic acid or chenodeoxycholic acid are end products of this pathway, see fig 1.

The alternative pathway begins with 27-hydroxylation of cholesterol by sterol 27-hydroxylase in the mitochondrion and chenodeoxycholic acid is regarded to be the most important final product in this pathway in man. Both routes make use of sterol 27-hydroxylase to form a C27 carboxylic acid, which is subject to chain shortening reactions in peroxisomes to produce C24 bile acids. The two end products, cholic and chenodeoxycholic acid are conjugated to either glycine or taurine before excretion into the bile as primary bile acids [11].

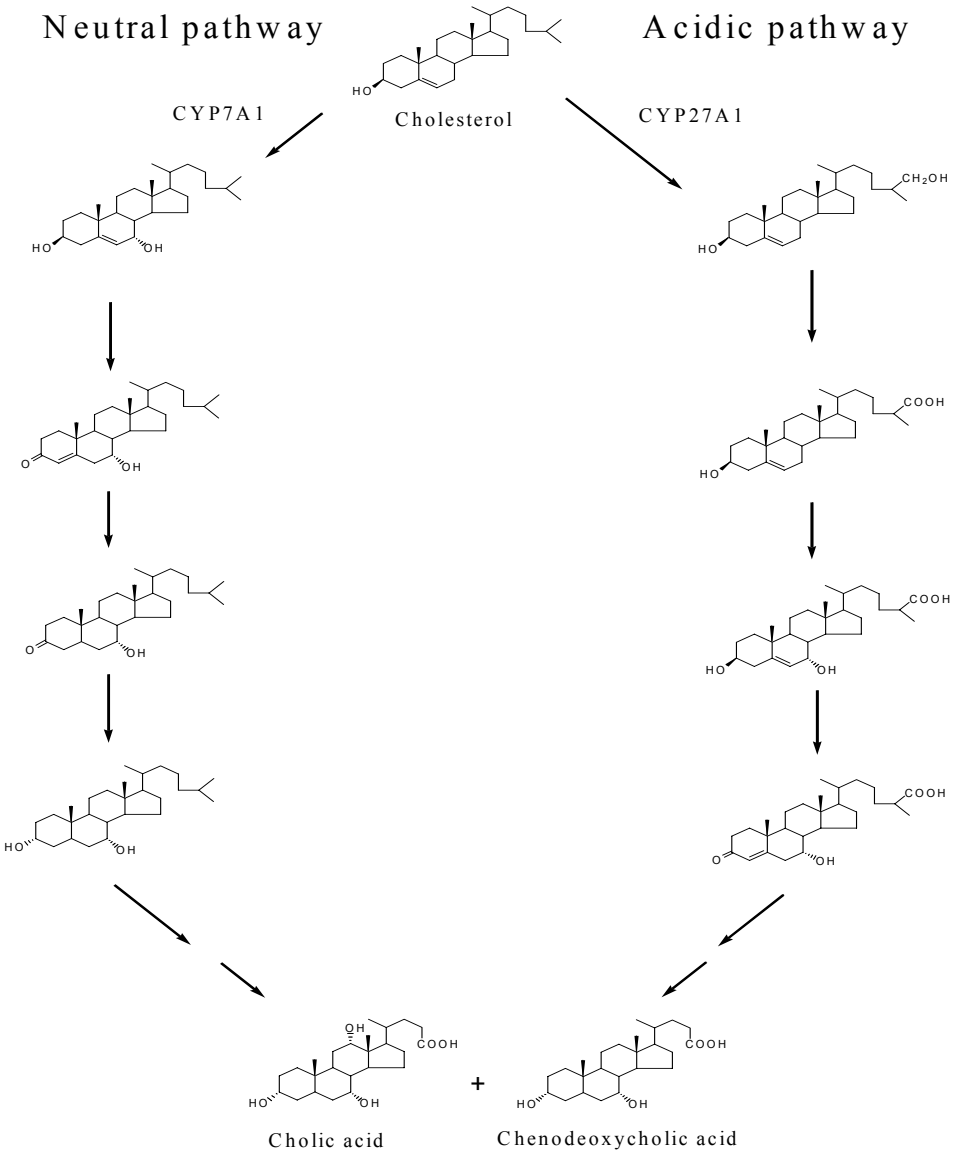


Fig. 1 Simplified overview of bile acid biosynthesis in humans.

In the intestine, bacteria may deconjugate and deoxygenate the bile acids to produce secondary bile acids, such as deoxycholate and lithocholate. These may be re-conjugated in the hepatocytes as they pass through the entero-hepatic circulation and are then excreted into the bile.

Regulation of bile acid synthesis.

Cholesterol 7α-hydroxylase is feedback inhibited by bile acids, with chenodeoxycholic acid as the most potent inhibitor in humans [12]. There are two important mechanisms by which bile acids suppress their own synthesis. One is by ligand-activation of the farnesoid X receptor (FXR), that heterodimerize with 9-cis retinoic X receptor (RXR) and induce transcription of Small Heterodimer Partner (SHP). SHP inactivates a transcription factor complex that leads to suppression of CYP7A1. Another mechanism is SHP-independent and involves a protein kinase C dependent phosphorylation and inactivation of hepatic nuclear factor 4α (HNF4α), leading to suppression of CYP7A1 [13, 14].

Cholesterol transport in the circulation

Cholesterol and other lipids are associated with lipoprotein particles in the plasma because of their hydrophobic nature. Ingested lipids are taken up by enterocytes and secreted as chylomicrons that are large lipoproteins with a high lipid/protein ratio. Chylomicrons reach the circulation via the lymphatic vessels, and are cleared from plasma by the liver as chylomicron remnants after some of the lipid content has been removed by lipoprotein lipase. Triglycerides and cholesterol are transported to peripheral tissues in VLDL-particles secreted by

the hepatocytes. VLDL is converted into a cholesterol rich LDL-particle through an intermediate IDL-particle by successive action by lipoprotein lipase and hepatic lipase that reduce the triglyceride content. Peripheral cells in need of cholesterol express LDL-receptors that recognize the ApoB-100 protein present in LDL. LDL-receptor expression is down regulated when the cholesterol level in the cell is sufficiently high. LDL particles in the vascular wall are vulnerable to oxidation, and oxidized LDL may be taken up through scavenger receptors expressed on macrophages. Scavenger receptors are not downregulated as is the LDL-receptor and thus the macrophage may become filled with cholesterol, a process leading to foam cell formation and initiation of atherosclerosis.

HDL has a high content of protein and phospholipids, and is produced by the liver as nascent HDL, consisting mainly of ApoA1 protein. HDL is able to bind to and receive cholesterol from peripheral cells and bring it back to the liver in a process called “reverse cholesterol transport” [15-17]. This is achieved by passive diffusion of cholesterol to different lipoproteins, but also trough an active membrane pump mechanism, mediated by ATP-binding cassette transporter A1 (ABCA1). ABCA1 interacts with the ApoA1 protein of HDL to actively pump out phospholipids and cholesterol to the HDL particles where free cholesterol is esterified by LCAT. In addition, another member of the ABC transporter family, ABCG1 that is highly expressed in macrophages, may preferentially efflux cholesterol to the larger and more lapidated HDL subtypes, HDL_2 and HDL_3, comprising the bulk of plasma HDL [18]. Tangiers disease is

caused by mutations that disrupts ABCA1, and this results in intracellular cholesterol accumulation and increased risk of atherosclerosis [19].

Intracellular cholesterol transport

Cellular cholesterol homeostasis is a balance between uptake, efflux, de novo synthesis, esterification and metabolism. Furthermore, since cholesterol is a very hydrophobic molecule, carrier proteins are believed to be necessary for the movement of cholesterol between the different organelles and the plasma membrane. In particular, transport of cholesterol to the sterol 27-hydroxylase in the inner mitochondrial membrane is of special interest, while it is the most important rate limiting factor for enzyme activity. These transport mechanisms are however not very well characterized.

Caveole

Caveolae are 50-100 nm invaginations of the plasma membrane, enriched in cholesterol and sphingolipids, and a specific protein called caveolin. In fibroblasts, caveolin-1 has been shown to cycle between the ER, Golgi apparatus and the plasma membrane. Due to these findings, caveolae were thought to play an important role in cholesterol transport from the cells. However, ABCA1-mediated cholesterol efflux is thought to occur in non-caveolae domains of the plasma membrane. The relative importance of these plasma microdomains are therefore not very clear [20].

StAR

In adrenal steroidogenic tissue, the steroidogenic acute regulatory protein (StAR) is responsible for cholesterol transport into the inner mitochondrial membrane. Disruption of StAR function results in a distinct clinical condition called congenital lipoid adrenal hyperplasia, resulting in severe impairment of steroid biosynthesis both in adrenals and gonads. Decreased substrate availability for P450scc (CYP11A), the side chain cleavage enzyme, that is rate-limiting for pregnenolone production, leads to reduced steroid hormone biosynthesis. Affected cells become filled with cholesterol and cholesterol esters that secondarily disrupts other cellular functions [21]. There is a very low expression of StAR protein in macrophages, and disruption of StAR function is not likely to affect sterol 27-hydroxylase activity in these cells.

Niemann-Pick

Niemann-Pick type C1 (NPC1), is a disease characterized by progressive neurodegeneration including ataxia, dystonia, seizures and dementia. The disease is caused by mutations in the NPC1 gene. The corresponding protein is involved in cholesterol transport from late endosome/lysosome derived from LDL, to trans-Golgi network, plasma membrane and endoplasmatic reticulum. In mice deficient in NPC1 there is an accumulation of cholesterol in the mitochondrial membranes with subsequent disturbances in cellular function,

measured as reduced ATP-synthesis [22]. It may be speculated that in cells with a disrupted NPC1, mitochondria receive excess cholesterol, that in the normal case would go to other organelles in the cell.

Other intracellular cholesterol transport proteins

MLN64 belongs to the same StAR-related lipid transfer (START)-domain containing gene family as StAR and is reported to be associated with late endosomes/lysosomes. The START-domain of MLN64 is capable of binding one cholesterol molecule in a hydrophobic pocket. Transfected COS-cells expressing a START-domain deficient MLN64 protein, accumulate cholesterol in lysosomes and has a reduced steroidogenic capacity, indicating that MLN64 normally is involved in transport of cholesterol to the mitochondria [23].

