NOTE TO USERS

Synthesis and secretion of apoC-I and apoE by human SW872 liposarcoma cells

by

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August 2004

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master's of Science (M.Sc.) of Nutrition

Your file Votre référence
ISBN: 0-494-12562-4
Our file Notre référence
ISBN: 0-494-12562-4

To my parents...

Abstract

Apolipoprotein C-I (apoC-I) plays an important role in the metabolism of plasma triglyceride levels and cholesterol metabolism. Little is known about the regulation of apoC-I production by human adipocytes. **Aim:** To investigate the effect of different tissue culture conditions on the synthesis and secretion of apoC-I and apoE in human SW872 liposarcoma cells and to study the effects of apoC-I overexpression in these same cells. **Methods:** SW872 cells were grown in DMEM/F-12 (3:1, v/v). QPCR was used to quantify mRNA synthesis. ELISAs were used to quantify intracellular and extracellular proteins. Colorimetric reaction kits were used to analyze intracellular cholesterol and triglyceride concentrations. **Results:** Maturation experiments revealed that after 17 days in culture, SW872 cells contained significantly more cholesterol (100%) and triglyceride (3-fold). Cell maturation was associated with significantly higher levels of apoE mRNA (+200%) but not apoC-I mRNA (-50%). The cells secreted more apoC-I (+110%) and apoE (+340%). Cellular apoC-I increased 620% and apoE increased 1540%. Treatment of cells during maturation with insulin (0, 10 or 1000 nM) significantly reduced the secretion of apoC-I and apoE (-14% and -56%, respectively) and intracellular apoC-I and apoE (-10% and -12%, respectively. Overexpression of apoC-I in SW872 cells resulted in increased cell number (+70%) and decreased lipids per cell (-32% triglyceride, -36% cholesterol) as compared to controls. **Conclusion:** These results suggest that apoC-I and apoE production is differentially regulated at the transcriptional level in adipocytes and that apoC-I and apoE play a role in the maturation of human adipocytes and may have an important role in mediating or regulating cell lipid accumulation. As well, overexpression of apoC-I in SW872 cells impedes cellular lipid accumulation and stimulates cellular proliferation.

Résumé

L'apolipoprotéine C-I (apoC-I) joue un rôle central dans la régulation des triglycérides plasmatiques et le métabolisme du cholestérol. Notre laboratoire a démontré que l'apoC-I est produite par le tissu adipeux humain mais sa fonction précise n'a pas encore été déterminée. **Objectif:** Déterminer quels facteurs peuvent influencer la synthèse et la sécrétion de l'apoC-I et examiner l'effet d'une surexpression de l'apoC-I par les cellules de liposarcomes SW872. **Méthodes:** Les cellules SW872 ont été cultivées dans du DMEM/Ham's F-12 (3:1, v/v). Les techniques de Q-PCR et ELISA ont été utilisées pour analyser l'ARNm et la masse d'apoC-I. Des réactions enzymatiques ont été effectuées pour mesurer les lipides cellulaires. **Résultats:** Les études de maturation ont démontré que les cellules cultivées durant 17 jours ont accumulé 3 fois plus de triglycérides et 100% plus de cholestérol que celles cultivées durant 4 jours. De plus, ces mêmes cellules ont exprimé plus d'ARNm d'apoE (+200%) et moins d'apoC-I (-50%). Pour les protéines sécrétées et cellulaires, il y a eu une augmentation significative d'apoC-I (+110% et +620% respectivement) et d'apoE (+340% et +1540% respectivement). En présence d'insuline (0nM, 10nM, 1000nM), il n'y a eu aucune différence significative dans l'accumulation de triglycérides ou de cholestérol. Par contre, une diminution significative d'ARNm d'apoC-I (-60%) a été obtenue. Il y a eu aussi des diminutions significatives pour l'apoC-I (-10% cellulaire et -14% sécrétée) et l'apoE (-12% cellulaire et -56% sécrétée). La surexpression de l'apoC-I dans les cellules SW872 a causé une augmentation significative du nombre cellulaire (+70%) et a inhibé l'accumulation des lipides (-32% triglycérides, -36% cholestérol) dans ces cellules. **Conclusion:** Ces résultats suggèrent que la production d'apoC-I et d'apoE est régulée différemment dans le tissu adipeux et que l'apoC-I et l'apoE sont des facteurs importants pour la maturation des cellules adipocytes et pourraient jouer un rôle important dans l'accumulation cellulaire de lipides. Aussi, nos résultats démontrent qu'une surexpression de l'apoC-I cause une augmentation de la prolifération cellulaire et une inhibition de l'accumulation de lipides cellulaires dans les adipocytes.

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Contributions of authors

Chapter 1 - Human subcutaneous and omental mRNA was obtained from Dr. Katherine Cianflone (MUHC). Differentiated and undifferentiated THP-1 macrophage mRNA was provided by Dr. Lise Bernier. HepG2 mRNA was obtained by Hanny Wassef.

Chapter 3 - All manipulations including cell culture, lipid extraction and assays, protein assays, and mRNA assays were performed solely by Hanny Wassef. Dr. Lise Bernier provided molecular biology advice. Dr. Jean Davignon contributed advice and financial support. Dr. Jeffrey S. Cohn provided advice, guidance, financial support, and text editing.

Chapter 4 - ApoC-I DNA constructs were kindly provided by Catherine Bouchard and Lucie Boulet. SW872 cellular transfections were performed by Jean-François Carmel. Cell culture, lipid extraction and assays, protein assays, mRNA assays, and assessment of apoptosis were performed solely by Hanny Wassef.

All chapters presented in this thesis were written by Hanny Wassef with editing provided by Dr. Jeffrey S. Cohn.

I – Introduction

1.1 Obesity and cardiovascular disease

Obesity is becoming a major medical problem in developing countries. The 2000/01 Canadian Community Health Survey reported that 32% of the population aged between 20 and 64 had an unhealthy BMI (greater than 27) (1). It was determined that between 1985 and 2000, over 57,000 deaths were attributed to overweight and obesity in Canada (2). Furthermore, it was estimated that in 1997, obesity cost the Canadian health care system over $1.8 billion (3).

It is well know that excess body fat is associated with increased prevalence of cardiovascular disease (4-10), hypertension (11-15), type 2 diabetes (16,17), dyslipidemia (18-19), gallbladder disease (20,21) and cancer (22-28). The mechanisms through which obesity causes disease are slowly being elucidated through both the *in vivo* and *in vitro* study of adipose tissue and adipocytes. It is interesting to note that the ability to store excess energy as fat was a crucial survival mechanism in times of food deprivation yet, in modern times, our sedentary lifestyles have caused our energy storage capabilities to be deleterious to our health.

1.2 Adipose tissue – an endocrine organ

Adipose tissue was, for many years, believed to act solely as a passive energy depot, storing or releasing fatty acids under the control of metabolic hormones. It is now widely accepted that adipose tissue acts as an endocrine

organ, synthesizing and secreting several proteins that play important roles in whole-body homeostasis. In 1994, Zhang *et. al.* discovered leptin, a protein found in adipocytes, which acts on the hypothalamus and functions as a metabolic signal for energy sufficiency (29-31). This molecule was the first circulating adipocyte-specific protein to be identified in a class of proteins that are now referred to as adipocytokines. Other important adipocytokines include tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), adiponectin, adipsin and resistin (31). This class of molecules is secreted by adipocytes and has important metabolic effects on other cells and tissues.

Adipocytes not only secrete adipocytokines, but they also synthesize and secrete other proteins, which are not adipose-tissue specific. Genome analysis of human adipose tissue, preadipocytes and adipocytes has identified hundreds of genes, which function in cell defense, cell division, cell signaling, cell structure, gene/protein expression and metabolism (32,33). Several of these genes have been identified on chromosomes 11, 19 and 22 (32).

1.3 Adipose tissue and apoC-I

Apolipoprotein (apo) C-I is an important plasma protein whose gene is situated on chromosome 19 with apoE, apoC-II and apoC-IV. As discussed in more detail in Chapter II, the role of apoC-I in lipid metabolism is varied and includes: a) activation of lecithin:cholesteryl acyltransferase (LCAT), an enzyme required for cholesteryl ester formation; b) inhibition of lipoprotein lipase (LPL), an enzyme required for triglyceride (TG) lipolysis; c) inhibition of binding of β-

VLDL to the LDL receptor-related protein (LRP), reducing hepatic clearance of triglyceride-rich lipoproteins (TRL); and d) inhibition of cholesteryl ester transfer protein (CETP), required for the transfer of cholesteryl esters from HDL to LDL (34). Recently, experiments in transgenic mice have suggested that apoC-I plays a significant role in the etiology of obesity and diabetes. It was demonstrated that ob/ob mice overexpressing human apoC-I were protected against obesity. ApoC-I was shown to inhibit fatty acid uptake in adipose tissue (35). This raises the interesting possibility that apoC-I has a central role to play in regulating lipid metabolism in adipose tissue.

Initial studies in our laboratory using human adipocytes kindly provided by Dr. Katherine Cianflone (MUHC) allowed us to quantify cellular apoC-I mRNA. Figure 1-1 shows that both subcutaneous and omental adipose tissue contain quantifiable amounts of apoC-I mRNA. The apoC-I mRNA present was comparable to that found in human hepatoma HepG2 cells. This is of significance because the liver is believed to be the principal source of circulating apoC-I (36,37).

1.4 Rationale, objectives and hypothesis

In view of the fact that apoC-I has been shown to affect adipocyte metabolism in mice and given the lack of information pertaining to apoC-I production by human adipocytes, we carried out the present study in which we investigated the effect of different tissue culture conditions on the synthesis and secretion of apoC-I and apoE by SW872 liposarcoma cells. The specific

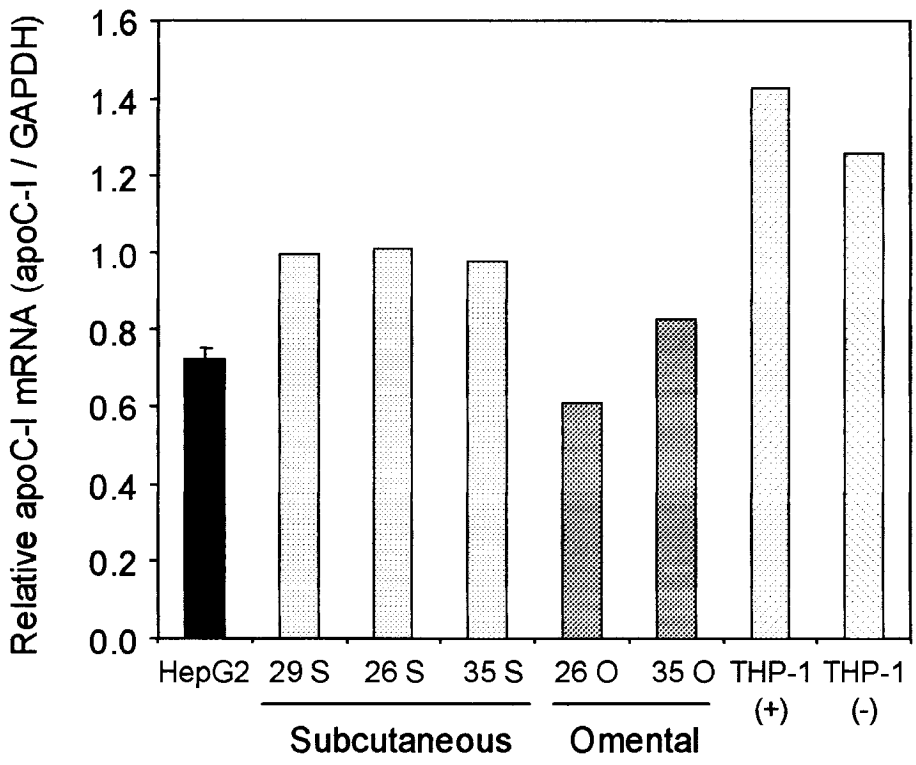

Figure 1-1. Apolipoprotein C-I mRNA quantification of HepG2 cells, subcutaneous and omental adipocytes and differentiated (+) and undifferentiated (-) THP-1 macrophages. ApoC-I mRNA were quantitated by reverse transcriptase PCR and were expressed relative to GAPDH mRNA. Cells were allowed to grow in the presence of their respective media for 48h. Cells reached 70% confluence after 2 days in culture at which time cells were collected for mRNA measurements. Data represent means ± SE (n = 8) for HepG2 and individual measurements for other cells.

objectives of the present Master's project were to: 1) quantify the synthesis and secretion of apoC-I by adipocytes in culture, 2) determine if factors such as cellular maturation, cellular cholesterol and triglycerides accumulation, and insulin alter adipocyte apoC-I production, 3) overexpress apoC-I in adipocytes and quantify its cellular effects, and begin to elucidate the physiological role of apoC-I in human adipose tissue. The hypothesis for this work was that apoC-I was synthesized and secreted by human adipose tissue and played an important role in the regulation of human adipose tissue lipid metabolism.