StarD4 and StarD5 are other START-domain containing proteins without a specific subcellular targeting signal, making them cytosolic cholesterol transporters. StarD4 appears to be positively regulated by SREBP2 as cholesterol feeding reduces mRNA levels of StarD4 in mice [24]. In the same study, StarD5 was not regulated by SREBP:s or LXR. Using Western blot techniques, StarD5 was recently localized to monocyte-macrophages, but not hepatocytes [25]. Since substrate availability appears to be a limiting factor for sterol 27-hydroxylase activity (cf below), StarD5 may be of some importance

for the capacity of macrophages to eliminate cholesterol in the form of 27-oxygenated products.

Oxysterols

Cholesterol can be oxidized at different positions producing oxysterols [26, 27]. Concentrations of oxysterols in the plasma are generally very low compared to cholesterol, less than 1:10 000, and detection therefore requires very sensitive methods. Formation of oxysterols can occur through auto-oxidation, with the allylic carbon at position C7 being most vulnerable. Enzymatic modification generates more stable products. Cytochrome P450 enzymes, further described below, are the most important enzymes involved in these reactions.

Two important examples are 27-hydroxylation of cholesterol by the sterol 27-hydroxylase (CYP27A1) and 24-hydroxylation by cholesterol 24-hydroxylase (CYP46A1). CYP46A1 is almost exclusively expressed in neurons whereas CYP27A1 is found in most cell types.

24-Hydroxylation is an important excretion pathway of cholesterol for neurons and a correlation has been shown to exist between neuron density and levels of 24-hydroxycholesterol in the circulation [7, 28]. Side-chain oxidized oxysterols are more water-soluble than cholesterol and can easily escape from the cells into the circulation, or the CSF [29, 30]. These oxysterols are also able to pass the blood-brain barrier.

27-Hydroxylation of cholesterol produces 27-hydroxycholesterol (27-OH) as a primary product, which is able to pass cell membranes much faster than cholesterol. Because 27-OH is transported in the same lipoprotein compartments as cholesterol [29], there is a strong correlation between total cholesterol and 27-OH in plasma [31]. Furthermore, sterol 27-hydroxylase has the capability to oxidize the product 27-OH at carbon 27 to produce 3β-hydroxy-Δ5-cholestenoic acid (CA) [32]. In similarity with fatty acids, CA can readily pass the cell membranes, and no lipoprotein acceptor is needed. Consequently, almost no CA is found intracellularly. In the plasma, CA is associated with albumin, and most of total body CA is produced in the lungs [31]. Once converted into an oxysterol, the former cholesterol molecule is destined to become a bile acid in the liver and consequently less likely to reappear in other LDL particles. Oxysterols have been shown to be ligands for nuclear receptors and have the potential to participate in regulation of lipid homeostasis of the cells [33, 34].

Cytochrome P450 enzymes

The cytochrome P450 enzymes emerged early in evolution and have diverged into one of the largest gene families, present in bacteria as well as in eukaryotic cells. Mammalian cytochrome P450: s are membrane bound as opposed to the bacterial variants that are soluble. Cytochrome P450 enzymes support the oxidative, peroxidative and reductive metabolism of xenobiotic substrates and are very important in phase 1 drug metabolism in the liver. All species of

cytochrome P450: s have a light absorbance peak at 450 nm when analyzing the reduced form treated with carbon monooxide. This absorbance spectrum is not shared by other heme containing proteins. Cytochrome P450 enzymes are monooxygenases, because they incorporate one atom of molecular oxygen into the substrate and one atom into water, differing from the dioxygenases that incorporate both atoms of molecular oxygen into the substrate. Breaking the oxygen-oxygen bond requires electrons donated to the heme iron by a prosthetic group. In the ER microsomal cytochrome P450: s the electron transfer is done by NADPH cytochrome P450 reductase that is anchored in the ER membrane, facing the cytosol. Mitochondrial inner membrane cytochrome P450 enzymes are supplied with electrons by ferredoxin (or adrenodoxin in the adrenals). Ferredoxin is in turn reduced by ferredoxin reductase, and NADPH is the source of electrons for ferredoxin reductase [35]. The interaction between mitochondrial P450:s and ferredoxin is dependent on four highly conserved amino acid residues in either protein, positively charged on the P450:s and negatively charged on ferredoxin [36].

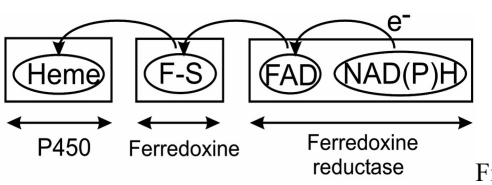


Fig 2. Electron transfer to heme.

Different gene regulatory mechanisms have evolved for cytochrome P450 enzymes, for example: Drugs of the fibrate class bind to PPARα and migrate to the nucleus where they heterodimerize with RXR and interact with response

elements on the gene coding for CYP4A. Aromatic hydrocarbons binds to a specific Ah- receptor, that migrates to the nucleus together with a protein called "arnt" and induces a number of P450 enzymes [37-39].

CYP27A1

The most abundant oxysterol in human plasma is 27-hydroxycholesterol (about 150 ng/mL plasma) closely followed by its oxidation product cholestenoic acid [40]. Cholesterol 27-aldehyde is an unstable intermediate in the conversion from 27-hydroxycholesterol to cholestenoic acid [41]. The lungs produce most of the cholestenoic acid present in plasma whereas 27-hydroxycholesterol has no such tissue specific origin. The sterol 27-hydroxylase is expressed by almost all cell types, but hepatocytes, macrophages and in particular lung alveolar macrophages have the highest expression [31, 32]. Sterol 27-hydroxylase resides in the inner mitochondrial membrane and requires NADPH, adrenodoxin, adrenodoxin reductase and molecular oxygen for proper function [42]. However, substrate supply is a limiting factor for the overall enzymatic production [43]. The sterol 27-hydroxylase enzyme is encoded by the CYP27A1 gene, which is located on chromosome 2 (2q33), and consists of 9 exons where intron 1 is particularly large, about 27k bp [44]. Potential Binding sites for transcription factors Sp1, Sp3 and hepatic nuclear factor 4α (HNF4α) have been found in the proximal promoter region.

The translated protein has a 33 amino acid mitochondrial signal sequence followed by a mature protein of 498 amino acids [32]. It contains an adrenodoxin binding domain, encoded by exon 6, and a heme-binding domain, encoded by exons 8 and 9 [45]. Mutations in the CYP27A1 gene that cause substantial reductions in sterol 27-hydroxylase activity, particularly mutations affecting the heme- or adrenodoxin-binding regions, is the basis for the disease cerebrotendinous xanthomatosis (CTX), see below.

Sterol 27-hydroxylase has broad substrate specificity with a preference for more polar steroids, and has the capacity to oxidize its own product 27-hydroxycholesterol into 3β-hydroxy-Δ5-cholestenoic acid [32, 46]. Plasma concentration of 27-hydroxylated cholesterol may be increased in some patients with atherosclerosis, and high amounts of 27-hydroxylated products are found within atherosclerotic plaques [47-50]. The latter finding is consistent with the possibility that CYP27A1 may be involved in a defence mechanism that protects from accumulation of excess cholesterol in foam cells in the vessel wall [51]. Physiologically, this mechanism may be of particular importance in tissues that are less vascularised, with limited access to lipoproteins, such as tendons, skin and the lens, clearly exemplified in CTX [52].

Regulation of CYP27A1 activity

In the human hepatocytes CYP27A1 does not seem to be regulated by chenodeoxycholic acid, its end product [12, 14]. Hydrophobic bile acids (eg.

lithocholate) have however been shown to have some suppressive effect on human CYP27A1 gene reporter activity in HepG2 cells. Bile acids appear to bind to a bile acid reponse element (BARE) located in the proximal promoter [53]. A few other mediators have been found capable of stimulating or inhibiting CYP27A1 gene expression or sterol 27-hydroxylase activity to some degree. For example, studies on rat hepatocytes have shown that dexamethasone stimulates and insulin and bile acids repress sterol 27-hydroxylase mRNA [54-56]. In HepG2 cells transfected with the CYP27A1 promoter, thyroxine (T4) and phorbol 12-myristate 13-acetate (PMA) decreased transcriptional activity, whereas dexamethasone, GH and IGF-1 increased the activity [57]. Pro-inflammatory mediators like LPS decreased sterol 27-hydroxylase activity in liver from hamsters, and IL-1 and TNFα decreased CYP27 mRNA in HepG2 cells [58]. IFNγ and immune complexes decreased sterol 27-hydroxylase mRNA and protein in human endothelial cells, monocyte-derived macrophages and THP-1 cells [59]. Vitamin D3 was found to suppress CYP27 mRNA in rat kidney [60], and HNF4α strongly stimulates CYP27A1 gene transcription in HepG2 cells [58, 61]. Szanto et al. showed that PPARγ-RXR and RAR-RXR heterodimer complexes are able to bind to a DR1 in the CYP27A1 promoter [62], inducing gene transcription. Important ligands for RXR and RAR are 9-cis-retinoic acid (9-cis RA) or all-trans retinoic acid (ATRA). Anti-diabetic drugs of the thiazolidinedione (TZD) class, rosiglitazone and pioglitazone, may

act as ligands for PPARγ [62, 63]. Induction of CYP27A1 leads to production of 27-hydroxycholesterol, that in turn may act as a ligand for LXRα [33] . LXRα responses include activation of ABCA1, ABCG1 and CD36. CD36 mediates uptake of oxLDL and the ABC-transporters are involved in the efflux of cholesterol from the cells. High amounts of PPARγ, LXRα and CYP27A1 mRNA were detected in histological preparations from human atherosclerotic lesions (mostly macrophages), and this finding is consistent with the possibility that these genes are linked to cholesterol metabolism and efflux *in vivo* [62]. Retinoids were shown to increase macrophage lipid efflux and induce genes associated with lipid efflux (ABCA1, ABCG1, CYP27A1 and LXRα). A retinoic acid response element (RARE) was located with in the proximal part of the CYP27A1 promoter [64]. A repressive cyclosporin A responsive element as well as an enhancer element might be found further upstream in the promoter, as well as a dexamethason responsive element [65]. The over all production from sterol 27-hydroxylase appears to be limited by the availability of cholesterol substrate to the inner mitochondrial membrane, as over expression of the StAR-protein was more efficient in elevating sterol 27-hydroxylase activity than over expression of CYP27A1 in primary rat hepatocytes [43].

Candidate factors for regulation of CYP27A1 activity

When the present work was initiated, the mechanism of regulation of CYP27A1 in human extra hepatic cells such as macrophages was not well characterized, but was thought to be of some importance in connection with atherosclerosis. It was regarded to be of interest to see if monocyte-derived macrophages (which could be assumed to resemble the cells in the early atherosclerotic lesion in humans better than cell-lines), would respond similar to the cells above. The following factors were considered to be of particular interest as possible modifiers of CYP27A1 activity: TGFβ1, GM-CSF, IL-10 and Probucol.

TGFβ1

TGFβ1 is a 25 kD homodimeric protein with anti-inflammatory and anti-atherogenic properties [66]. Numerous cells, including platelets, macrophages, epithelial cells and fibroblasts, secrete it as a biologically inactive form. TGFβ1 also has the ability to inhibit growth of most epithelial and haematopoietic cells and to regulate the production of extra cellular matrix. The latter effect could be of importance in stabilising atherosclerotic plaques and be of importance in fibrosis development [67]. Conversely, disruption of the TGFβ1 gene in T-cells results in accelerated atherosclerosis [66].

The signalling pathway of TGFβ1 goes through the binding of its specific cell surface receptor, leading to phosphorylation of an intracellular protein called

Smad on the cytoplasmic side of the membrane. Phosphorylated Smad heterodimerize with another member of the Smad family and enters the nucleus [68], where the heterodimer recognizes a specific DNA-sequence, although interaction with other transcription factors may be necessary to alter gene transcription [69, 70].

GM-CSF

GM-CSF is considered to be an inflammatory cytokine opposing many effects of TGFβ1, for example ApoE secretion and cholesterol efflux [71]. In AD, GM-CSF is increased in the CSF whereas TGFβ1 is decreased [72]. GM-CSF is also regarded to be a patogenetic factor in the rare disease pulmonary alveolar proteinosis (PAP) [73]. In PAP, neutralizing autoantibodies against GM-CSF lead to a disease characterized by accumulation of cholesterol and proteins in the alveoli of the lungs. The link between inactivation of GM-CSF and the cholesterol accumulation is not known. Hypothetically, this accumulation in the alveolar macrophages in PAP patients could be due to a possible positive regulatory effect on CYP27A1 by GM-CSF. Low levels of GM-CSF in the lungs would then result in a reduced capacity to excrete cholesterol from the cells due to diminished production of 27-hydroxycholesterol and cholestenoic acid.

IL-10

In addition to TGFβ1, the cytokine IL-10 is also considered to be an anti-inflammatory mediator. In contrast to the pro-inflammatory cytokines that are associated with atherosclerosis [74, 75], the anti-inflammatory cytokines may have beneficial effects on the development of atherosclerosis. It would therefore be of interest to test the possibility that IL-10 has an effect on CYP27A1 when added to monocyte-derived macrophages. If IL-10 has a similar effect as TGFβ1, this could mean that CYP27A1 is controlled by a more generalized anti-inflammatory mechanism, which would further link inflammation to atherosclerosis.

Probucol

The lipophilic antioxidant Probucol is a drug that was previously used as a cholesterol-lowering agent, fig 3.

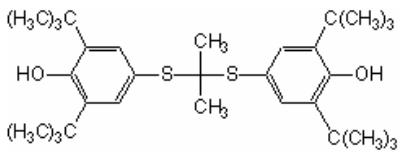


Fig 3. Structure of Probucol.

It had a beneficial effect in decreasing total cholesterol and reducing the size of xanthomas, but unfortunately it also lowered HDL in human subjects. In some cases, the over-all effect of Probucol has in fact been suspected to be atherogenic rather than anti-atherogenic [76]. Recently, it was shown that Probucol is a specific inhibitor of ABCA1 in the plasma membrane, inhibiting

both apoA1 binding to the cell membrane and the apoA1 mediated cellular lipid release [77]. It was suggested that Probucol mediates a transfer of ABCA1 protein from the plasma membrane to the interior of the cell. In addition to lowering the total cholesterol level, a few beneficial clinical effects have been reported in some patients receiving Probucol, including stabilization of cognitive function in AD, and size reduction of tendon xanthomas [78, 79]. The mechanisms behind these effects are not known. The possibility must be considered that Probucol stimulates CYP27A1. If this is the case, more 27-hydroxylated cholesterol is produced and exported from the cell and this could explain the xanthoma reducing effects. Another possibility could be that ABCA1 inhibition leads to increased concentration of free cholesterol, which is a suitable substrate for sterol 27-hydroxylase. As a consequence production and efflux of 27-hydroxylated cholesterol would increase. Probucol has also been shown to increase apoE secretion, which may be related to cholesterol efflux [80]. Lastly, Probucol's strong antioxidative effect may reduce formation of oxidized LDL in plasma, resulting in reduced uptake by macrophage scavenger receptors, which may lead to tendon xanthoma size reduction.

CTX

Cerebrotendinous xanthomatosis (CTX) is a rare inborn disorder caused by a lack of functional sterol 27-hydroxylase. About 50 different mutations in the first eight exons CYP27A1 gene have been defined in a total of about 300

patients thus far diagnosed with CTX worldwide [81]. CTX is a heterogeneous progressive disease with a broad spectrum of symptoms, from very mild to very severe, and no predictable genotype-phenotype correlation exists. These patients are commonly affected by tendon xanthomas, juvenile cataract, xanthelasma, premature atherosclerosis and osteoporosis. Infantile-onset diarrhoea may be the earliest symptom of CTX. Childhood-onset cataract and neonatal cholestasis may be early findings. In addition they have progressive neurological dysfunction with epilepsy and dementia as a consequence of xanthomas in the brain. Brain MRI spectroscopy may show diffuse cerebral and cerebellar atrophy and white matter signal abnormalities [81]. The most effective treatment consists of bile acids, in particular chenodeoxycholic acid, which may result in disappearance of the xanthomas [45, 82, 83].

Three-dimensional structural imaging of the sterol 27-hydroxylase has revealed that a majority of the known CTX mutations disrupt the heme- or adrenodoxin-binding sites, critical for enzyme activity [45, 83]. Almost all cases of CTX reported so far, have mutations in exons of both alleles in CYP27A1. Sugama et al. reported a patient with a heterozygote mutation in CYP27A1, with biochemical findings compatible with CTX. However, the clinical picture was very dissimilar, and may represent a previously undescribed neurodegenerative disease [84]. Recently a novel case of CTX was diagnosed in our laboratory. The patient had unusually mild clinical symptoms but was a clear case of CTX as judged from the biochemical investigations.

The biochemical basis for CTX is a lack of enzymatic activity in sterol 27-hydroxylase. This lack has two important primary consequences. First, CYP27A1 is the primary enzyme in the acidic pathway of bile acid production, and lack of this enzyme in particular reduces the levels of the end product chenodeoxycholic acid, the major inhibitor of CYP7A1. As CYP7A1 is the rate-limiting enzyme for overall bile acid production, release of inhibition leads to a

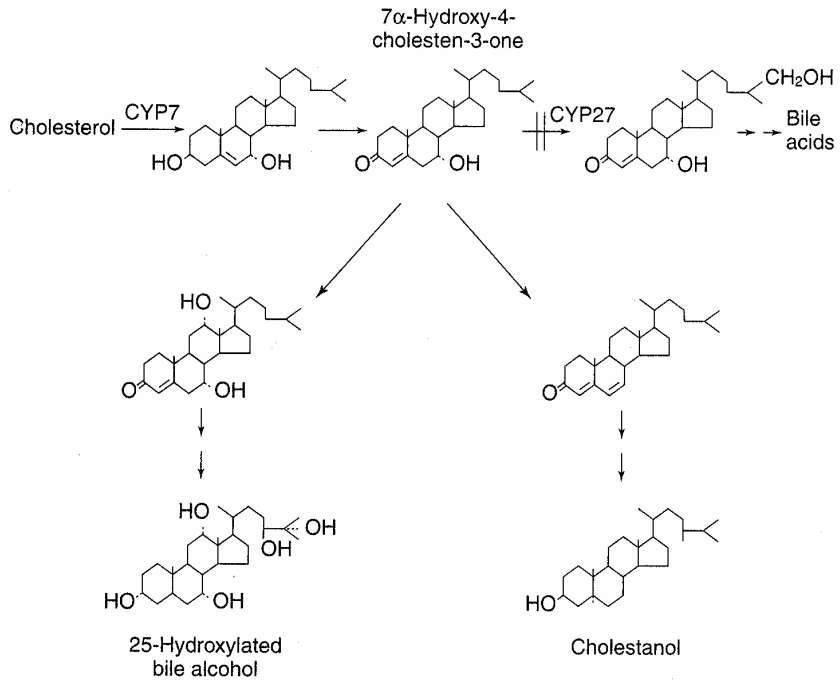


Fig 4. CTX situation with a blocked CYP27 pathway leads to increased levels of bile acid intermediates and cholestanol.

very high CYP7A1 activity. Second, 27-hydroxylation of bile acids in the liver is important for subsequent side chain shortening and production of 24 carbon

bile acids. Lack of CYP27A1 activity leads to the accumulation of C27-bile alcohols. The combined consequence of sterol 27-hydroxylase deficiency is therefore the daily production in the liver of gram amounts of 25-hydroxylated bile alcohols that are excreeted from the body in bile, faeces and urine, fig 4. It may be mentioned that a minor part of the 25-hydroxylated bile alcohols may be converted into the bile acid cholic acid, by a pathway bypassing CYP27A1. This pathway does not produce chenodeoxycholic acid, however, which is the main inhibitor of CYP7A1 in man.

Following these primary biochemical events, the heavy production of bile alcohols has further consequences. The increased production of bile acid intermediates will consume cholesterol, and as a consequence cholesterol synthesis is up regulated.