II - Literature Review

2.1 Adipocytes and adipose tissue

On average, adult humans have approximately 15kg of adipose tissue. In cases of extreme obesity, this can increase to over 100kg (38). Importantly, adipose tissue is itself not a monocellular tissue. Approximately one third of human adipose tissue consists of adipocytes, while the remaining two thirds is made up of small blood vessels, nerve tissue, fibroblasts, immune cells, stromal vascular cells and preadipocytes at different stages of differentiation (39,40). The body has various depots of fat, with abdominal subcutaneous fat and visceral fat garnering greatest research attention because of their strong association with cardiovascular disease. In a recent study determining the contributions of human visceral adipose tissue and human subcutaneous adipose tissue to the metabolic complications of obesity, it was found that visceral adipose tissue plays a greater role in the etiology of obesity (41). More recently, Klein *et. al.* (42) investigated the effect of abdominal subcutaneous liposuction on insulin action and risk of coronary heart disease in obese women with or without type 2 diabetes. It was determined that although both groups of women lost greater than 18% of their total fat through liposuction, there were no significant effects on insulin sensitivity in liver, muscle or adipose tissue, nor were there any significant effects on the plasma concentrations of C-reactive protein (CRP), IL-6, TNFα, adiponectin, nor on blood pressure, blood glucose or lipid concentrations.

2.2 Adipose tissue metabolism

The primary function of adipose tissue is to store excess energy derived from food. Under the control of insulin, fatty acids and glucose are cleared from circulating blood in the postprandial period. Fatty acids released from TRL through the action of lipoprotein lipase are taken up by adipose tissue. It is believed that plasma membrane fatty acid transporters such as CD36, FATP1, FATP2, FATP5 and aP2 are responsible for fatty acid entry into adipocytes and that GLUT1 and GLUT4 are glucose transport proteins for bringing glucose into adipocytes. Inside adipocytes, fatty acids are esterified to glycerolphosphate to form triglyceride through the action of DGAT. In times of energy deficiency, lipolysis occurs whereby free fatty acids and glycerol are released from the triglyceride pool. Catecholamines such as epinephrine and norepinephrine, or hormones such as glucagon and glucocorticoids activate hormone sensitive lipase and monoacylglycerol lipase that release fatty acids from the stored triglyceride pools. The fatty acids are then released from adipose tissue, become attached to albumin, and are transported in the blood to the liver, muscle or other tissues, where they are oxidized for ATP synthesis (43-49).

2.3 Adipocytokines

Gene expression profiling studies have shown that there are a large number of proteins synthesized by visceral adipose tissue, which act as endocrine and paracrine factors. This interesting class of molecules (i.e. adipocytokine, *aka* adipokines) is thought to be the link between adiposity and

increased risk of insulin resistance, type 2 diabetes and cardiovascular disease. Yang *et. al.* (50) obtained omental adipose tissue from non-obese women and identified 8300 expressed genes, 5200 being known genes and 3030 corresponding to expressed sequence tags (ESTs). Secretory proteins such as hormones, cytokines, growth factors and apolipoproteins were identified, as were receptor proteins such as the leptin receptor, TNF receptor, and IL receptors. Other proteins included 32 appetite-regulating proteins, 12 autocrine/paracrine proteins and 74 transcription factors that play important roles in adipocyte differentiation. Similarly, Fain *et. al.* (51) compared the release of adipocytokines by visceral and subcutaneous adipose tissue of obese humans. It was found that visceral adipocytes from morbidly obese humans (BMI > 45) released more adipocytokines such as leptin, adiponectin, IL-6 and TNFα than visceral adipocytes from obese individual (BMI = 32). Interestingly, aside from leptin and adiponectin, the amounts of certain adipocytokines such as PGE_2, IL-6 and IL-8 released from adipocytes were less than visceral adipose tissue explants. These results suggest that the bulk of adipocytokines secreted by adipose tissue may come from cells other than adipocytes.

Recently, it was suggested that stromal vascular cells, which are comprised of pre-adipocytes and immature fat cells (52), have the ability to produce IL-1β, PGE_2, TNFα and IL-6 (53). In addition, there is new data suggesting that the pro-inflammatory activity of adipose tissue is dependent on macrophages present in the tissue. Because changes in adipose tissue mass are correlated with changes in endocrine and metabolic functions of adipose

tissue, Weisberg *et. al.* (54) studied those genes whose expression correlated with adiposity. In parametrial and epididymal adipose tissue from 24 mice with varying degrees of adiposity, 1304 transcripts were significantly associated with increased body mass. Of the 100 transcripts found to correspond most strongly to adiposity, 30% encoded proteins characteristically found in macrophages. Also, macrophages identified in adipose tissue were the predominant source of TNFα and it was determined that these cells were of bone marrow origin (54). Xu *et. al.* (55) also showed that the upregulation of macrophage specific genes during increased body mass does not occur in the liver, spleen or muscle, but was specific to adipose tissue. The possibility therefore exists that macrophages found in adipose tissue could arise from the transformation of preadipocytes, since their phenotypes are very similar. Under the right conditions, preadipocytes rapidly transform into macrophages and express several macrophage specific antigens (56).

Leptin was the first adipocytokine to be identified in 1994 by Zhang *et. al.* (29). The leptin gene was identified as the gene responsible for obesity in ob/ob mice. Although the synthesis of this protein occurs in many tissues such as brown adipose tissue and the stomach (57), subcutaneous adipose tissue is the principal site of circulating leptin (51). Plasma levels of leptin are directly proportional to adiposity and can be affected by insulin, glucocorticoids, estrogen androgens free fatty acids and TNFα (58). Among its many functions, leptin's primary role is to mediate energy expenditure by acting on the hypothalamus (30,31).

Other adipokines of note include TNFα, IL-6, adiponectin and resistin. TNFα, expressed by adipose tissue and stromal vascular cells, is referred to as a pro-inflammatory cytokine (51,59). TNFα acts through two TNFα receptors (TNFR), TNFR1 and TNFR2 (60). Although TNFα inhibits LPL activity (61) and promotes lipolysis in humans through the activation of mitogen activated protein kinases (MAPKs) p44/42 and JNK (61), increased levels of TNFα have been found in obese women (63). As well, TNFα has been found to inhibit the expression of genes that play important roles in the storage of glucose, NEFA and adipogenesis such as LPL, DGAT, HSL, CEBPα, PPARγ and RXRα (61). Most importantly, TNFα impairs insulin signaling and is thought to play a major role in the development of type 2 diabetes (64). Interestingly, it also affects the expression of other adipokines such as IL-6 and adiponectin (31,61,65).

It is estimated that 30% of IL-6 found in the plasma originates from adipose tissue, with visceral adipose tissue being the predominant source (51,66). Circulating levels are significantly higher in patients with obesity and insulin resistance (67). Principally, IL-6 decreases insulin signaling in peripheral tissue by reducing the expression of insulin receptor signaling components (31,68). Interestingly, IL-6 can downregulate adiponectin gene expression *in vitro* (69). Adiponectin, unlike IL-6 and TNFα, is downregulated in patients with obesity and insulin resistance (70). In fact, overexpression of adiponectin in mice improves insulin sensitivity (71). Also, it is believed that adiponectin might enter damaged vascular endothelium and decrease the development of vascular atherogenic changes through its inhibition of intracellular adhesion molecule-1

(IAM-1), vascular cellular adhesion molecule-1 (VCAM-1) and E-selectin (70). Resistin is believed to only be secreted by adipocytes (72,73). Injecting resistin into mice causes reduced glucose tolerance and insulin action whereas resistin-deficient mice have improved fasting blood glucose levels (71,74). Furthermore, resistin is also linked to cardiovascular disease through its upregulation of VCAM-1 and monocyte chemoattractant chemokine (MCP-1) as demonstrated in endothelial cells incubated with human recombinant resistin (75).

2.4 Adipocyte differentiation and adipogenesis

Importantly, adipocyte cellular functions are dependent on cellular differentiation. Adipocytes originate from multipotent mesenchymal stem cells that also have the potential to become muscle, bone or cartilage cells given the right conditions (76). The molecular events that determine how the stem cells become committed adipocytes are still unclear however. A significant amount of work has been performed with cell lines, which has elucidated the mechanisms of adipogenesis after commitment of the stem cell to preadipocytes. One difficulty in studying adipocyte differentiation is that it is not easy to separate pre-adipocytes from other adipose tissue cells such as fibroblasts and stromal vascular cells. As well, primary culture has a limited lifespan and a large amount of adipose tissue is required for adipocyte isolation. For these reasons, the majority of what is currently known about adipocyte differentiation has been obtained from animal cell lines.

Adipogenesis is a complex process involving changes in gene expression, cellular morphology and hormone sensitivity. In most murine cell lines such as 3T3-L1 cells and in cultured human primary adipocytes, differentiation only occurs when cells are treated with a chemically-defined medium. The medium usually contains a cocktail of chemicals, most importantly: insulin, dexamethasone (a synthetic glucocorticoid agonist that stimulates the glucocorticoid pathway); and isobutylmethylxanthine (a cAMP-phosphodiesterase inhibitor that stimulates the cAMP-dependent protein kinase pathway) (77,78). Several transcription factors involved in adipogenesis have been identified, yet most attention has been paid to peroxisome proliferators-activated receptor-gamma (PPARγ) and CCAAT enhancer-binding protein-alpha (C/EBPα). PPARγ is part of a family of nuclear hormones which also include PPARα and PPARβ/δ (79). PPARγ has two isoforms PPARγ_1 and PPARγ_2. PPARγ_2 has 30 more amino acids at the N terminus as compared to PPARγ_1 (80). PPARγ_2 is the isoform primarily produced by adipocytes (81). It forms an oligodimer with 9-cis-retinoid acid-activated retinoid X receptor (RXR) and then binds to specific sites in the promoter area of target genes called PPAR response elements (PPREs) (82). It has been found that PPARγ is necessary for adipogenesis (83). Its expression is highest during pre-adipocyte differentiation. As well, PPARγ-heterozygous-null mice have a reduced amount of adipose tissue (84). Many ligands activate PPARγ, including eicosanoid metabolites such as prostaglandins (PGJ2, PGH1 and PGH2), fatty acids, and fatty acid-derived compounds (85). Insulin-sensitizing drugs called thiazolidinediones (TZD) have been used

extensively in molecular studies because they are known PPARγ agonists (86). Many lipogenic genes have been identified that are under the transcriptional control of PPARγ including LPL, fatty acid translocase CD36, fatty acid transport protein, acetyl-CoA carboxylase, fatty acid synthase and ATP-citrate lysase (87). As well, PPARγ downregulates leptin and resistin expression (87). Because of its role in adipogenesis and lipogenesis, PPARγ mRNA is believed to increase with obesity, however, this remains controversial (88,89).

C/EBPα is a transcription factor crucial to the development of white adipose tissue (90). Overexpression of C/EBPα in 3T3-L1 preadipocytes promotes differentiation, whereas decreased expression of C/EBPα in 3T3-L1 cells inhibits differentiation (91,92). This protein is expressed in early stages of differentiation and binds to the promoter region of several adipocyte genes including aP2, SCD1, Glut-4, PEPCK, leptin and the insulin receptor (93). Interestingly, growth arrest of 3T3-L1 preadipocytes is crucial to the initiation of several rounds of mitotic clonal expansion and subsequent activation of differentiation (94). Wang *et. al.* (95) have shown that C/EBPα interacts directly with cyclin-dependent kinases 2 and 4 and induces growth arrest.

2.5 Human SW872 liposarcoma cells

Although many advances in adipocyte research have been made using animal cell line, significant differences remain between animal and human tissue.

We have therefore chosen the human SW872 liposarcoma cell as a model to study adipocyte metabolism. These malignant tumor cells were originally from a fibrosarcoma removed from a 36 year male Caucasian. After immortalization, the cells were made commercially available (96). These cells have a fibroblastic phenotype, yet in DMEM/F-12 medium supplemented with 10% fetal calf serum, they readily accumulate lipids and display a mature adipocyte phenotype (97, Chapter III). As seen in Figure 2–1, immature cells appear long and fibroblastic and mature cells have lipid-filled vesicles.