One important bile acid intermediate that accumulates, 7α-hydroxy-4-cholesten-3-one (C4) reaches high plasma levels (50- 250 times normal) in CTX patients. Most of the C4 is transformed into water soluble bile alcohols and excreted from the body, but a small fraction may be converted into cholestanol in the tissues by the following sequence of reactions, fig 5:

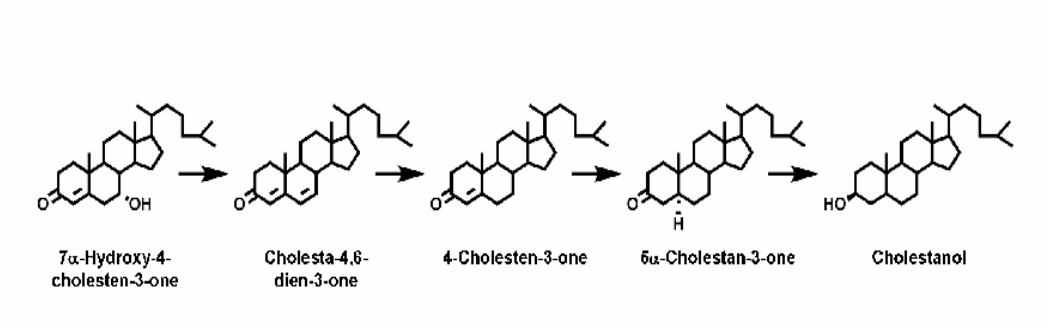


Fig 5. Sequence of reactions from C4 to cholestanol.

Cholestanol is a structural analogue of cholesterol that lacks the double bond in the Δ5-position. Cholestanol is very insoluble and accumulates in the tissues and xanthomas together with cholesterol.

In an in vivo study in CTX patients utilizing 7α-^{3}H-labelled cholesterol [85], results were obtained consistent with the possibility that about 70% of the cholestanol accumulating in these patients is derived from 7α-hydroxylated intermediates in bile acid synthesis. It seems likely that 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one are the most important of these intermediates.

In addition to cholestanol, also cholesterol is accumulating in patients with CTX, despite normal levels of this steroid in the circulation. Four different mechanisms may be considered:

- The lack of CYP27A1 activity leads to reduced efflux of 27-hydroxycholesterol and cholestenoic acid from the cells. Since

cholestanol is also a substrate for the sterol 27-hydroxylase, there is also a reduced efflux of 27-hydroxycholestanol.

- 27-Hydroxycholesterol is an inhibitor of HMGCoA reductase under in vitro conditions. Theoretically a lack of this inhibitor may result in increased synthesis of cholesterol.
- 27-Hydroxycholesterol is a ligand for LXR (although rather weak). A lack of this compound may result in lower activation of LXR and thus a lower activity of ABCA1 and a reduced reverse cholesterol transport.
- Cholestanol may have specific effects on cholesterol homeostasis in the cells leading to cholesterol accumulation.

The fact that treatment with chenodeoxycholic acid results in a reduction in the size of the xanthomas (even in the brain) is not consistent with the possibility that any of the first three factors are most critical for the development of xanthomas. With respect to the last factor there are some experimental data suggesting that cholestanol has a lower capacity to inhibit cholesterol synthesis than cholesterol itself. A reduced feed-back inhibition on HMGCoA reductase was thus observed in a cholestanol feeding experiments on rodents, and in one study a stimulatory effect on cholesterol synthesis was observed [86-88]. If accumulated cholestanol does not suppress cholesterol synthesis, the SREBP-SCAP sensing mechanism may respond to a dilution of the cholesterol pool with cholestanol with a compensatory increase in cholesterol synthesis. It may thus be speculated that a local cholestanol induced increased cholesterol synthesis in

patients with CTX is the critical factor behind the accumulation. Such an accumulation may be most important in tissues in which the sterol 27-hydroxylase pathway for cholesterol removal is of some importance under normal conditions. It was considered to be of interest to test the above hypothesis in a suitable in vitro system.

Accumulation of cholesterol and cholestanol in brain xanthomas of CTX patients is of special interest, since the most destructive symptoms arise here. For unknown reasons cerebellum seems to be the most severely affected part of the brain.

Possible mechanisms behind accumulation of cholestanol in the brain of CTX-patients

An enduring mystery of CTX is the origin of the cholestanol present in the brain of these patients. The simplest mechanism – direct blood-to-brain passage of cholestanol formed extracerebrally – is based on the possibility that cholestanol is able to cross the blood-brain barrier. In support of such a mechanism Buchmann and Claussen showed that rabbits fed a diet enriched with cholestanol for 8 weeks had brain cholestanol about twice that of animals fed a control diet [89]. Additionally, Byun et al. showed that feeding mice with 1% cholestanol for 8 months lead to a significant enrichment of this sterol in

cerebellum [90]. It is thus clear that cholestanol can to some extent, pass the blood-brain barrier. In theory an alternative pathway could be the passage of a circulating precursor such as 7α-hydroxycholesterol or 7α-hydroxy-4-cholesten-3-one into the brain, where it may be subsequently converted to cholestanol.

Aims of the study

- To investigate whether or not there is an induction of CYP27A1 at the transcriptional and/or enzyme level during differentiation of human monocytes into macrophages.
- To investigate whether or not different cytokines, hormones and other factors are able to affect CYP27A1 during and after differentiation of human monocytes. TGFβ1, GM-CSF, IL-10 and Probucol were considered to be of particular interest.
- To clarify whether or not there is an effect on CYP27A1 and 27-OH levels in patients with pulmonary alveolar proteinosis, who have low levels of GM-CSF and accumulation of cholesterol in alveolar macrophages.
- To define the molecular defect behind a novel unique case of sterol 27-hydroxylase deficiency (CTX).
- To clarify the molecular mechanism behind accumulation of cholestanol in the brain of CTX-patients.

Materials and Methods

Materials

Transforming growth factor-b1 (TGF-b1), granulocyte-macrophages colony stimulating factor (GM-CSF) and IL-10 were obtained from Sigma Chemical Company (St. Louis, MO); Probucol was obtained from Calbiochem; macrophage serum-free medium (SFM) was purchased from Life Technologies Inc., Ficoll-Paque™ from Pharmacia Corp. All other reagents and chemicals were high-purity standard commercial products. Deuterium labelled 27-hydroxycholesterol and unlabelled 3β-hydroxy-5-norcholestenoic acid were synthesised as previously described [40], Ultraspec reagent for total RNA isolation was purchased from Nordic Biosite, RPAIII™ kit, pTRI-β-actin-Human as internal control template from Ambion and Nick columns were bought from Amersham-Pharmacia.

The manufacturer does not provide the exact composition of macrophage serum-free medium. We checked for the components that are of relevance to our study and found that the medium contains no lipoproteins, small amount of cholesterol (1ug/ml) and small amount of albumin (3g/L).

The CYP27 promoter constructs used in paper 3 were generous gifts from Prof. Eran Leitersdorf (Hadassah university hospital, Jerusalem, Israel).

Methods

Culturing of monocyte-derived macrophages and isolation of total RNA, protein and DNA

Human monocytes from healthy donors were isolated from buffy coats obtained from a blood bank. Mononuclear cells were isolated by centrifugation in Ficoll-Paque^{TM}, washed and suspended in Minimum Essential Medium supplemented with benzyl penicillin (400 U/ml) and streptomycin (0.2 mg/ml) for at least 3 hours at 37° C at a concentration of 4-5x10^6 in 10 ml dishes. The non-adherent lymphocytes were removed and the adherent monocytes (approximately 1-2x10^6) per 100 mm dish were incubated for approximately one week in Macrophage serum-free medium (SFM) with or without addition of fetal calf serum (FCS) 10 %, in order to differentiate them into macrophages. Different test substances were added to the media during, or after differentiation into mature macrophages. The medium was changed every 48 hours. Medium removed from the dishes was stored at -20° C until analysis of 27-oxygenated products. Adherent cells were lysed and removed by addition of 2 mL of Ultraspec. The cells were then harvested in 12 ml Falcon tubes and homogenised and stored at -70° C until isolation of total RNA and quantification using RNA protection assay. The purity of RNA was assessed by the 260 to 280 nm ratio and RNA concentration was determined at 260nm.

Alternatively, Trizol (Invitrogen) 1 mL was used to detach and lyse the cells in the dishes. 2 mL Falcon tubes were then used to store the homogenate at -70° C until isolation of total RNA, DNA and protein, according to the manufacturers manual.

Measurement of 27-oxygenated steroids in culture media by combined gas chromatography mass spectrometry

Measurement of 27-oxygenated steroids was carried out as described previously [91]. In brief, 200 ng of 3β-hydroxy-5-norcholestenoic acid and 100 ng of [$^2H_5$]-27-hydroxycholesterol were added to 1 to 10 mL of cell medium. The medium was acidified with hydrochloric acid and extracted with 20 mL of diethyl ether. The ether phase was washed with water until neutral and the solvent was removed under reduced pressure. The residue was dissolved in 0.5 ml of chloroform and fractionated on a Bond-Elut NH_2 cartridge. The neutral lipid fraction, containing 27-hydroxycholesterol, was eluted from the column using 4 mL of chloroform: isopropanol (2:1) while the fatty acid fraction containing 3β-hydroxy-Δ5-cholestenoic acid was eluted using 4 mL of 2% acetic acid in ether. The fractions were dried under a stream of argon. The fatty acid fraction was methylated with diazomethane and both fractions were converted into trimethylsilyl ethers.

A Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970 MSD mass spectrometer was used for the analysis. [$^2H_5$]-27-hydroxycholesterol was used as an internal standard for 27-hydroxycholesterol, and 3β-hydroxy-Δ5-norcholestenoic acid was used as the internal standard for 3β-hydroxy-Δ5-cholestenoic acid.

Quantification of (CYP27A1) mRNA by ribonuclease protection assay

Ribonuclease protection assays were performed using the RPA III^{TM} kit according to the protocol provided. In brief, RNA-probes for human CYP27A1 (297 bp) and human β-actin (245 bp) were labelled with ^{32}P-UTP using T_3 polymerase and purified on NICK-columns. Sample RNA and probes were co-precipitated and dissolved in hybridization buffer. The samples were allowed to hybridize with the probes at 46° C overnight. After RNase treatment, the samples were precipitated and re-dissolved in loading buffer. The protected fragments (CYP27 297 bp, β-actin 245 bp) were separated using a denaturing 5 % polyacrylamide urea gel. These fragments were detected and quantified using a Fuji bas 1800 phospho-imager.