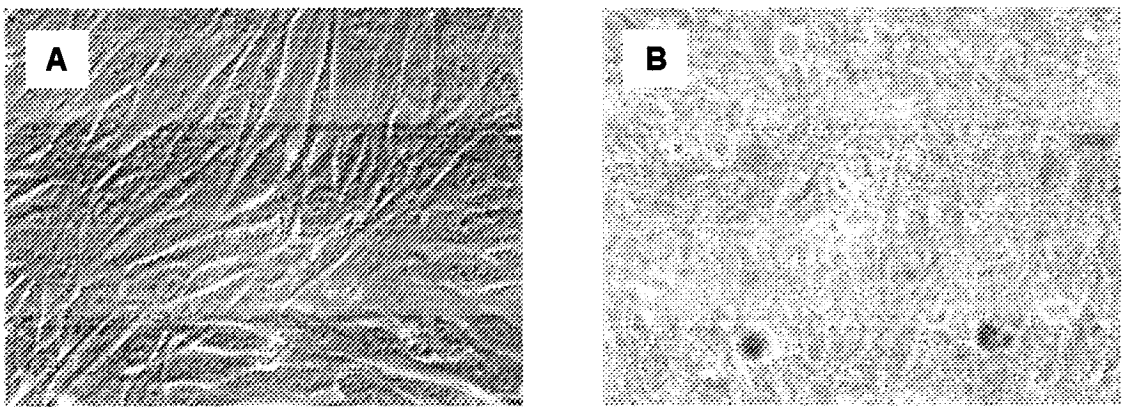

Figure 2-1. A) immature human SW872 liposarcoma cells grown for 2 days in DMEM/F-12 (20x magnification). B) mature human SW872 liposarcoma cells grown for 15 days in DMEM/F-12 (stained with oil Red-O)

2.6 Lipids and lipoproteins

Because of the hydrophobic nature of lipids and the aqueous environment of the blood in which they must travel, lipids are shuttled throughout the circulatory system packaged in lipoproteins. Lipoproteins are generally spherical particles consisting of a neutral lipid core surrounded by a monolayer of phospholipids,

unesterified cholesterol and apolipoproteins. Lipoproteins are made by the liver and the intestine, and they transport triglyceride and cholesterol to different tissues in the body for either energy use or storage (98). Several different classes of lipoproteins exist in human plasma and are distinguished primarily by their density. They also have distinct sizes, electrophoretic mobilities and apolipoprotein compositions (99). The structure and composition of human lipoproteins have been presented in many excellent reviews (99-101). In brief, the largest lipoproteins are the chylomicrons (>100nm in diameter). They are synthesized by the intestine and consist mainly (85-95%) of triglyceride. They contain apoA-I, apoB-48 and apoA-IV (100). Within the plasma compartment, LPL hydrolyses their triglycerides to release fatty acids as well as apoA-I and apoA-IV. This gives rise to a triglyceride-depleted, cholesterol-enriched chylomicron remnant. Chylomicron remnants acquire apoE and cholesterol esters and are taken up by the liver. The liver then secretes very low-density lipoproteins (VLDL) (30-90nm in diameter), which are rich in triglyceride and contain apoB-100. In the circulation, LPL hydrolyses triglyceride giving rise to smaller VLDL remnants. The lipoproteins acquire apoCs, apoE and cholesterol esters from HDL and return to the liver or are further hydrolyzed to form low-density lipoproteins (LDL). LDL is the major cholesterol transporter in human plasma. HDL is secreted from the liver and intestine. It contains 1 to 4 molecules of apoA-I and acquires cholesterol from peripheral tissue to be retuned to the liver for degradation or utilization (101).

Lipoprotein	Size (diameter in nm)	Density (g/ml)	Site of Synthesis	Composition (%)				
				Protein	Triglyceride	Phospholipid	Cholesteryl Ester	Cholesterol
Chylomicron	> 100	< 0.980	Intestine	1 - 2	85 – 95	3 - 6	2 - 4	1 - 3
VLDL	30 - 90	0.980 - 1.006	Liver	7 - 10	50 – 65	15 -20	16 - 22	4 - 8
IDL	25 - 35	1.006 - 1.019	Liver	10 - 12	25 – 30	25 - 27	32 - 35	8 - 10
LDL	20	1.019 - 1.063	Liver	20 - 22	4 – 8	18 – 24	45 - 50	6 - 8
HDL	8 - 12	1.063 - 1.210	Liver & Intestine	45 - 55	2 – 7	26 - 32	15 - 20	3 - 5

Table 2-1. Lipoprotein characteristics. Adapted from (ref. 100)

2.7 Apolipoproteins

Lipoprotein metabolism is largely regulated by apolipoproteins, which reside on the surface of lipoprotein particles. Apolipoproteins act as ligands for cell-surface lipoprotein receptors. They also function as cofactors for enzymes of lipid metabolism and help maintain the structure of lipoproteins (99). Their importance is reflected by the fact that patients with apolipoprotein defects have specific dyslipidemias, which predispose individuals to atherosclerosis and ultimately cardiovascular disease. The major apolipoproteins are listed in Table 2–2. ApoA-I is found on HDL and acts as a structural protein, as well as an activator of LCAT. ApoA-II also resides on HDL. It acts as a structural protein for HDL and is an activator of hepatic lipase. ApoA-IV is found on HDL and chylomicrons and is an activator of LPL and LCAT. ApoB-100 is the principal structural protein of VLDL, intermediate-density lipoprotein (IDL) and LDL and is a ligand for binding to the LDL receptor. ApoB-48 is a component of

chylomicrons and chylomicron remnants and is an important structural protein for these lipoproteins.

ApoC-II is found on chylomicrons and VLDL and is an essential cofactor for LPL. It is a 79 amino acid protein with a molecular weight of 8824 Da. It has three amphipathic α-helical structures, which are believed to be the lipid binding regions. It is secreted in the plasma at a concentration of about 4 mg/dl (99,102). Mutations of the apoC-II have been identified in families with familial hyperchylomicronemia. The mutations result in a truncated or non-secreted form of apoC-II (103). Overexpression of human apoC-II in mice led to hypertriglyceridemia due to the accumulation of VLDL triglyceride (104). Therefore, it would seem that in low concentrations apoC-II activates LPL whereas at high concentrations, apoC-II inhibits VLDL lipolysis (103). ApoC-III is found on chylomicrons, VLDL and HDL and is an inhibitor of VLDL lipolysis (34). The gene for apoC-III is located on the long arm of chromosome 11 as part of the 15kb cluster which includes apoA-I, apoA-IV and the recently discovered apoA-V (105). ApoC-III is in the opposite transcriptional orientation of the other genes in the cluster. It functions as an inhibitor of VLDL lipolysis through the inhibition LPL. It also interferes with remnant lipoprotein clearance and affects LCAT activity. ApoC-III has been extensively studied because of its strong association with hypertriglyceridemia. For example, overexpression of human apoC-III in mice causes severe hypertriglyceridemia (106). Also Hong *et. al.* (107) showed increased S2 allele frequency of the *Sst* I polymorphism in the 3' non-coding region of the apoC-III gene to be strongly associated hypertriglyceridemic

subjects as compared to controls. ApoC-IV protein is undetectable in human plasma. It was predicted to be a 127 amino acid protein with two amphipathic α-helices (103). Overexpression of human apoC-IV in mice caused hypertriglyceridemia as a result VLDL triglyceride accumulation (108). Apo(a) resides on lipoprotein(a) (Lp(a)) and is an inhibitor of plasminogen activation. ApoE resides on remnants, VLDL, LDL and HDL. It acts as a ligand for binding to the LDL receptor and apoE receptor (109). Several authors (99,102) have extensively reviewed apolipoprotein structure and function. ApoE and C-I will be described in more detail in the following sections.

Apolipoprotein	Chromosome	Molecular Weight (Da)	Lipoprotein Association	Plasma Concentration (mg/dl)	Major Function
apoA-I	11q23-q24	29,016	Chylomicron, HDL	140	Structural Protein of HDL / Activates LCAT
apoA-II	1q21-q23	17,400	Chylomicron, HDL	35	↑ hepatic lipase activity
apoA-IV	11q23-q24	46,000	Chylomicron, HDL		Fatty acid uptake ?
apoB-48	2p23-p24	241,000	Chylomicron	0.1	Chylomicron secretion
apoB-100	2p23-p24	513,000	VLDL, IDL, LDL	7 – 95	Ligand for LDLr
apoC-I	19q13.2	6,605	Chylomicron, VLDL, IDL, HDL	7.9 [124]	Activates LCAT / inhibits CETP
apoC-II	19q13.2	8,824	Chylomicron, VLDL, IDL, HDL	3	Activates LPL
apoC-III	11q23-q24	8,750	Chylomicron, VLDL, IDL, HDL	4	Inhibits LPL
apoE	19q13.2	34,200	Chylomicron remnants, VLDL, IDL, HDL	5	Ligand for LDLr

Table 2–2. Properties of major human apolipoproteins. Adapted from (ref. 100)

2.8 Apolipoprotein E

ApoE is secreted ubiquitously, yet the majority of plasma apoE is believed to be made by the liver (110). This arginine-rich glycoprotein of 299 amino acids has a molecular weight of 34.2 kDa and has an 18 amino acid signal peptide which is cleaved. Three isoforms of apoE exist, apoE2, apoE3 and apoE4, yielding six possible phenotypes (2/2, 3/3, 4/4, 3/2, 4/2, 4/3). The difference of isoforms lies in the cysteine to arginine substitutions at positions 112 and 158. ApoE3 has a cysteine at position 112 and an arginine at position 158 and is present in 77% of the population. ApoE2 has an arginine to cysteine substitution at position 158. It is the least common of the isoforms and is only found in 8% of the population. ApoE4 has a cysteine to arginine substitution at position 112 and is present in 15% of the population. These amino acid substitutions cause changes in the electrophoretic charge of the protein relative to apoE3, apoE2 is -1 and apoE4 is +1 (99). The plasma concentration of apoE ranges from 3 to 7 mg/dl in normolipidemic subjects and can be 20 to 60 mg/dl in subjects with certain dyslipidemias (reviewed in 99 and 111).

The function of apoE is varied. Primarily, apoE is responsible for the cellular uptake of TRL such as IDL, β-VLDL, chylomicron and chylomicron remnants. It does so through its interaction with the LDL receptor (LDLr), the LDL receptor-related protein (LRP), and the VLDL receptor (VLDLr). It is also associated with HDL and mediates its cholesterol uptake. ApoE also has the ability to bind heparin. (99)

ApoE has been extensively studied because of it strong association with atherosclerosis as reviewed by Davignon *et. al.* (111), Curtiss *et. al.* (112) and Ribalta *et. al.* (113). The affinity of the different isoforms for the receptors affects TRL clearance. ApoE2 has the lowest affinity of the three isoforms (~ 100 times less) to the LDL receptor causing the improper clearance of remnant lipoproteins (β-VLDL). Consequently, β-VLDL is slowly catabolized, resulting in hypertriglyceridemia and hypercholesterolemia. This condition is commonly referred to as type III hyperlipoproteinemia and is characterized by cutaneous xanthomas, coronary and peripheral atherosclerosis (111,114). Interestingly, carriers of apoE2 have lower total cholesterol, lower LDL-cholesterol and higher HDL-cholesterol levels. ApoE4 has the lowest plasma concentration of the three isoforms. It is associated with a decreased fractional catabolic rate of VLDL, IDL and LDL. It increases the flux of cholesterol to the liver, which downregulates the LDLr, causing plasma cholesterol concentration to increase. ApoE4 is also strongly linked to Alzheimer's disease. It is believed that astroglia and microglia are the apoE producers in the brain and that neurons carry the apoE receptors. The interaction between apoE and receptor affects cholesterol levels of the brain. ApoE can affect amyloid clearance and fibril formation by direct binding of amyloid β peptide. The amyloid β peptide is characteristic of plaque found in the brain of Alzheimer's patients and its formation is dependent upon cholesterol content. It is therefore hypothesized that apoE could play direct or indirect roles in the formation of amyloid β peptides (115).

The apoE-null mouse has established apoE as anti-atherogenic since apoE-deficient mice rapidly develop atherosclerosis. Similarly heterozygous apoE (+/-) mice rapidly develop atherosclerosis when fed an atherogenic diet. The lipoproteins of apoE-knockout mice are more susceptible to oxidation. Finally, it was demonstrated that bone marrow transplant in apoE (-/-) mice increase plasma apoE levels to 10% of normal and reduce plasma lipid levels and risk of atherosclerosis (111)

2.9 Apolipoprotein C-I

The apoC-I gene is located 5.5 kb downstream of the apoE gene (116). It synthesizes a 57 amino acid glycoprotein with a molecular mass of 6605 Da, which is initially secreted with a 26 amino acid signal peptide (117). It has a high content of lysine and contains no histidine, tyrosine, cysteine or carbohydrate. The protein has two amphipathic α-helices at position 7 to 24 and 35 to 53, which are believed to be lipid-binding regions (118). ApoC-I is principally secreted by the liver, but it has also been found to be expressed by the lungs, spleen, testes, and skin. (36). The approximate plasma concentration of apoC-I is 6 mg/dl in normolipidemic subjects (119). Among its many roles, apoC-I has been shown to activate LCAT (120), inhibit CETP (121), LPL (122) and impede the apoE-mediated binding of β-VLDL to the LDL receptor (123).

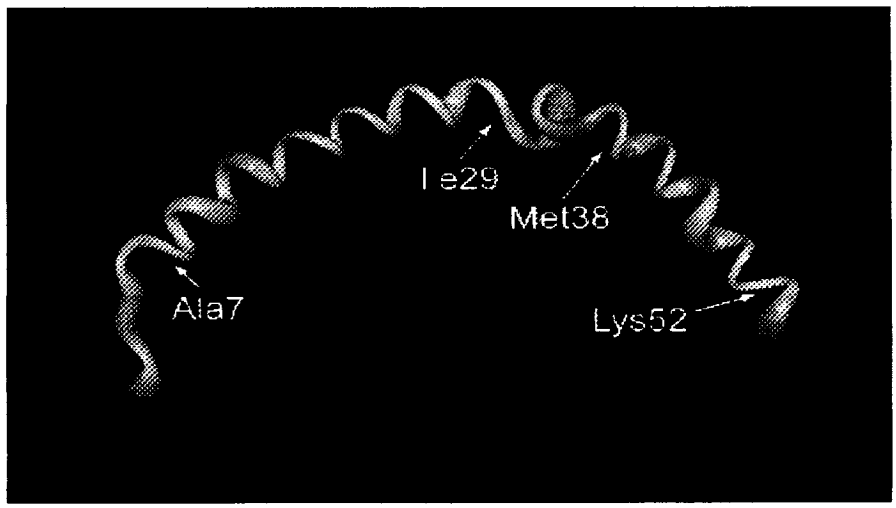

Figure 2–2. Structure of apoC-I showing the two α-helices (delineated by Ala7 and Ile29, and Met38 and Lys 52), which are purported to be the lipid-binding regions.

In normolipidemic subjects, Cohn *et. al.* (124) determined plasma concentration of apoC-I to be approximately 8.0 mg/dl, more than 92% of which was associated with HDL. In hypertriglyceridemic subjects and combined hyperlipidemic subjects, total plasma apoC-I concentration is increased and is largely associated with VLDL.

ApoC-I deficiency in man is extremely rare and has only been reported in a single case (125). The 70-year-old woman was also deficient in apoC-II, which was believed to be the reason for the observed familial chylomicronemia. More common, is the *Hpa* I polymorphism of the apoC-I gene, located 317 bp 5' to the transcription initiation site (126). Using a reporter gene assay, Xu *et. al.* (126) showed that the *Hpa* I polymorphism is associated with a 1.5-fold increase in apoC-I gene transcription. As well, it was found that the H1 allele of the *Hpa* I polymorphism was associated with the apoE3 isoform in European-Americans whereas the H2 allele was associated with apoE2 and apoE4. In apoE3/3

individuals, the H2 allele was associated with lower fasting plasma triglyceride levels and apoB levels and higher HDL levels. ApoC-I levels are not however associated with apoC-I allele genotype in normolipidemic subjects. Plasma apoC-I levels are related to apoE genotype suggesting that apoE genotype is a stronger influence of apoC-I levels than the *Hpa* I polymorphism (127).

Overexpression of human apoC-I in mice has provided significant insight into the potential roles of apoC-I *in vivo*. Mice overexpressing human apoC-I have 2- to 3-fold higher plasma triglyceride levels than control mice (128). ApoC-I overexpression decreases the uptake of apoB-containing lipoproteins and lead to increased circulating VLDL, IDL and LDL levels (129,130). When crossed with LDLr (-/-) mice, apoC-I (+/+)/LDLr (-/-) mice have severely elevated cholesterol and triglyceride levels (52±19mM and 36±19mM, respectively), as compared to apoC-I (+/+) mice (8.4±0.9mM and 0.5±0.2mM respectively). In these experiments, it was determined that apoC-I inhibits VLDL clearance through the LDLr pathway (131), however, it was later determined that VLDL clearance inhibition is through the VLDLr pathway (132). Other characteristics of apoC-I overexpressing mice were cutaneous abnormalities, including hair loss, epidermal hyperplasia and atrophic sebaceous glands, and impaired whole body non-esterified fatty acid clearance (133,134).

To determine its role independent of apoE, Conde-Knapp *et.al.* (135) crossed human apoC-I (+/+) mice with apoE (-/-) mice. These mice had unaffected HDL lipids, unchanged VLDL triglyceride production rates and unchanged remnant particle clearance. HDL particles were enriched with apoC-I

and depleted of apoA-II. Most strikingly, male mice had a 14-fold increase of triglyceride and a 2-fold increase of cholesterol compared to apoE (-/-) mice. It was determined that the purified apoC-I had a minimal effect on LPL activity, but a marked inhibition of hepatic lipase activity.

Genotype	Weight (g)	Cholesterol	Triglyceride	FFA (mM)	Glucose	Insulin (ng/ml)
WT	23.7	1.6	0.2	0.6	9.5	1.0
WT / apoC-I (+/+)	19.6	7.0	9.1	1.1	6.9	1.1
ob/ob	43.8	4.4	0.4	1.0	17.9	9.8
ob/ob / apoC-I (+/+)	21.1	10.9	9.4	1.7	3.4	1.5
ob/ob	61.6	4.1	0.28	0.85	10.2	19.9
ob/ob / apoC-I (+/-)	46.0	9.6	7.6	1.4	19.9	29.6

Table 2–3. Characteristics of apoC-I overexpressing mice. Adapted from (35,136)

ApoC-I's most impressive attribute was determined when human apoC-I (+/+) mice were crossed with ob/ob (leptin-deficient) mice. Characteristically, ob/ob mice are obese and diabetic. It was found that when crossed with apoC-I overexpressing mice, body weight was similar to wild type mice and animals regained insulin sensitivity (Table 2–3). Closer analysis of these mice revealed increased plasma cholesterol and triglyceride levels, and decreased free fatty acid uptake by adipose tissue which resulted in smaller fat pads (35). Although striking, Muurling *et. al.* (136) suggested that this model was too extreme due to the severe skin abnormalities and absent subcutaneous fat. Less-extreme human apoC-I (+/-)/ ob/ob mice were created. These mice showed reduced

body weight, increased plasma cholesterol levels, triglyceride levels and free fatty acids. Surprisingly, mice had increased glucose and insulin as compared to the ob/ob controls. They also developed hepatic steatosis.

2.10 The apoE/apoC-I/apoC-IV/apoC-II gene cluster

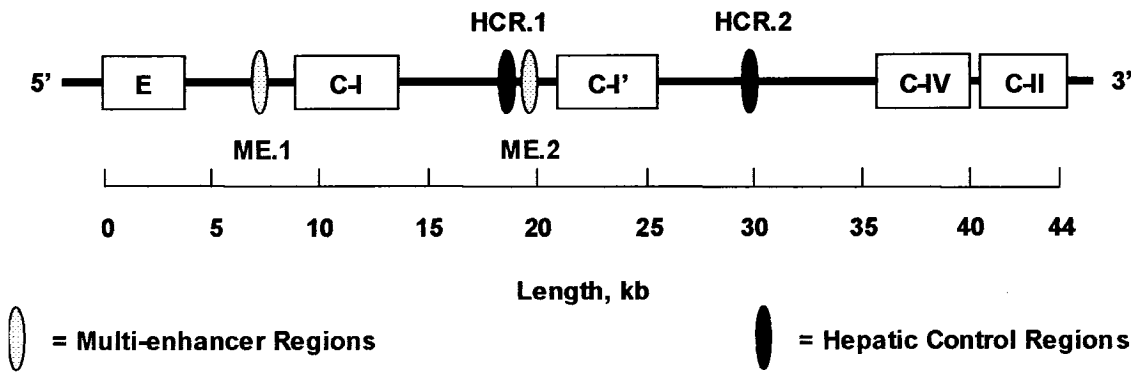

Figure 2–3. The apoE/apoC-I/apoC-IV/apoC-II gene cluster (reproduced from ref. 137)

The gene for apoC-I resides in a 45kb cluster of genes on the long arm of chromosome 19 which also contains apoE, pseudo-apoC-I, apoC-IV and apoC-II (36). In the liver, transcriptional regulation of the apoE/apoC-I/apoC-IV/apoC-II cluster (Figure II–3) is under the regulation six proximal regulatory elements in the apoE promoter area. Additionally, two hepatic control regions (HCR), HCR-1 and HCR-2 are necessary for the expression of apoE and apoC-I in the liver (111). It was recently determined that two other regulatory regions are required for the expression of apoE in macrophages and adipocytes. Multi-enhancer regions (ME) 1 and 2 are present 3.3 and 15.9 kb 3' of the apoE gene (137). It has been shown that apoE is synthesized by 3T3-L1 adipocytes and production

of apoE has been confirmed using human adipose tissue biopsies (138). Both tissue types contain apoE mRNA, and they both synthesize and secrete apoE. In 3T3-L1 cells, apoE is expressed in a differentiation-dependent manner and its regulation is related to intracellular free cholesterol levels (138). Lafitte *et. al.* (139) have demonstrated that the nuclear receptors LXRα and LXRβ and their oxysterol ligands are key regulators of adipocyte apoE expression through their interaction with conserved LXR response elements present in ME-1 and ME-2. ApoC-I mRNA or protein has never been quantitated in adipose tissue or adipocytes. As mentioned above, it has been shown in mice overexpressing human apoC-I that there is impaired fatty acid uptake by the adipose tissue and severely reduced obesity, especially when crossed with ob/ob mice (35). It is therefore likely that apoC-I is synthesized and secreted by adipose tissue and adipocytes and that it might play an important role in adipose tissue lipid metabolism.

Synthesis and Secretion of ApoC-I and ApoE During Maturation of Human SW872 Liposarcoma Cells [1]

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[1] Supported by an operating grant from the Canadian Institutes of Health Research (MOP-14684) and a CIHR/Pfizer Rx&D Investigator Salary Award to JSC (DSC-64146). The financial support of Pfizer Canada to JD is also gratefully acknowledged.

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Supplementary key words: adipose tissue, atherosclerosis, cholesterol, insulin, obesity

Abbreviations: apo, apolipoprotein; CETP, cholesterol ester transfer protein; DMEM, Dulbecco's Modified Eagle's Medium; ELISA, enzyme-linked immunosorbent assay; FBS, foetal bovine serum; LCAT, lecithin cholesterol acyltransferase; LPDS, lipoprotein-deficient serum; LRP, LDL-like receptor protein; PBS, phosphate-buffered saline; TRL, triglyceride-rich lipoprotein(s).

Published in : Journal of Nutrition (2004); 134(11): 2935-2941.

3.1 Abstract

Little is known about the regulation of apoC-I production by human adipocytes. The aim of the present study therefore was to investigate the effect of different tissue culture conditions on the synthesis and secretion of apoC-I and apoE in human SW872 liposarcoma cells. After 3 to 4 d in culture (0.5 x 10^6 cells per well, DMEM/F-12 medium with 10% FCS), cells reached confluence and became growth arrested. The molar ratio of apoE:apoC-I in the cell was 8.9 ± 0.6 and in the medium was 6.6 ± 0.5. After 17 d in culture, SW872 cells contained significantly more cholesterol (100%) and triglyceride (3-fold), and secreted more apoC-I (4 vs. 17 d: 0.11 ± 0.01 vs. 0.23 ± 0.01 pmol.10^6 $cells^{-1}.24h^{-1}$, $P < 0.001$) and apoE (0.7 ± 0.1 vs. 3.1 ± 0.3 pmol.$10^6$$cells^{-1}.24h^{-1}$, $P < 0.001$). Cellular apoC-I increased 7-fold and apoE increased 16-fold. Cell maturation was associated with significantly higher levels of apoE mRNA but not apoC-I mRNA. Increases in cell lipids, apoC-I and apoE were not dependent on the presence of extracellular lipids, since similar changes occurred in cells incubated with lipoprotein-deficient serum or in cells incubated without serum. Treatment (7 d) of cells during maturation with insulin (10 or 1000nmol/L) significantly reduced the secretion of apoC-I and apoE. These results demonstrate that in maturing SW872 cells, cholesterol and triglyceride accumulation in the presence or absence of extracellular lipids, is associated with increased apoC-I and apoE production. Furthermore, apoC-I and apoE production are differentially regulated at the transcriptional level, and long-term treatment with insulin has an inhibitory rather than stimulatory effect on apoC-I and apoE production.

3.2 Introduction

Adipose tissue is not simply a depot of fat, but is an endocrine organ capable of secreting proteins that can affect whole-body metabolism and homeostasis (160). Several of these proteins (e.g., adiponectin, leptin, PAI-1, apolipoprotein (apo) E and apoC-I) have been implicated in the etiology of insulin resistance and atherosclerosis, and may be responsible for the direct link between increased adiposity and cardiovascular disease (161,162).