Quantification of mRNA by real-time PCR.

RNA quantification was in some experiments performed through single-plex real time PCR analysis on an ABI PRISM 7000 Sequence Detection System

(Applied Biosystems, Foster City, CA, USA). cDNA samples from PAP patients and matched controls were assayed using FAM-labled probe mixtures, Assay-on-demand™ (Applied Biosystems, Foster City, CA, USA) for human CYP27A1 (Assay ID: Hs00168003_m1). Human Cyclophilin A (Assay ID: Hs99999904_m1) was used as internal standard. All samples were analysed in triplicates and results were calculated in accordance with the user manual.

Measurements of luciferase activity in hepG2 cells

HepG2 cells were grown to 40-80% confluence in 60 mm dishes and subjected to transient transfection with CYP27A1 promoter constructs in a pGL2 vector also containing β-galactosidase. The cells were then cultured with or without addition of human TGF-β1 10 ng/mL for 24 h and then harvested for determination of luciferase activity. Transfection efficiency was corrected for by measuring β-galactosidase expression and correction for differences in protein concentration was also made to correct for differences in cell number.

Quantification of human TGF-β1 in human plasma samples

Assays were performed using the human TGF-β1 Immunoassay kit by Quantikine™ (R&D Systems, Inc. Minneapolis, USA) according to the protocol provided. In brief, 5 mL of fasting citrate plasma was collected from healthy volunteers in the morning. Each sample was centrifuged at 3300xg 15' and the

supernatant transferred to a new tube and again centrifuged at 10000xg 10' to remove all platelet contamination. Activation of TGF-β1 was performed by addition of 2.5 M acetic acid/10 M urea and samples were then put on a 96-well plate precoated with TGF-β1 receptors. An enzyme-linked polyclonal antibody specific for TGF-β1 was used for detection and quantification against a standard curve at 450 nm wavelength in a microplate reader.

Statistical analysis.

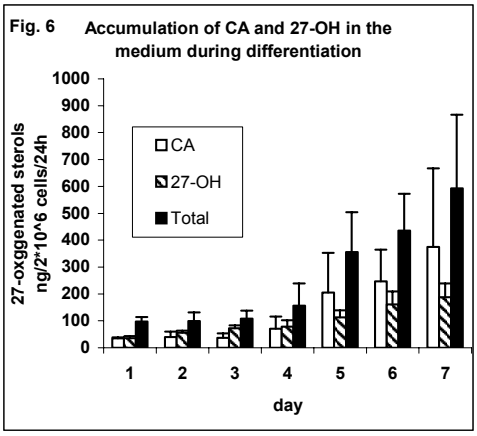
Statistical significance among the experiments was evaluated by Student's t-test.

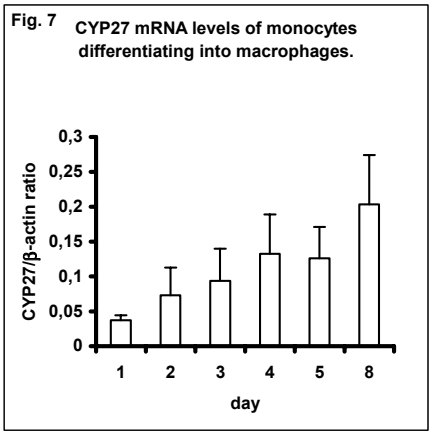
Results and Discussion

It is clear that CYP27A1 has a role in human cholesterol homeostasis since a lack of it causes development of xanthomas and eventually premature atherosclerosis. This is not the case in a mouse model with a disrupted CYP27A1 gene, demonstrating marked species differences [92, 93]. In view of the anti-atherogenic potential of this enzyme and the possibility to find new therapeutic strategies, it was considered to be of interest to clarify the regulation of CYP27A1 in human extrahepatic cells, in particular in macrophages, which are of key importance in the development of atherosclerosis. When this study was initiated, very little information was available about this regulation.
In an attempt to find regulatory mechanisms for the human CYP27A1, monocytes were cultured and differentiated into macrophages. Assessment of activity and transcription of CYP27A1 were made both during differentiation into macrophages as well as after addition of different hormones and cytokines.

Effect of differentiation of monocytes into macrophages (Paper 1)
During differentiation of human monocytes into macrophages there was a marked increase in sterol 27-hydroxylated products in the media as well as mRNA for CYP27A1 in the harvested cells (Fig. 6 and 7). The increase started

at about 3 or 4 days after initiation of the cell culture.





This marked increase was observed regardless of whether or not fetal calf serum (FCS) was present in the culture media. Addition of FCS reduced the amount of 27-hydroxylated cholesterol in the media. The latter effect may be due to the lipoprotein particles available in the media that may shift cholesterol efflux away from the sterol 27-hydroxylase pathway towards the HDL pathway.

The monocyte to macrophage transition involves induction of many genes involved in lipid metabolism. Recently it was reported that activation of PPARγ-RXR / RAR-RXR may be crucial to CYP27A1 induction, and a specific response element for PPAR retinoids in the proximal promoter region of CYP27A1 was defined [62]. 27-Hydroxycholsterol has been shown to be a ligand to LXR that induces transcription of lipid-associated genes like ABCA1 and ABCG1 that may be important in cholesterol efflux [33].

From the above experiments it seems well documented that there is a marked induction of CYP27A1 during differentiation of human monocytes into macrophages. This large induction, corresponding to a 5-10 fold increase in 27-hydroxylated products in culture media from mature macrophages relative to monocytes, may be regarded as a defence mechanism against cholesterol overload. Such a defence mechanism may be needed in view of the fact that the monocyte also starts to express scavenger receptors CD36 during its differentiation to a macrophage. The latter are able to ingest oxidized LDL and other cholesterol rich components like cellular debris, leading to accumulation of intracellular cholesterol.

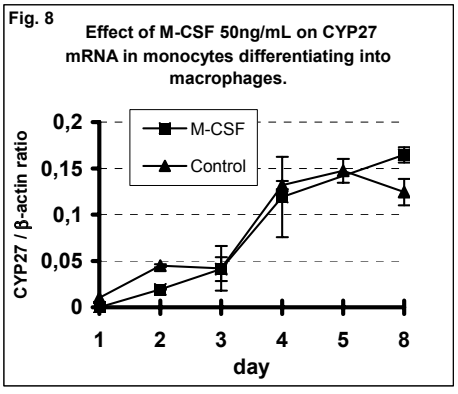
The recent observation that macrophages express the intracellular START-domain containing cholesterol transport protein StarD5 is of interest for sterol 27-hydroxylase activity, since substrate supply is considered to be an important limiting factor for enzymatic production from this mitochondrial enzyme [25, 43]. Theoretically upregulation of StarD5 in foam-cell macrophages in atherosclerotic lesions would be expected to increase reverse cholesterol transport through generation of oxysterol LXRα ligands and direct cholesterol metabolism. It is not know whether StarD5 is induced during the differentiation of monocytes to macrophages.

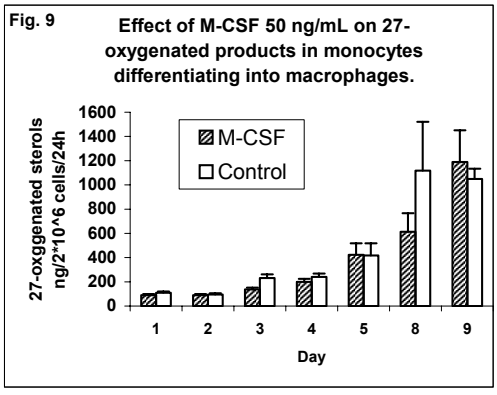
Different factors tested for effect on CYP27A1

In the attempts to find regulatory factors for CYP27A1, different substances (hormones and cytokines) were assayed for effect on sterol 27-hydroxylase. Some of the factors tested had previously been shown to regulate CYP27A1 in cell lines and in hepatocytes of different non-human species.

The components were tested in different concentrations with or without fetal calf serum (FCS) supplementation. Addition of FCS had an inhibitory effect on the production of 27-hydroxylated cholesterol species in the cell media.

Human monocyte-colony stimulating factor (M-CSF) was thought to be necessary for differentiation of monocytes and induction of CYP27A1. In contrast to the expectation, however, there was no difference in production of 27-hydroxylated cholesterol in the media or mRNA whether M-CSF was added or not (Fig. 8 and 9).





Several other important hormones and cytokines were tested in the monocyte to macrophage experimental system with or without FCS 10 %. The following

substances were tested: Insulin, growth hormone (GH), Dexamethasone, interleukin 6 (IL-6), testosterone, estrogen, vitamin D3 and thyroid hormone (T3). None of these factors had any significant effect on sterol 27-hydroxylase activity over a range of different concentrations. A typical set of experiments is shown in fig 10 a-e.