ApoE and apoC-I are two proteins that play a central role in regulating plasma lipid metabolism (34,140). ApoE mediates the uptake of triglyceride-rich lipoproteins (TRL) by hepatic receptors (163), whereas apoC-I has an inhibitory effect (103). The genes for these two proteins are adjacent to each other on chromosome 19 within a 45kb cluster containing the pseudo apoC-I, apoC-IV and apoC-II genes (36). The majority of plasma apoE and apoC-I is made by the liver. However, a number of tissues are able to produce these proteins, e.g., the brain, spleen, lung, adrenals, ovary, kidney, and muscle (164-166). Adipose tissue also has the capacity to synthesize apoE and apoC-I. Zechner et al. (138) have shown that differentiated mouse 3T3-L1 adipocytes and human adipose tissue biopsies contain apoE mRNA, and they both synthesize and secrete apoE. In 3T3-L1 cells, apoE is expressed in a differentiation-dependent manner and its regulation is related to intracellular free cholesterol levels. Lafitte et al. (139) have demonstrated that the nuclear receptors LXRα and LXRβ and their oxysterol ligands are key regulators of adipocyte apoE expression through their

interaction with conserved LXR response elements present in multi-enhancer regions ME.1 and ME.2 of the apoE/CI gene cluster.

ApoC-I is a 57-amino acid protein (6.613 kd mol. wt.) which, like apoE, regulates both systemic and cellular lipid metabolism (34,103). *In vitro* experiments have demonstrated that apoC-I has the capacity to activate lecithin:cholesterol acyltransferase (LCAT), inhibit lipoprotein lipase, hepatic lipase and phospholipase A_2, and inhibit cholesterol ester transfer protein (CETP) activity. ApoC-I also inhibits the binding and/or uptake of triglyceride emulsions or VLDL by the LDL receptor, the LDL-like receptor protein (LRP) and the VLDL receptor (34,103). In mice, over-expression of human apoC-I leads to elevated plasma cholesterol and triglyceride concentrations, impaired hepatic uptake of VLDL particles, reduced whole body NEFA uptake and impaired uptake of fatty acids by adipose tissue (35,129,133,134).

In view of the aforementioned evidence that apoC-I plays an important role in regulating fatty acid uptake by adipose tissue, and given the lack of information pertaining to apoC-I production by adipocytes, we carried out the present study in which we investigated the effect of different tissue culture conditions on the synthesis and secretion of apoC-I and apoE by SW872 liposarcoma cells. The human liposarcoma SW872 cell line has been used in previous studies as a human adipocyte cell model (167-169). Compared to mouse 3T3-L1 adipocytes, SW872 cells have the advantage of being of human origin and of not requiring an incubation cocktail (e.g., dexamethasone, insulin and IBMX) to differentiate into mature adipocytes. SW872 cells, when initially

plated, have an immature adipocyte phenotype, and they constitutively express important adipocyte genes such as PPARα, PPARγ, LRP, LPL, CETP, CD36 and adipsin (169).

3.3 Materials and methods

Materials. Human SW872 liposarcoma cells were obtained from American Type Culture Collection (Rockville, MD). Dulbecco's Modified Eagle's Medium (DMEM) high glucose was purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS), dexamethasone and bovine insulin were purchased from Sigma (St-Louis, MO). Bovine lipoprotein-deficient serum (LPDS) was obtained by ultracentrifugation at density 1.25 kg/L using solid KBr. LPDS was dialyzed against physiological saline (0.15 mol/L) and was stored at 4°C.

Cell culture. SW872 cells were cultured in high glucose DMEM supplemented with $NaHCO_3$ (3.7 g/L), 100 µmol/L non-essential amino acids, 50 kU/L penicillin, 50 mg/L streptomycin, and 10% FBS, in a humidified incubator (37°C, 5% CO_2). Media were replaced every 2-3 d. Cells (0.5×10^6) were seeded into 6-well plates containing 2 mL of medium. Maturation of SW872 cells was studied by allowing cells to grow for a maximum of 19 d. Media were changed every 48 h and analyses were conducted at 4, 10 and 17 d. Cells reached 100% confluence after 4 d. At each time point, media were removed and cells were washed twice with phosphate-buffered saline (PBS) before further manipulation. Cell maturation was studied in the absence of lipoproteins by incubating cells with 10% LPDS (100 µmol/L non-essential amino acids, 50 kU/L penicillin, 50 mg/L streptomycin). Cells were also studied in the absence of serum, whereby the medium was supplemented with a growth-promoting cocktail containing 15 mmol/L $NaHCO_3$, 15 mmol/L Hepes, 33 µmol/L biotin, 17 µmol/L pantothenate, 0.2 nmol/L triiodothyronine, 1.25 µmol/L bovine insulin, 0.1 mmol/L

NaOH, 0.1 µmol/L dexamethasone, 100 µmol/L non-essential amino acids, 50 kU/L penicillin and 50 mg/L streptomycin, (individual components from Sigma, St-Louis, MO). To study the effect of insulin, cells were grown for 7 d post confluence in FCS-containing medium, washed with PBS, and incubated with either 0 nmol/L, 10 nmol/L or 1000 nmol/L bovine insulin-supplemented medium (10% FCS) for a subsequent 7 d.

Cell analysis. Cells were washed with PBS and total cellular lipids were extracted using three one-hour incubations with 1.0 mL hexane/isopropanol (3:2, vol/vol). Solvent extracts were centrifuged (10 min at 2000g), and supernatants were transferred and dried under nitrogen. Total cholesterol and triglyceride were quantified by enzymatic reaction using reagents from Roche Diagnostics (Indianapolis, IN). Cellular lipid accumulation was also monitored microscopically after lipid staining with Oil Red O (170). Media were collected and apolipoproteins were quantified by sandwich enzyme-linked immunosorbent assay (ELISA) (124,171) using goat anti-human polyclonal antibodies obtained from Biodesign International (Saco, ME) and HRP conjugated goat anti-human antibodies obtained from Academy Bio-Medical Company inc. (Houston, TX). ELISAs were specific for human apolipoproteins and zero values were obtained when fresh media containing bovine serum were assayed. ApoC-I and apoE detected in cell media therefore represented a measure of secreted apolipoprotein. Re-uptake of human apolipoproteins was negligible since media contained an excess of bovine apoC-I and apoE provided by the FCS. To quantify cellular apolipoproteins, SW872 cells were washed with PBS, harvested

and dissolved in a lysis buffer (50 mmol/L TRIS-Base, 150 mmol/L KCl and 1% Tween). Cellular apolipoproteins were then quantified by ELISA. To measure total cell proteins, cells were washed with PBS and solubilised using 0.1 mol/L NaOH. Total cell proteins were quantified according to Lowry et al (145), using bovine serum albumin as a standard. RNA was extracted according to the manufacturer's instructions with Trizol© obtained from Invitrogen (Burlington, Ontario) and then quantified using a spectrophotometer. A reverse transcriptase reaction was then carried out and the resultant cDNA was amplified using real-time PCR with the SYBR green mastermix purchased from Qiagen (Mississauga, Ontario) and analysed with Mx4000 software. S14 (used as a standard), apoC-I, apoE, PPARα, PPARβ and PPARγ primers were designed using the Primer 3 software (172), and were made by Invitrogen (Burlington, Ontario).

Statistics. Values in the text are means ± SEM. One-way ANOVA were performed using SigmaStat 2.0 (Jantel Corporation, San Rafael, Ca). Data not normally distributed were analyzed nonparametrically using Kruskal-Wallis one-way ANOVA on ranks. Post-hoc multiple comparison tests included Tukey's test (parametric) or Dunn's method (nonparametric). Differences were considered to be significant at $P < 0.05$.

3.4 Results

Human SW872 cells were cultured in the presence of 10% FCS and cell maturation was monitored for 17 d. They were initially elongated and fibroblastic in appearance when first plated. After several days in culture, they became more rounded. At 100% confluence, they became growth arrested, as evidenced by a plateau (3 to 4 d after plating) in the number of cells per dish (Figure 3-1A). Cellular protein increased gradually with time (Figure 3-1B). Cells became vacuolized and Oil Red O staining provided evidence of lipid accumulation (data not shown). Seventeen days after plating, cells contained about 100% more cholesterol and 3-times more triglyceride compared to when they reached confluence at day 4 (Figure 3-1C).

Cell maturation was associated with a significant increase in cellular levels of PPARβ/δ mRNA (Figure 3-2A). Neither PPARα nor PPARγ mRNA levels increased significantly with time, although PPARγ mRNA levels were already elevated in confluent cells at 4 d. This was consistent with the fact that SW872 cells resemble differentiated adipocytes, and express other functionally important adipocyte genes including LRP, LPL, CETP, and CD36 (169). At d 17, SW872 cells had significantly higher LRP mRNA levels (0.46 ± 0.05 vs. 0.19 ± 0.03, $P < 0.001$) and higher LPL mRNA levels (0.05 ± 0.01 vs. 0.04 ± 0.01, $P < 0.01$) compared to d 4. Cellular levels of apoE mRNA increased significantly during cell maturation however, levels of apoC-I mRNA decreased significantly (Figure 3-2B).

Levels of both apoC-I and apoE were higher in the medium than in cells. Both cellular and medium levels of apoC-I (Figure 3-3A) and apoE (Figure 3-3B) increased significantly during cell maturation. From d 4 to d 17, cellular apoC-I and apoE increased 7-fold and 16-fold, while medium apoC-I and apoE increased 2-fold and 4-fold, respectively. At d 4, the molar ratio of apoE:apoC-I in the cell was 8.9 ± 0.6 and in the medium, 6.6 ± 0.5. At d 17, the molar ratio of apoE:apoC-I in the cell was 21.6 ± 2.6 ($P < 0.001$), and in the medium, 13.3 ± 1.6 ($P < 0.001$). SW872 cells therefore contained and secreted more apoE than apoC-I. SW872 cells secreted 6-7 molecules of apoE for each molecule of apoC-I at 100% confluence (i.e. d 4) and secreted 13-14 molecules of apoE for each molecule of apoC-I after 17 d in culture.

In order to determine the extent to which lipoproteins in the medium were able to affect the production of apoC-I and apoE during maturation of SW872 cells, experiments were carried out in which cells were grown in the presence of 10% FCS or in the presence of 10% FCS devoid of lipoproteins (i.e., in 10% lipoprotein-deficient serum, LPDS). As demonstrated before (Figures 3-1 to 3-3), maturation in the presence of 10% FCS was associated with an increase in cellular lipids (Figure 3-4A), an increase in apoE mRNA but not apoC-I mRNA (Figure 3-4B), and significant increases in both cellular and medium apoC-I (Figure 3-4C) and apoE levels (Figure 3-4D). Cellular triglyceride and apoE mRNA levels were lower in LPDS-incubated cells, however this was not associated with any reduction in levels of apoC-I or apoE compared to FCS-incubated cells.

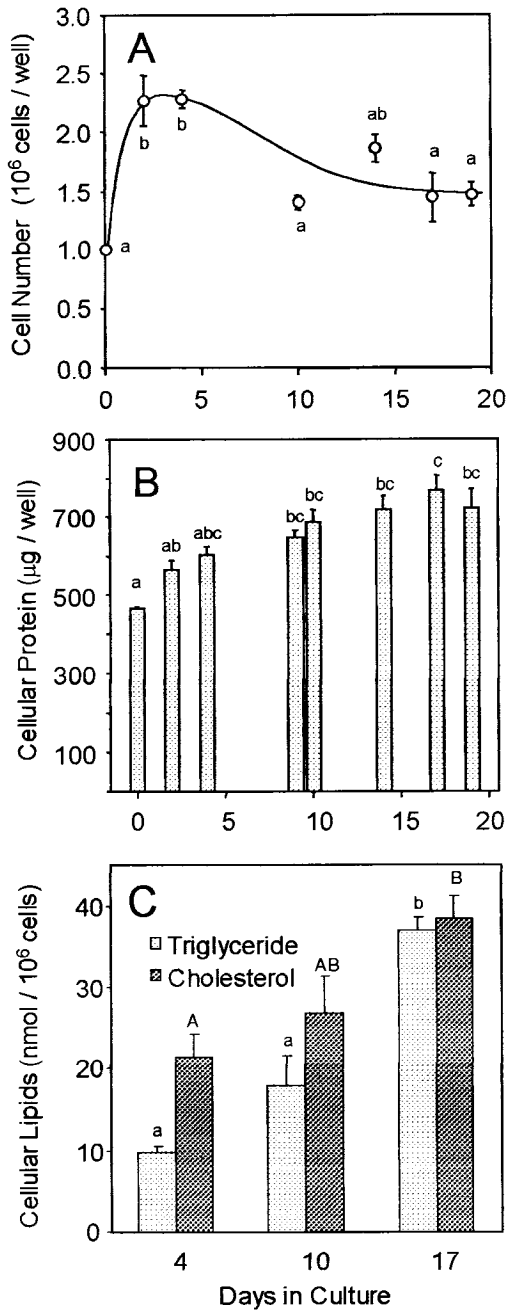

Figure 3-1. Time-course of maturation of SW872 liposarcoma cells. At day 0, cells were plated into six-well tissue culture plates (0.5 x 10^6 cells / well). They were allowed to grow in the presence of DMEM/F-12 (3:1,v/v) and 10% FCS. The medium was changed every 48h. Cells reached confluence after 3 to 4 d in culture and became growth arrested. Number of cells per dish at different time-points is shown in panel A. Cellular protein per dish is shown in panel B and cellular lipid is shown in panel C. Data in A & B represent means ±SEM of three wells. Data in C represent means ± SEM (n = 5). Means for a variable without a common letter differ ($P < 0.05$), by one-way ANOVA and the post-hoc Tukey's test.

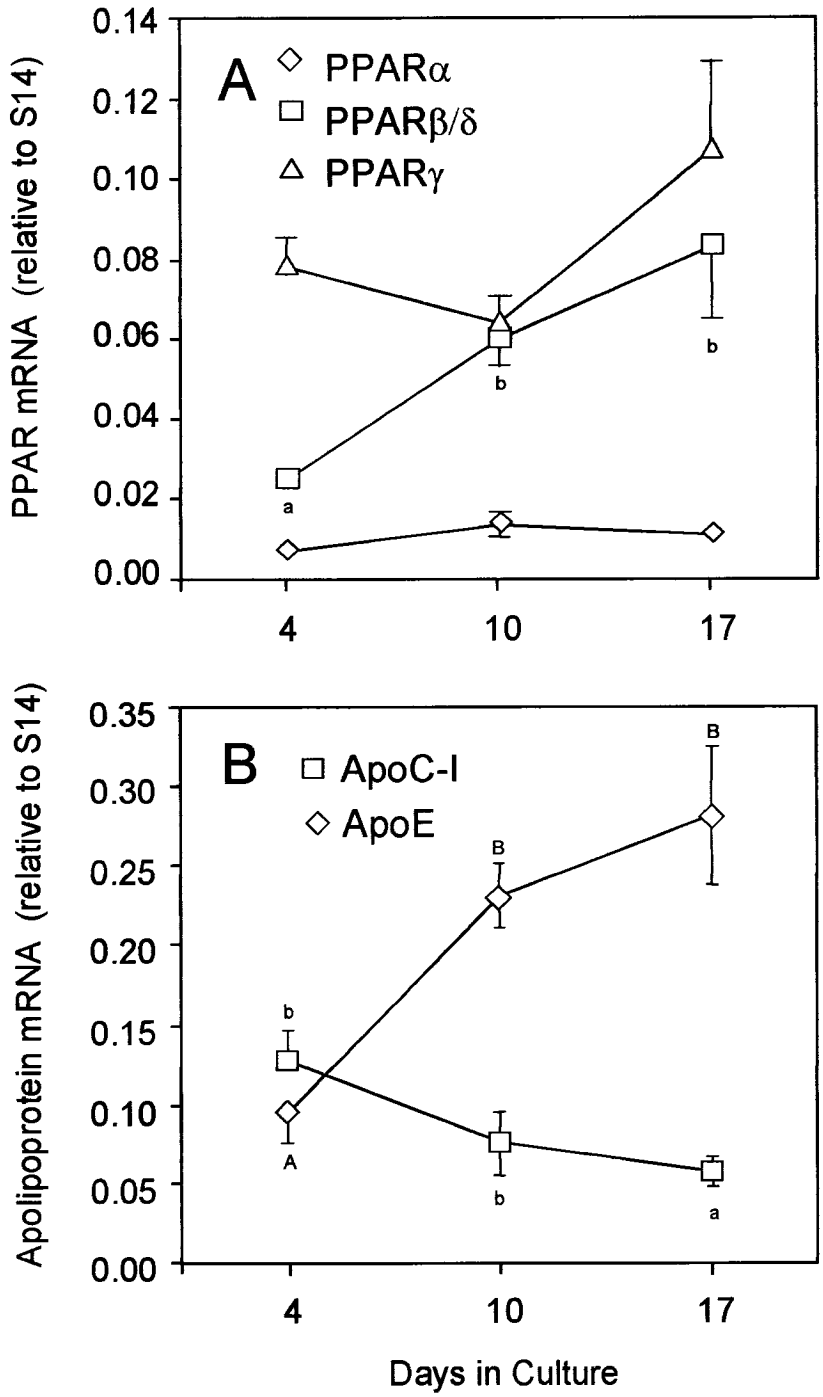

Figure 3-2. Change in cellular mRNA levels during maturation of SW872 cells. PPAR mRNA (panel A) and apolipoprotein mRNA (panel B) were quantitated by real-time RT-PCR and were expressed relative to S14 mRNA. Data are shown for PPARα, PPARβ/δ, and PPARγ in panel A, and data for apoC-I and apoE are shown in panel B for cells grown for 4, 10 or 17 d. Results represent means ± SEM (n = 6). Means for a variable without a common letter differ ($P < 0.05$), by one-way ANOVA and the post-hoc Dunn's method.

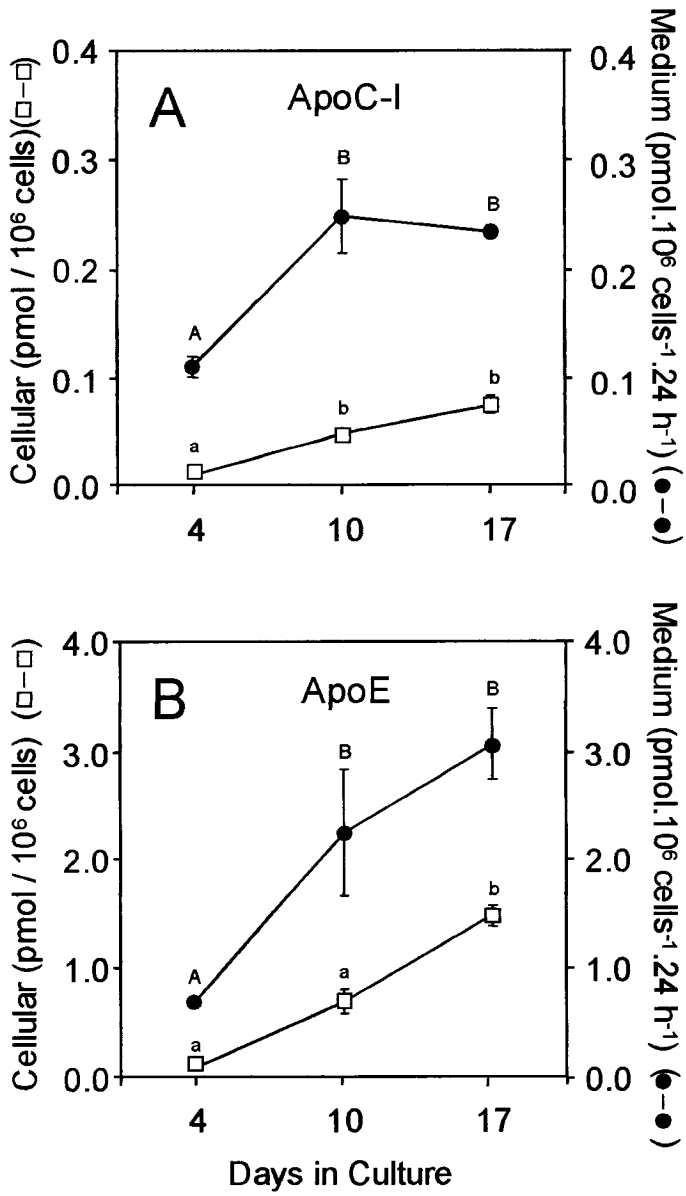

Figure 3-3. Effect of SW872 cell maturation on cellular and medium apoC-I (panel A) and apoE levels (panel B). Apolipoproteins were measured by ELISA in cells dissolved in lysis buffer or in medium recovered from cells after 48h of incubation. Cellular apolipoprotein levels were expressed in units of pmol per 1 x10^6 cells and medium apolipoprotein levels were expressed in pmol.10^6 cells-1.24h-1). Results represent means ± SEM (n = 6). Means for a variable without a common letter differ ($P < 0.05$), by one-way ANOVA and the post-hoc Dunn's method.

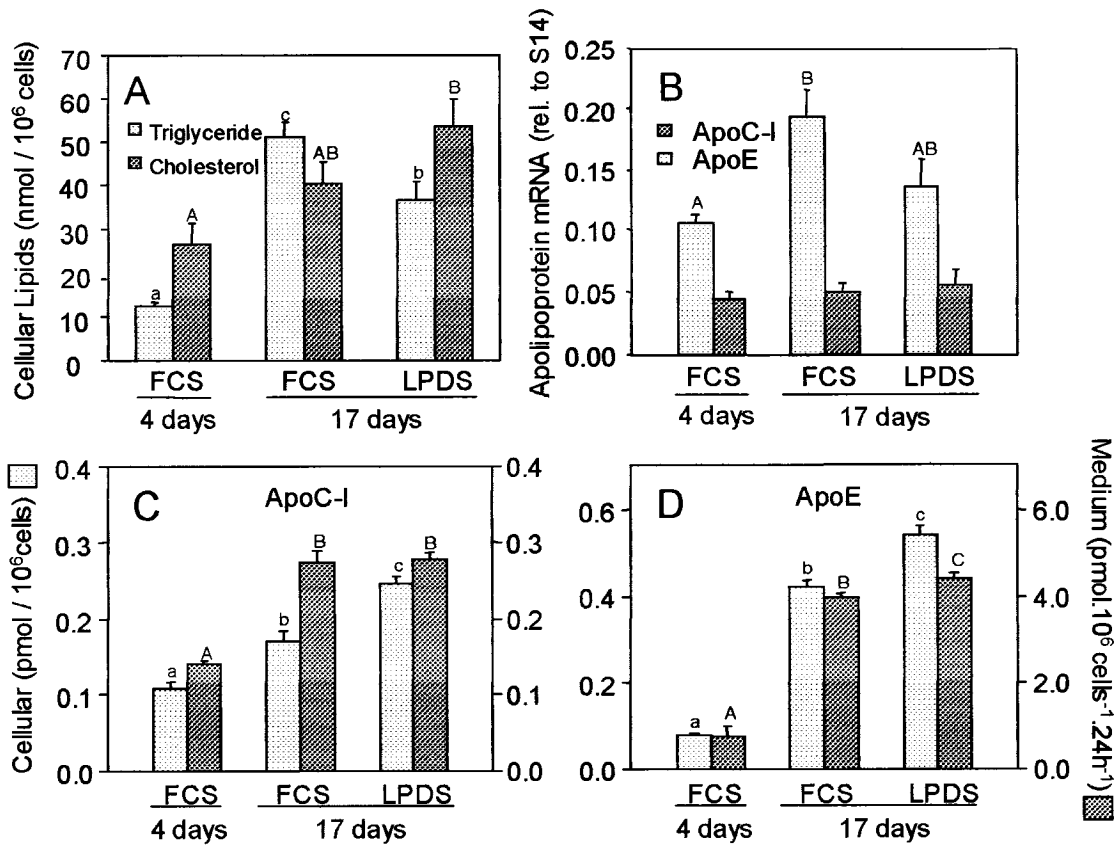

Figure 3-4. SW872 cell maturation and apoC-I and apoE production in the presence or absence of serum lipoproteins. Cells were plated into six-well tissue culture plates (0.5 x 10^6 cells / well) in the presence of DMEM/F-12 (3:1,v/v) and 10% FCS. At 100% confluence (d 4), cells were allowed to continue to mature in the presence of 10% FCS or in the presence of 10% LPDS. Cells and media were harvested 13 d after reaching confluence (i.e., at d 17). Cellular triglyceride and cholesterol levels are shown in Panel A. ApoC-I and apoE mRNA levels were determined by real-time RT-PCR and expressed relative to S14 mRNA (panel B). Cellular and medium apoC-I levels were determined by ELISA (panel C). Cellular and medium apoE levels are given in panel D. Results represent means ± SEM (n = 6). Means for a variable without a common letter differ ($P < 0.05$), by one-way ANOVA and the post-hoc Tukey's test.