Fig. 10a

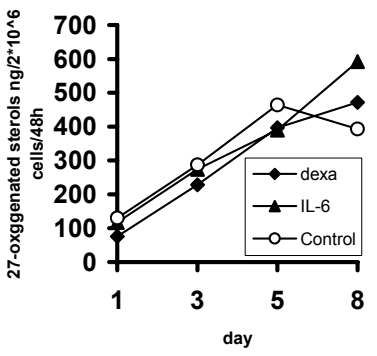


Fig. 10b

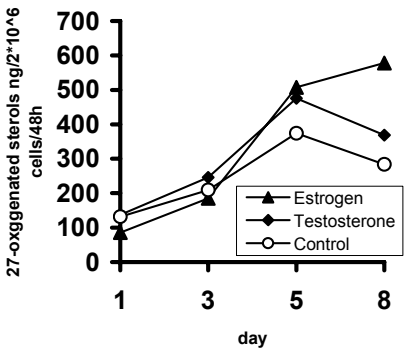


Fig. 10c

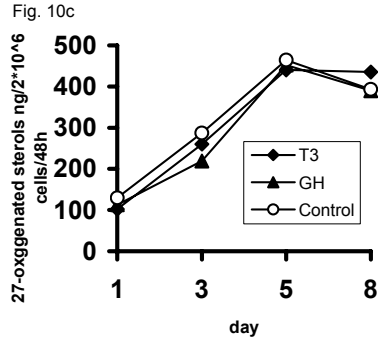


Fig. 10d

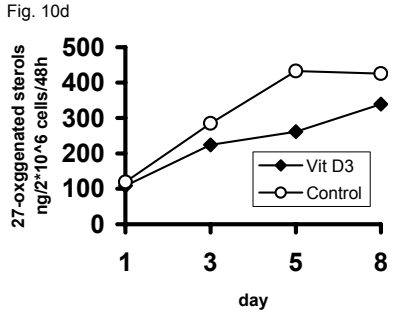
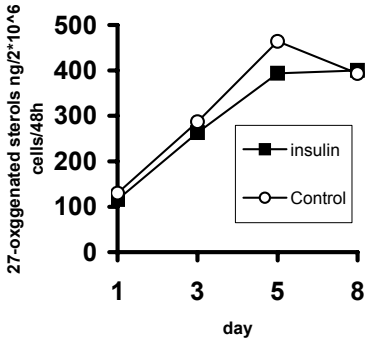


Fig. 10e



Effect of TGFβ1 on the activity of CYP27A1 (Paper 2)

TGFβ1 was shown to have a stimulatory effect on CYP27A1 gene expression and sterol 27-hydroxylase production in a dose dependent manner when added to mature human monocyte-derived macrophages. Addition of 1 ng/mL TGFβ1 to the cell media for a 48 h period roughly doubled the production of 27-hydroxylated products in human monocyte derived macrophages, fig 11.

Fig. 11 **Effect of TGFbeta1 1 ng/mL on 27-oxygenated products in monocyte derived macrophages.**

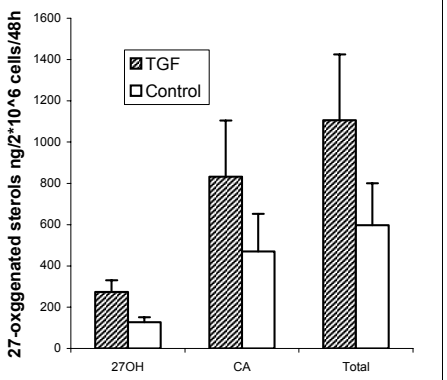
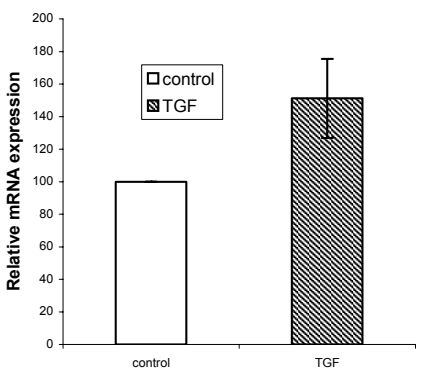
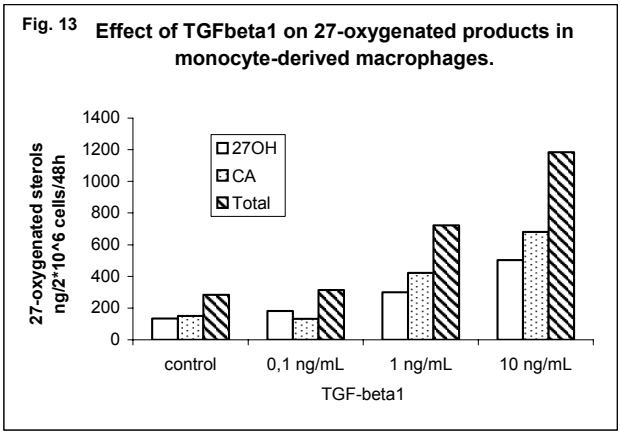


Fig. 12 **Effect of TGFbeta1 1 ng/mL on CYP27/betaAktin mRNA ratio in monocyte derived macrophages**

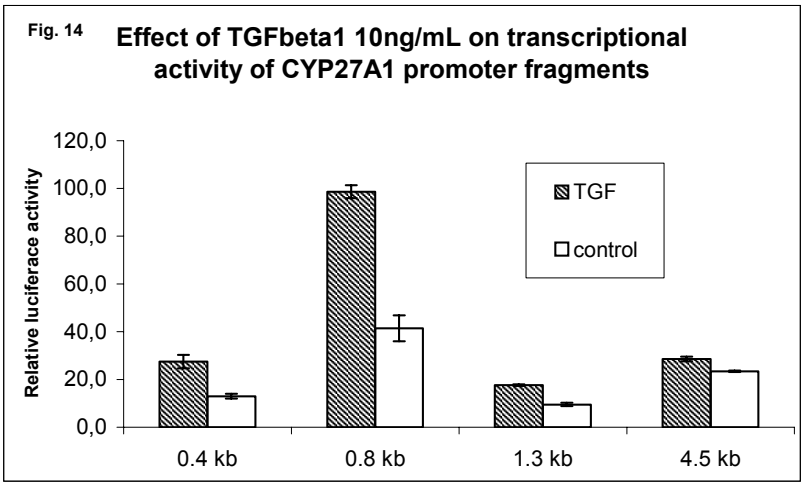


Levels of CYP27A1 mRNA normalized to β-actin mRNA increased about 50 % in the same experiments, fig 12. At the highest exposure, 10 ng/mL, the increase was about threefold, and a stimulatory effect was achieved at a level between 0,1 and 1 ng/mL, fig 13.



The effect of TGFβ1 was also tested in a luciferase expression system. Promoter fragments from CYP27A1 of different lengths from 0,4 kb to 4,5 kb were inserted into a pGL2 luciferase vector also containing β-galactosidase for transfection efficiency measurements. Transfection of HepG2 cells with this vector was followed by incubation with 10 ng/mL TGFβ1 for 24 h to assess the specific effect of TGFβ1 on the CYP27A1 promoter. A threefold increase in luciferase activity was measured when the full length promoter fragment (4,5 kb) was used together with TGFβ1. A further dissection of the promoter into

smaller fragments in this luciferase system revealed that TGFβ1 had a similar stimulatory effect in all of the four promoter fragment lenghts tested, fig 14.



To test whether TGFβ1 is related to CYP27A1 activity in vivo, we sampled citrate plasma from apparently healthy adult volunteers that had been fasting overnight. Samples were centrifuged twice to remove all platelet contamination because platelets contain much TGFβ1. However, no correlation was found between plasma levels of TGFβ1 and 27-hydroxycholesterol, whether or not corrected for total cholesterol. Consequently, if present in vivo the TGFβ1 effect on CYP27A1 may be confined to the microenvironment and is not reflected in changes in plasma levels of 27-hydroxycholesterol.

TGFβ1 is believed to exert many of its effects through Smad-proteins that heterodimerize after phosphorylation by the activated TGFreceptor in the

membrane. Smad complex enters the nucleus and interacts with specific DNA sequences to induce gene expression. The increase in luciferase activity for all four promoter constructs is consistent with the presence of a TGFβ1 responsive element in the proximal region of the promoter (0 to –400 bp).

Patients with pulmonary alveolar proteinosis (PAP) and the effect of GM-CSF (Paper 3)

PAP is a disease characterized by accumulation of protein and lipid in the alveoli of the lungs. The underlying defect is regarded to be the production of autoantibodies towards GM-CSF, disrupting GM-CSF signalling. The pulmonary macrophages of PAP patients have high cholesterol content, and it could be speculated that they in some way resemble macrophage foam cells of atherosclerotic lesions. CYP27A1 is present at high levels in human lung, and it was considered to be of interest to test if it may have a role in the cholesterol accumulation in PAP.

If GM-CSF is a normal stimulator of sterol 27-hydroxylase in the lung and the antibodies towards GM-CSF occurring in patients with PAP reduces the activity of the enzyme, this may be the explanation for the accumulation of cholesterol. The hypothesis was shown to be wrong. The levels of 27-hydroxycholesterol and cholestenoic acid in the circulation and in the brochioalveolar lavage (BAL) fluid from PAP patients were found to be increased rather than decreased. The

accumulation of cholesterol can thus not be due to reduced CYP27A1 activity. GM-CSF added to monocyte-derived macrophages was shown to inhibit rather than stimulate enzymatic activity (Fig. 15). The inhibitory effect was not due to reduced transcription, since the CYP27A1 mRNA levels in monocyte-derived macrophages increased rather than decreased after addition of GM-CSF, fig. 16. It seems likely that the increased transcription rate of the CYP27A1 gene could be a compensatory response to the reduced activity.

Fig. 15 **Effect of GM-CSF (ng/mL) on production of 27-oxygenated sterols by monocyte-derived macrophages**

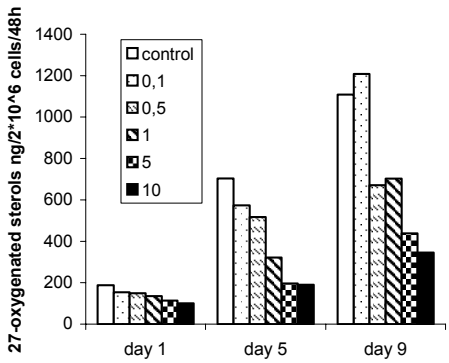
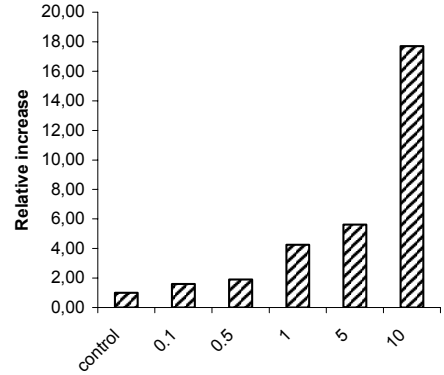


Fig. 16 **Effect of GM-CSF (ng/mL) on mRNA levels CYP27A1/cyclophilin A**



The increased levels of 27-hydroxycholesterol and cholestenoic acid in bronchioalveolar lavage fluid and in the circulation of the PAP patients may have two explanations: 1) The inhibitory effect of GM-CSF on CYP27A1 activity is released as a consequence of the antibodies present in the patients; 2) The increased production of 27-oxygenated products is secondary to the accumulation of cholesterol (increased substrate availability). Evidence has been

presented that the CYP27A1 enzyme present in macrophages is not normally saturated with its substrate [91], and also that the rate of influx of cholesterol to the mitochondria may be rate-limiting [43]. Whether or not there was an increased expression of CYP27A1 at the protein level in patients with PAP could not be determined due to a lack of materials. It was shown, however, that the CYP27A1 mRNA levels in the macrophages isolated from the BAL fluid were similar to those in the corresponding control cells. Thus, increased cholesterol availability is likely to be the most important explanation for the increased production of 27-oxygenated metabolites in PAP patients.