In view of the small effect of the absence of lipoprotein lipids in the medium on apoC-I and apoE production during SW872 maturation, an experiment was carried out with serum-free medium containing a cocktail of compounds (i.e., insulin, dexamethasone, triiodothyronine, biotin, and

pantothenate) designed to promote the maturation of SW872 cells and also the production of apoC-I and apoE. The cells accumulated both triglyceride and cholesterol (Figure 3-5A). At 100% confluence (d 4), they contained similar levels of triglyceride and cholesterol as SW872 cells incubated in medium containing 10% FCS (Figure 3-1C). At d 10 and 17, however, they contained 4-times and 7-times more triglyceride, respectfully and also slightly more cholesterol than cells in standard medium. Unfortunately, samples were only duplicates and no error bars or statistical measurements could be obtained. The lipid accumulation was associated with an increase in apoE mRNA, though not apoC-I mRNA (Figure 3-5B). Increase in cellular apoE mRNA with growth-promoting medium was about 3-times greater than that which occurred with standard medium (Figure 3-5B). Cellular and medium apoC-I and apoE increased significantly (Figures 3-5C & 3-5D) and this increase was also greater in magnitude than that observed in the presence of FCS-containing medium (Figures 3-3A & 3-3B).

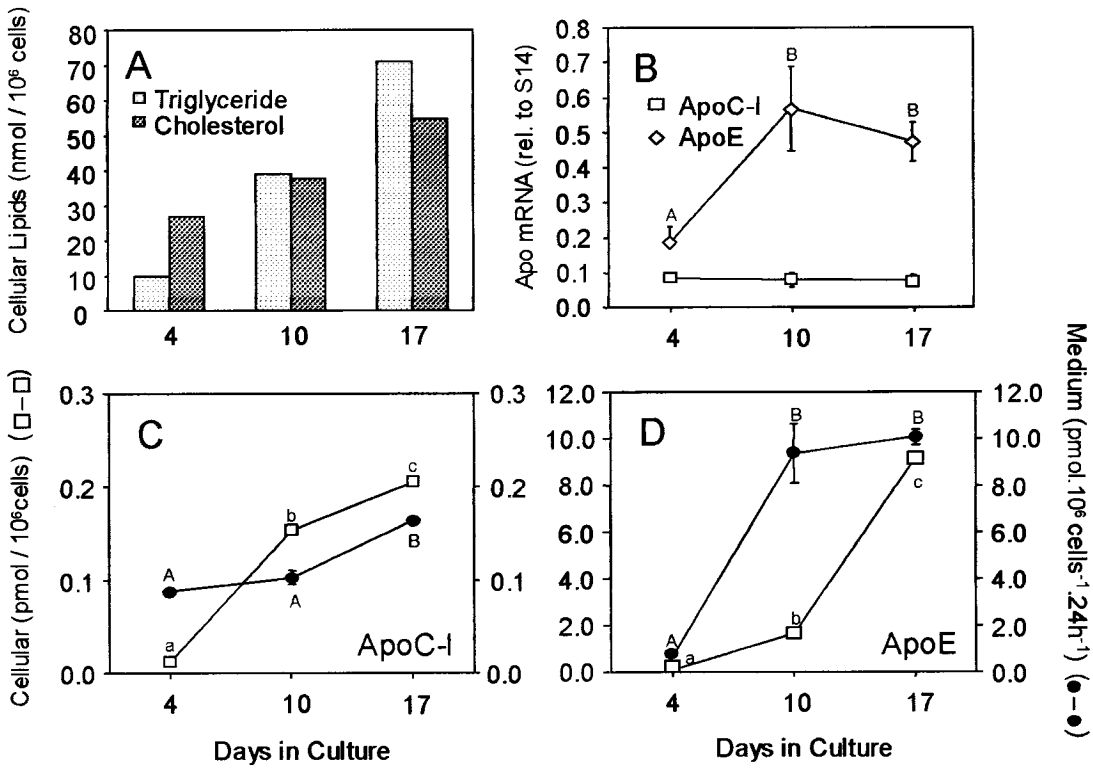

Figure 3-5. Effect of serum-free medium supplemented with a growth-promoting cocktail on SW872 cell maturation and production of apoC-I and apoE. Cells were plated into six-well tissue culture plates (0.5 x 10^6 cells / well) in the presence of DMEM/F-12 (3:1,v/v) containing 1.25µmol/L insulin, 0.1µmol/L dexamethasone, 0.2nmol/L triiodothyronine, 33µmol/L biotin, and 17µmol/L pantothenate. Cells and media were harvested 4, 10 and 17 d after plating. Cellular triglyceride and cholesterol levels are expressed in units of nmol/10^6 cells (panel A); mean data are shown for 2 dishes at each time-point. ApoC-I and apoE mRNA levels by real-time RT-PCR were expressed relative to S14 mRNA (panel B). Cellular and medium apoC-I levels are shown in panel C and cellular and medium apoE levels are shown in panel D. Results in panels B, C & D represent means ± SEM (n = 6). Means for a variable without a common letter differ ($P < 0.05$), by one-way ANOVA and the post-hoc Tukey's test.

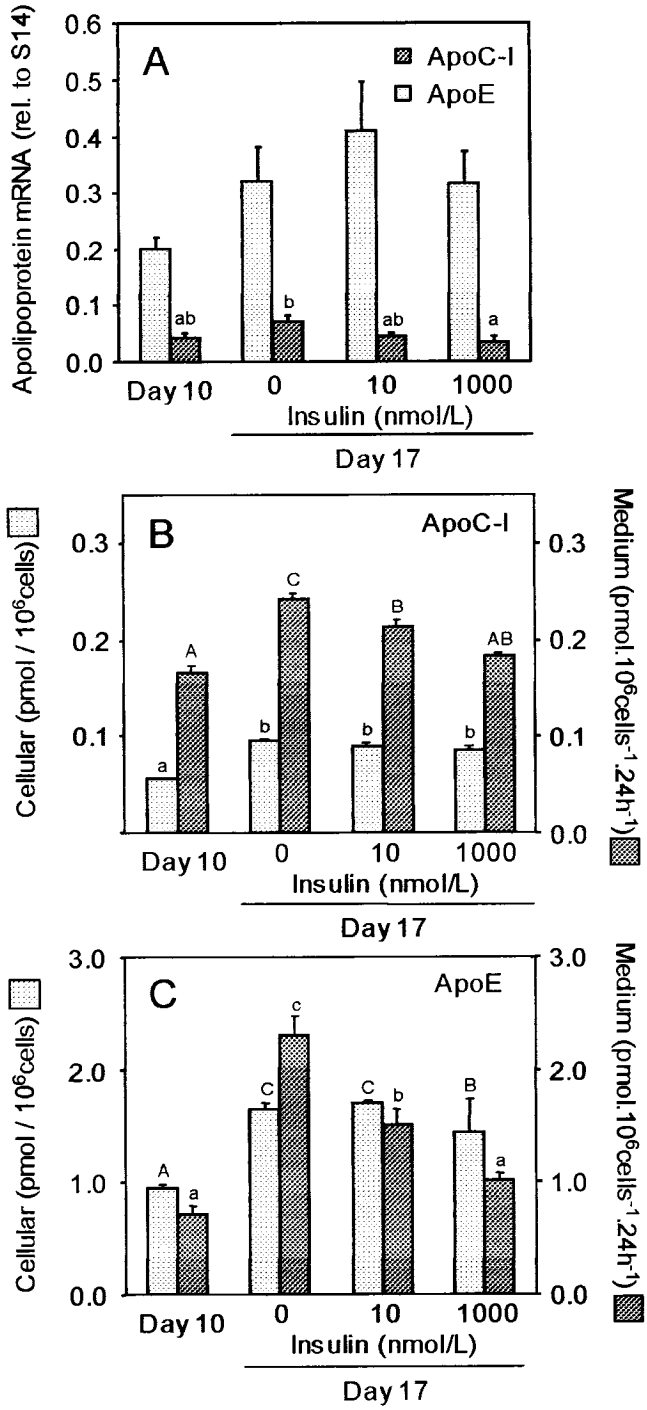

Figure 3-6. Effect of insulin on SW872 production of apoC-I and apoE during maturation. Cells were grown in DMEM/F-12 (3:1,v/v) containing 10% FCS for 10 d before being incubated for an additional 7 d in medium containing either 0, 10 or 1000nmol/L insulin. ApoC-I and apoE mRNA levels were determined by real-time RT-PCR and were expressed relative to S14 mRNA (panel A). Cellular and medium apoC-I levels (panel B) and apoE levels (panel C) were determined by ELISA. Results represent means ± SEM (n = 6). Means for a variable without a common letter differ ($P < 0.05$), by one-way ANOVA and the post-hoc Tukey's test.

Since the maturation-promoting medium (described above) contained a large amount of insulin, experiments were carried out to investigate the effect of insulin alone on SW872 apoC-I and apoE production (Figure 3-6). Insulin at concentrations of 10 and 1000 nmol/L in medium containing 10% FCS did not affect apoE mRNA, but the higher concentration reduced apoC-I mRNA levels (Figure 3-6A). This was associated with a significant decrease in medium apoC-I and apoE (Figures 3-6B & 3-6C), with no difference in cellular apoC-I and a small 20% decrease in cellular apoE in incubations with 1000 nmol/L insulin (Figure 3-6B).

3.5 Discussion

The results of the present study have shown that both apoC-I and apoE are synthesized and secreted by SW872 cells. At confluence, after 3 to 4 d in culture, cells contained approximately 1 molecule of apoC-I for every 9 molecules of apoE, and the medium contained about 1 molecule of apoC-I for every 7 molecules of apoE. When SW872 cells were allowed to mature for 17 d in culture in the presence of 10% FCS, cellular lipid accumulation (2-fold increase in cholesterol, 4-fold increase in triglyceride) was associated with a significant increase in both cellular and medium levels of apoC-I and apoE. Increase in the synthesis and secretion of apoE was greater than that of apoC-I (Figure 3-3) and after 17 d in culture, the molar ratio of apoE:apoC-I in the cell was 21.6 ± 2.6 and in the medium was 13.3 ± 1.6. Similar changes were observed when cells were incubated with lipoprotein-deficient serum (Figure 3-4) or when cells were incubated without serum (Figure 3-5), demonstrating that increased production of both apoC-I and apoE was closely linked to the accumulation of cellular cholesterol and triglyceride, independent of the presence or absence of lipid in the medium.

Zechner et al. (138) have previously shown that differentiation of mouse 3T3-L1 preadipocytes into mature adipocytes results in increased production of apoE. More recently, gene chip experiments have shown that apoC-I expression is also significantly increased during adipocyte differentiation (53,173). The present results extend these observations, by showing that human SW872 cells, which already display a differentiated adipocytic phenotype, i.e., they express

measurable amounts of PPARγ mRNA (Figure 3-2) and other adipocytic genes in the basal state (169), increase their apoE production as they accumulate lipid. Cell maturation is also associated with an increase in synthesis and secretion of apoC-I, independent of any additional increase in PPARγ mRNA (Figure 3-2). These data suggest that increased apoC-I and apoE production is not only a feature of adipocyte differentiation, but is a characteristic of the transformation of SW872 cells from immature to mature adipocytes. ApoC-I and/or apoE may play an important role in the regulation of adipocyte lipid accumulation, consistent with their documented effects on lipid uptake and/or efflux in other cell types. For example, apoC-I is known to be an inhibitor of lipoprotein receptors of the LDL-receptor family (34,103), and to have an inhibitory effect on the tissue uptake of free fatty acids (35,134). ApoE might be important in regulating receptor-mediated uptake of adipocyte lipid (140), as supported by the observation that adipose tissue mass is significantly reduced in obese ob/ob mice that are also made deficient in apoE (174). ApoE can also stimulate LRP-mediated selective uptake of HDL cholesteryl esters by adipocytes (169). On the other hand, newly-synthesized endogenous apoE mediates cholesterol efflux from macrophages (175,176), and one can hypothesize that apoE plays a similar role in maturing adipocytes. Clearly, these different possibilities can only be substantiated by future experiments.