The mechanism behind the primary cholesterol accumulation remains to be explained, however.

In any case it is evident that the increased production of 27-oxygenated metabolites of cholesterol in the PAP patients is consistent with the anti-atherogenic role of CYP27A1, counteracting a local accumulation of cholesterol in some specific cells.

Effect of IL-10

IL-10 at a level of 10 ng/mL did not have any significant effect on CYP27A1 in mature monocyte-derived macrophages exposed for 48 h in SFM.

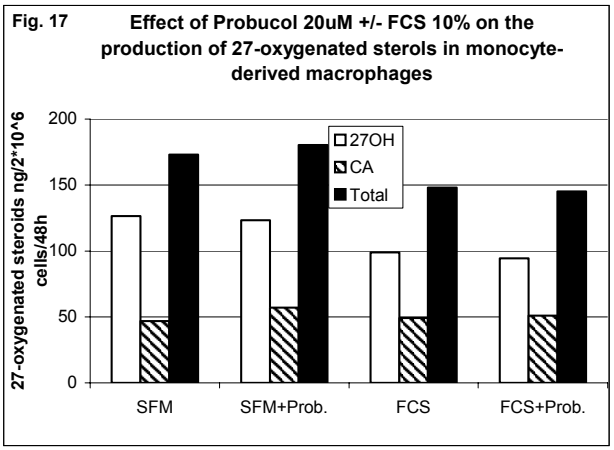
This implies that IL-10, having anti-inflammatory and anti-atherogenic properties [75], does not exert any of its anti-atherogenic effects through CYP27A1. The stimulatory effect shown for TGFβ1 and the inhibitory effect of

GM-CSF on CYP27A1 seem to be specific for these cytokines and are probably not due to general inflammatory responses.

Effect of Probucol

The most important mechanism for cholesterol efflux from the cells in the body is regarded to occur via ABCA1 and ABCG1 to HDL particles, but this mechanism may not be sufficient in tissues that are less vascularized. That the complementary CYP27A1 mediated mechanism may be of importance in such tissues is exemplified in CTX. These patients have pronounced xanthomas in their tendons due to accumulation of cholesterol and cholestanol as a consequence of the defective sterol 27-hydroxylase. The cholesterol-lowering drug Probucol has a unique capacity to reduce xanthoma size in spite of lowering HDL cholesterol levels [94]. Thus it was considered to be of interest to test the hypothesis that the effects of Probucol are mediated by effects on CYP27A1. Very recently it was shown that Probucol appears to inhibit ABCA1 in macrophages [77, 94]. Theoretically such an effect would be expected to increase the intracellular levels of free cholesterol, providing more substrate for CYP27A1. Probucol was added to monocyte-derived macrophages to see if it had any effect on the production of 27-hydroxylated metabolites. Concentrations of 10 μM and 20 μM were used as it was previously reported that maximal ABCA1 inhibition could be achieved by 10 μM. Experiments were performed

both in presence and absence of FCS 10%. However no effect of Probucol on production of 27-hydroxylated metabolites could be shown regardless of whether or not FCS 10% was added, fig 17. In accordance with the previous results (paper 1), a 20-25% reduction in the production of 27-hydroxylated metabolites was observed when FCS was added.



In conclusion, Probucol does not seem to affect sterol 27-hydroxylation under the above experimental conditions. A possible explanation could be that cholesterol efflux to HDL2 and HDL3 in macrophages is mediated mostly by ABCG1 and not by ABCA1 [95, 96].

Characterization of a patient with CTX (Paper 4)

Cerebrotendinous xanthomatosis (CTX) is a rare but treatable disease that is caused by a defective sterol 27-hydroxylase enzyme, see specific section above. Mutations in CYP27A1 is the cause of almost all cases presented to this date. Here we describe the molecular characterization of an adult CTX patient with a

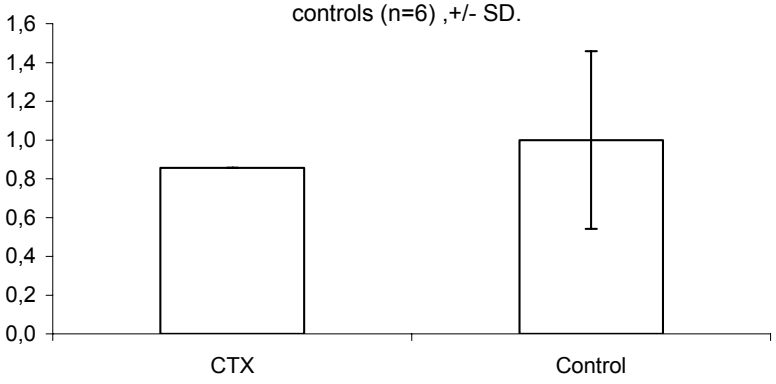
mild phenotype. She debuted in her teens with bilateral Achilles tendon xanthomas as the only CTX manifestation. Normal plasma cholesterol levels and normal routine laboratory measurements and her mild symptoms made her undiagnosed for several years. In her early thirties, suspicions of sitosterolemia warranted a more advanced lipid analysis to be done, although her tendon xanthomas were still the only clinical manifestation of CTX. Gas chromatography mass-spectrometry measurements showed that she had not sitosterolemia, but a typical sterol pattern consistent with CTX with increased bile alcohols in both plasma and urine, as well as increased cholestanol and markedly reduced 27-hydroxycholesterol in plasma. Chenodeoxycholic acid and simvastatin therapy was initiated and bile alcohols and cholestanol levels normalized. An MRI investigation excluded the presence of brain xanthomas and her neurological and psychiatric status was normal.

Genomic DNA was prepared from leucocytes from plasma samples and used for sequencing the exons of the CYP27A1 gene. One allele appeared to be normal, while the other contained a previously not described mutation in exon 8. It was a two base substitution (C478A and C479A), likely to disrupt the heme-binding domain of the enzyme.

The mutated protein was expressed in HEK293 cells and was found to have a great reduction in enzymatic activity compared to the normal enzyme, proving that this mutation is indeed causing the enzymatic defect. In the reaction, 5β-

cholestane-3α,7α,12α-triol was used as substrate and 5β-cholestane-3α,7α,12α, 27-tetrol was the product formed by sterol 27-hydroxylase. However, since the other allele was found to be normal, and the mother of the patient who shared the mutation in exon 8, was in good health, we continued to sequence other parts of the CYP27A1 gene for presence of possible mutations that together with the mutation in exon 8 would cause disease in this patient. The promoter (up to 4500 bp) was found to be similar to the reference sequence and this was the case also with intron-exon boundaries and the polyA site in the 3' end. Larger exon deletions or insertions that could possibly be missed in the sequencing effort was excluded by amplifying DNA exons 3 through 9 and running the resulting transcript on a gel. We also sequenced the complete cDNA without finding any alterations. The possibility of an altered regulation leading to low level of transcription of one allele or increased degradation of mRNA from the normal allele was excluded by sequencing revealing a 1:1 ratio between the two mRNA species in the patient. We also compared the CTX patient's RNA-levels with those of healthy controls. The latter investigation showed no significant difference between the patient and the controls, fig 18.

Fig. 18 Relative amount of CYP27A1 mRNA from monocyte derived macrophages determined by RealTime PCR. CTX and controls (n=6) ,+/- SD.



Northern blot analysis was not successful and Western blot indicated that the protein levels for sterol 27-hydroxylase was only slightly reduced in the CTX patient.

Furthermore, the father, mother and sister of the patient all had reduced plasma levels of 27-hydroxycholesterol (27OH), fig 19, the primary product of the sterol 27-hydroxylase, indicating that the father may harbour the second mutation leading to an almost complete absence of 27-hydroxycholesterol (27OH) in the patient. This finding was objectified by comparing the ratio 27OH / 24OH in all family members and relating them to a control material. 24OH is produced only by brain tissue, and levels are more stable than those of 27OH, which may be dependent on atherosclerotic burden. Treatment with statins does not change this ratio to any larger extent. All members of the family except the

patient (who had an extremely reduced ratio) had clearly reduced ratios, indicating that both the mother and the father are carriers of separate defects that both reduce 27OH production.

Figure 19

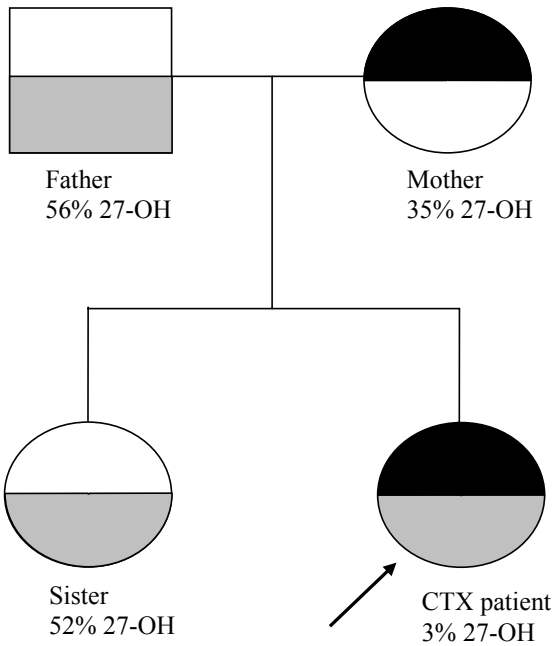


Fig 19. Plasma levels of 27-hydroxycholesterol (27-OH) in the family of the CTX-patient as percentages of the average in a reference group.

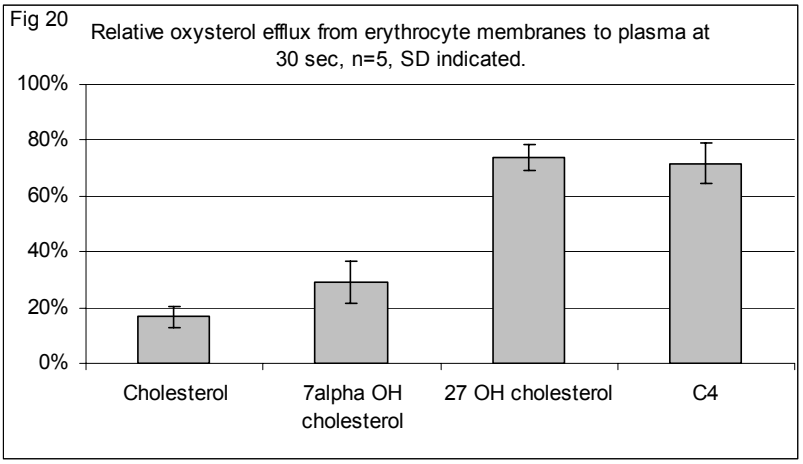
The unknown defect in the father and the sister was not identified in the present work, however we speculate that an intracellular cholesterol transporter may be involved.

To conclude, only one mutation was found in this CTX patient, making it a rare case of heterozygous CTX. The as yet unknown mutation may, when revealed, give new insight into cholesterol metabolism in the cells.

Possible mechanisms behind accumulation of cholestanol in the brain of CTX-patients

To elucidate the mechanism behind cholestanol accumulation in the brain of CTX patients, a model of the blood-brain barrier was applied as well as cell-culturing experiments. We demonstrated that 7α-hydroxylated bile acid intermediates, that are present at 50-250 fold normal levels in the plasma of CTX patients are able to cross the artificial barrier much more rapidly than is cholestanol or cholesterol. In particular 7α-hydroxy-4-cholesten-3-one was about 100-fold more efficiently transferred across the cultured porcine brain endothelial cells than was cholestanol.

The efficient transport of 7α-hydroxy-4-cholesten-3-one across the endothelial cells may be due to an active process or a consequence of the physicochemical properties of the oxysterol. In preliminary experiments the latter possibility was tested by the rate of transfer of radiolabelled 7α-hydroxy-4-cholesten-3-one from erythrocyte membranes to plasma lipoproteins. The rate of the detachment of 7α-hydroxy-4-cholesten-3-one from the erythrocytes was considerably higher than that of 7α-hydroxycholesterol and cholesterol, and of a similar rate as 27-hydroxycholesterol, fig 20.



In previous work the latter oxysterol has been shown to be very efficiently transferred in this system. It may be concluded that at least part of the efficient flux of 7α-hydroxy-4-cholesten-3-one over endothelial cells is likely to be a consequence of the physicochemical properties.

Culturing experiments using human cell lines of astrocytic, microglial, and neuronal origin, as well as human monocyte-derived macrophages showed that 7α-hydroxy-4-cholesten-3-one was efficiently converted to cholestanol when added to the media. These results are consistent with the observation that treatment with chenodeoxycholic acid, which normalizes the level of bile acid precursors in CTX patients, results in a reduction of cholestanol-containing xanthomas, even in the brain.

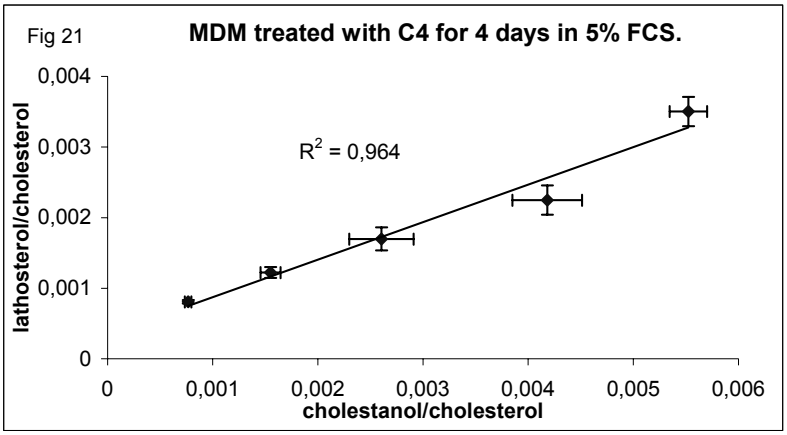
Possible mechanism for accumulation of cholesterol in patients with CTX

In addition to cholestanol, also cholesterol is accumulating in xanthomas in patients with CTX. As shown in paper 4 most of the cholestanol present in the brain of patients with CTX is likely to be a metabolite of 7α-hydroxy-4-cholesten-3-one fluxing over the blood-brain barrier.

As discussed in Introduction, it seems less likely that the lack of the sterol 27-hydroxylase is the most important direct explanation for the accumulation of cholesterol. Treatment with chenodeoxycholic acid is thus known to shrink xanthomas over time, despite a complete absence of sterol 27-hydroxylase. The fact that treatment with chenodeoxycholic acid causes a normalization of the levels of cholestanol in parallel with the shrinkage of the xanthomas is consistent with the possibility that the accumulation of cholesterol is secondary to the accumulation of cholestanol.

The structural similarity between cholesterol and cholestanol would suggest that both these compounds have about the same capacity to inhibit cholesterol synthesis. According to some in vivo experiments (cf. Introduction) this is not the case, however. It is shown in paper 4 that loading of monocyte-derived macrophages with cholestanol caused an expansion of the total steroid pool (cholesterol + cholestanol) with very little change in the cholesterol levels. This finding suggests that cholestanol is less effective than cholesterol to suppress cholesterol synthesis. The levels of lathosterol in the cell is believed to reflect

the rate of synthesis of cholesterol, and in the experiments shown in Paper 4 the accumulation of cholestanol in the macrophages did not significantly affect the levels of this precursor to cholesterol. When exposing cultured monocyte-derived macrophages to 7α-hydroxy-4-cholesten-3-one with a subsequent low accumulation of cholestanol, there was a significant increase in the levels of lathosterol that was correlated to this accumulation, fig 21.



A possible explanation is that accumulation of cholestanol leads to a dilution of the cholesterol pool which may trigger a compensatory SREBP-dependent cholesterol synthesis. The findings are in consonance with the previous in vivo experiments with rats and rabbits in which cholestanol caused an increased cholesterol synthesis. Further experiments are required, however, to evaluate if this mechanism is of major importance for the accumulation of cholesterol in patients with CTX.

Future perspectives

- Our hypothesis that a dilution of the cholesterol pool with cholestanol triggers a local de novo synthesis of cholesterol in macrophages and glial cells of patients with CTX will be further tested in different cell systems (monocyte-derived macrophages, astrocytes and microglial cells). These studies will also involve measurements of mRNA and protein levels of SREBP1 and 2.
- At present it is not possible to exclude the possibility that specific transport mechanisms exist for the flux of some oxysterols across the blood-brain barrier. The flux of different oxysterols across endothelial cells and their mobility from erythrocyte membranes to plasma will be studied more in detail. In particular we will study the possibility that specific mechanisms exist for the transport of oxysterols with a 3-oxo-Δ^4-structure.
- An animal model for accumulation of cholestanol in the brain is still lacking. Our hypothesis that most of the cholestanol in the brain of patients with CTX originates from 7α-hydroxy-4-cholesten-3-one fluxing across the blood-brain barrier will be tested with use of CYP27A1 deficient mice that are treated with 7α-hydroxy-4-

cholesten-3-one in diet or intraperitoneally. If the hypothesis is correct, xanthomas may appear in the brain of these mice. The markedly increased production of 7α-hydroxy-4-cholesten-3-one in patients with CTX is due to the very high activity of CYP7A1. We will treat CYP27A1 deficient mice with cholestyramine to obtain an upregulation of CYP7A1 and study if brain xanthomas appear.

- The inhibitory effect of GM-CSF on CYP27A1 will be studied more in detail

General summary

- CYP27A1 was found to be markedly induced during differentiation of human monocytes into macrophages with increased levels of mRNA and increased production of 27-oxygenated products of cholesterol. This induction has the potential to counteract the simultaneous induction of scavenger receptors.
- The cytokine TGFβ1 was found to have a stimulatory effect on transcription and enzymatic activity of CYP27A1 in monocyte-derived macrophages. Experiments with different promoter constructs suggest that the stimulatory effect of TGFβ1 on the transcription is mainly due to responsive element(s) in the proximal region of the promoter. The possibility is discussed that part of the antiatherogenic effect of TGFβ1 may be mediated by its effect on CYP27A1.
- Patients with pulmonary alveolar proteinosis have a local accumulation of cholesterol in the lung, known to contain a particularly high level of CYP27A1. This accumulation was associated with increased levels of the two CYP27A1 products 27-hydroxycholesterol and cholestenoic acid. The increased levels of these products were regarded to reflect a CYP27A1 mediated defence towards a local accumulation of cholesteol. The most important factor for the increased metabolism of cholesterol is likely to be availability of substrate for the enzyme rather than induction of enzyme.

All the above findings support the contention that CYP27A1 is an antiatherogenic enzyme. A selective upregulation of CYP27A1 would represent a new antiatherogenic strategy, that would improve reverse cholesterol transport, particularly in macrophages.

In a patient with a mild form of sterol 27-hydroxylase deficiency (CTX) a heterozygote mutation in exon 8 of Cyp27A1 was identified. Despite our sequencing efforts, no other mutation was found in the CYP27A1 gene. Reduced levels of 27-hydroxycholesterol in the plasma of other family members indicates that another genetic defect is present, and that this defect together with the mutation in exon 8 is causing CTX in this patient.

Using a model system of the blood-brain barrier, it was shown that the bile acid precursor 7α-hydroxy-4-cholesten-3-one, which is accumulated in the circulation of CTX patients, passes the blood-brain barrier at a rate about 100-fold higher than that of cholesterol and cholestanol. Cultured human astrocytes, microglial cells, neurogenic cells, and moncyte-derived macrophages were able to convert 7α-hydroxy-4-cholesten-3-one into cholestanol. It is suggested that most of the cholestanol present in brain xanthomas of CTX patients accumulate as a consequence of a flux of 7α-hydroxy-4-cholesten-3-one over the blood-brain barrier.

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