The apoC-I and apoE genes are adjacent to each other on the same chromosome (36) and are known to be regulated by common control regions (128,139,177). Thus, apoC-I and apoE gene expression in the liver is driven by

two enhancer regions, hepatic control regions (HCR) 1 and 2, which are respectively ~9kb and ~20kb downstream of the apoC-I gene (156,177). Macrophage and adipose tissue apoC-I and apoE gene expression is in turn directed by two multienhancer regions (ME.1 and ME.2), which are situated either side of the apoC-I gene (137). Furthermore, two LXR response elements located within ME.1 and ME.2 appear to be responsible for the induction of macrophage apoC-I and apoE gene expression by ligands for LXR and RXR (178). Based on these results, we initially hypothesised that any change in the level of apoC-I mRNA would be mimicked by a similar change in apoE mRNA. Furthermore, we expected that changes in apoC-I or apoE mass in maturing SW872 cells would be mimicked by similar changes in mRNA levels. In fact, parallel changes in apoC-I and apoE mRNA were not observed in either the maturation experiments or those with insulin. Increased levels of apoE mRNA but not apoC-I mRNA were consistently observed in cells incubated in the presence or absence of extracellular lipids (Figures 3-2B & 3-5B), while insulin treatment was associated with a decrease in apoC-I mRNA but not apoE mRNA (Figure 3-6A). These data provide clear evidence for differential regulation of the apoC-I and apoE genes in SW872 cells, and suggest the presence of regulatory elements in the apoE/apoC-I/apoC-II gene cluster, which are specific for individual apolipoproteins. At the same time, our data suggest that the production of these apolipoproteins was regulated post-transcriptionally, as evidenced by the increase in apoC-I protein in the absence of increased apoC-I mRNA during maturation (e.g., Figure 3-5), and the decrease in secreted apoE in

the absence of any change in apoE mRNA during insulin treatment (Figure 3-6). This is consistent with the post-transcriptional regulation of apoC-I and apoE production in human HepG2 cells (155,179) and of apoE production in macrophages (180,181). Post-translational degradation of apoC-I and apoE by specific proteases has been shown to occur in hepatocytes and macrophages (179), however it remains to be determined whether similar proteases are responsible for regulating levels of apoC-I and apoE secretion in human adipocytes.

Insulin stimulates the uptake of glucose and free fatty acids by adipose tissue. It can increase LPL activity, induce the translocation of fatty acid transport proteins to the surface of adipocytes and stimulate adipocyte lipid accumulation (182,183). In view of the relationship observed between lipid accumulation and increased apoC-I and apoE production in SW872 cells during maturation, we expected that insulin treatment would further stimulate apoC-I and apoE production. Surprisingly however, long-term incubation of cells with insulin was found to cause a decrease in apoC-I and apoE secretion, concomitant with a decrease in apoC-I mRNA, though not apoE mRNA (Figure 3-6). These data suggest that insulin by itself does not induce transcription of the apoC-I or apoE gene in adipocytes, corresponding with results obtained with cultured rat hepatocytes (184) and human hepatoma cells (185,186), in which insulin had no effect on apoE mRNA levels, but a small and reproducible lowering effect on apoE secretion. From these results, it can also be deduced that the ability of the serum-free growth-promoting medium to increase apoE and

apoC-I production in SW872 cells (Figure 3-5) was probably not a direct consequence of its high insulin concentration (1.25 μmol/L).

In conclusion, the results of the present study demonstrate that SW872 liposarcoma cells synthesize and secrete both apoC-I and apoE. When these cells are incubated for an extended period of time in the presence of 10% FCS, they accumulate cholesterol and triglyceride, and produce significantly increased amounts of these two apolipoproteins. Lipid accumulation and increased apoC-I and apoE production can also occur over time in the absence of extracellular lipids. In addition, evidence has been obtained for the differential regulation of apoC-I and apoE production at the transcriptional as well as post-transcriptional level, and long-term insulin treatment was found to have an inhibitory rather than stimulatory effect on apoC-I and apoE production. These results suggest that apoC-I and apoE play a role in the maturation of human adipocytes and may have an important role in mediating or regulating cell lipid accumulation. Further work is required to define this regulatory function and determine whether these apolipoproteins are essential for adipocyte maturation.

Overexpression of apoC-I in human SW872 liposarcoma cells

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The material presented in this chapter has not yet been submitted for publication.

4.1 Abstract

ApoC-I overproduction has never been studied in human adipocytes. Therefore, the aim of the present study was to investigate the effect of apoC-I transfection in human SW872 liposarcoma cells on adipocyte lipid metabolism. Transfected cells, vector-only cells and untransfected cells were allowed to mature for 3 d in culture. The average overexpression of apoC-I mRNA was 8.3 ± 7.3-fold compared to untransfected cells. Transfected cells were not growth arrested and continued to proliferate in spite of cell-cell contact unlike the vector-only and untransfected cells. Average cell number increased 70% and average cell protein increased 84% as compared to untransfected cells. In the transfected cells, cellular cholesterol decreased 36% and cellular triglyceride decreased 32%, as compared to untransfected cells. These results suggest that not only does apoC-I overexpression in human SW872 liposarcoma cells impede growth arrest, but it also suggests that overexpression of apoC-I maintains adipocytes in an immature phenotype, resulting in less triglyceride and cholesterol accumulation.

4.2 Introduction

Apolipoprotein (apo) C-I and E are two proteins that play a central role in regulating plasma lipid metabolism and atherosclerosis (34,141). Previous work from our laboratory has shown that both proteins are synthesized and secreted by human SW872 liposarcoma cells and that their regulation is dependent on cellular lipid accumulation (97, Chapter III). The precise role(s) of both proteins in adipose tissue lipid metabolism has yet to be clearly determined.

It has been shown that newly secreted rat chylomicrons and VLDL enriched with human apoC-I were more slowly taken up by the perfused rat liver (141,142). It has been further demonstrated that transgenic mice expressing human apoC-I in the liver had 2- to 3-fold increases in their plasma triglyceride levels as compared to control mice (128) and that this was due to reduced uptake of apoB-containing lipoproteins by the liver (129). The most predominant effect was on VLDL (131). It has been demonstrated that there is a decrease of non-essential fatty acid (NEFA) clearance in mice over-expressing apoC-I (134). More recently, it was determined that the effects of apoC-I were concentration dependent. When mice were homozygous for human apoC-I, they had a 2- to 3-fold reduction in whole body fatty acids uptake, resulting in reduced adiposity (134). These mice had elevated serum cholesterol and triglyceride, cutaneous abnormalities including hair loss, epidermal hyperplasia and atrophic sebaceous glands (133). Most surprisingly, fatty acid uptake was severely impaired in adipose tissue, resulting in reduced subcutaneous fat and smaller adipocytes

(35). The reduced fat pad weight was most noticeable when the mice had an ob/ob background (35). Further studies with heterozygous apoC-I mice with an ob/ob background found less reduction in fat pad weight, with no effect on subcutaneous fat accumulation but a significant reduction of visceral adipose tissue (136). More recently, it has been shown that human aortic smooth muscle cells incubated with human apoC-I-enriched HDL were apoptotic (143).

Since it has clearly been demonstrated that apoC-I affects adipose tissue lipid metabolism (35, 136), we carried out experiments overexpressing apoC-I in the human SW872 liposarcoma cell line to more clearly outline its role(s) in adipose tissue lipid metabolism and cellular maturation. The overexpression of apoC-I has never been studied in an adipose tissue cell line nor has it been shown how endogenous apoC-I overproduction affects adipocytes.

4.3 Materials and methods

Materials. Human SW872 liposarcoma cells were obtained from American Type Culture Collection (Rockville, MD). Dulbecco's Modified Eagle's Medium (DMEM) high glucose was purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Sigma (St-Louis, MO).

DNA construct and cellular transfection. The apoC-I and vector-only constructs were kindly provided by Catherine Bouchard and Lucie Boulet. Briefly, the pUCI-A4 plasmid was obtained from the ATCC. This plasmid includes the pUC8 plasmid with multiple cloning sites and an ampicillin resistance marker as well as the complete human apoC-I coding sequence (419 bp). The apoC-I insert includes the signal peptide and a 56 nucleotides non-coding sequence at the 5' end and a 110 nucleotides non-coding sequence at the 3' end. The signal peptide is necessary to allow the protein to be secreted by the cells. The plasmid purchased was inserted into E. coli and grown in Luria-Bertani (LB) medium supplemented with 50 μg/ml ampicillin (Stratagene, Montreal, Qc). The bacteria were grown and the plasmid DNA was excised using a QIAprep Spin Miniprep Kit (Qiagen, Ont, Ca), and an EcoRI/HindIII digestion. This step was performed to assure ourselves that the entire apoC-I gene was present. The gene fragments were isolated and run on an agarose gel for verification (1.2% agarose gel, TAE 1x buffer, 40 min. at 80 volts). Once assured that the plasmid contained the desired gene, the entire apoC-I gene was

inserted into a pcDNA3 vector which contains CMV , T7, and Sp6 promoter regions, multiple restriction sites, and ampicillin and neomycin resistance genes (Invitrogen Life Technologies, Ont, Ca). This step was necessary since the pUC8 plasmid is not functional in mammalian cells. To insert the apoC-I gene into pcDNA3, both pUC8 and pcDNA3 were linearised (pcDNA3 with BamHI/EcoRV and pUC8 with HindIII). The cohesive end of the linear pUC8 was transformed into a blunt-end and the apoC-I insert was subsequently removed using BamHI. Using T4 DNA ligase (Gibco, Grand Island, NY), the BamHI sites were ligated and the inserted apoC-I gene was in the same transcriptional orientation as the pcDNA3 promoters. The new plasmids, either with or without (i.e. negative control) the apoC-I gene, were put into XL1 Blue bacterial cells for replication. After a suitable quantity of colonies were obtained, the apoC-I gene was once again excised from the plasmid using a maxi-prep technique (Quiagen, Ont, Ca). To verify that the inserted gene was in the proper reading frame, the plasmid containing the gene was sequenced in the Molecular Biology Service department of the IRCM.

The pcDNA3 plasmids, either empty or containing the apoC-I gene, were transfected into SW872 cells using the Invitrogen Lipofectamine Plus™ Reagent kit as per the manufacturer's instructions (Invitrogen Life Technologies, Ont, Ca). Transfections of SW872 cells were performed by Jean-François Carmel. Briefly, 1 μg of DNA construct was mixed with 100 μl of serum-free DMEM/F-12 medium and 6 μl Plus™ Reagent and was incubated at room temperature for 15 minutes. 4 μl of Lipofectamine™ Reagent was added and the mixture was incubated at

room temperature for 15 minutes. Following the incubation, the mixture was added to SW872 cells (75% confluence) and volume was completed to 1 ml with serum-free DMEM/F-12 medium. Cells were incubated for 3 hours at 37°C and 5% CO_2. After the 3 hour incubation, the 1 ml of serum-free DMEM/F-12 medium and 200 µl of FBS were added.

Cell culture. Transfected SW872 cells were cultured in high glucose DMEM supplemented with $NaHCO_3$ (3.7 g/L), 100 µM non-essential amino acids, 50 U/ml penicillin, 50 µg/ml streptomycin, 10% FBS, and 0.3 mg/ml neomycin in a humidified incubator (37°C, 5% CO_2). Media were replaced every 2-3 days. Cells (0.5×10^6) were seeded into 6-well plates containing 2ml of medium.

Lipid extraction and assays. Cells were washed with PBS and total cellular lipids were extracted using three one-hour incubations with 1.0ml hexane/isopropanol (3:2, vol/vol). Solvent extracts were centrifuged (10 min at 3000 rpm), and supernatants were transferred and dried under nitrogen. Total cholesterol and triglyceride were quantified by enzymatic reaction using reagents from Roche Diagnostics (Indianapolis, IN).

Protein assays. Media were collected and apoC-I were quantified by sandwich enzyme-linked immunosorbent assay (ELISA) (124,144) using goat anti-human polyclonal antibodies obtained from Biodesign International (Saco, ME) and HRP conjugated goat anti-human antibodies obtained from Academy

Bio-Medical Company inc. (Houston, TX). ELISAs were specific for human apolipoproteins and zero values were obtained when fresh media containing bovine serum were assayed. ApoC-I detected in cell media therefore represented a measure of secreted human apolipoproteins. Reuptake of human apolipoproteins was negligible since media contained an excess of bovine apoC-I provided by the FCS. To quantify cellular apolipoproteins, SW872 cells were washed with PBS, harvested and dissolved in a lysis buffer (50mM TRIS-Base, 150mM KCl and 1% Tween). Cellular apolipoproteins were then quantified by ELISA. To measure total cell proteins, cells were washed with PBS and solubilised using 0.1 M NaOH. Total cell proteins were quantified according to Lowry *et. al.* (145), using bovine serum albumin as a standard.

mRNA assays. RNA was extracted according to the manufacturer's instructions with Trizol© obtained from Invitrogen (Burlington, Ontario) and then quantified using a spectrophotometer. A reverse transcriptase PCR was then done. The resultant cDNA was amplified using real-time PCR with the SYBR green mastermix purchased from Qiagen (Mississauga, Ontario) and analysed with Mx4000 software. S14 (used as a standard) and apoC-I probes were obtained from Invitrogen (Burlington, Ontario).

Assessment of apoptosis. A DNA laddering assay was used to measure cellular apoptosis. Genomic DNA was extracted from cells using Trizol© as per the manufacturer's instruction. 5μl of DNA was applied to a 2 % agarose gel and

run at 120 volts for 30 minutes. The gel was stained with ethidium bromide and photographed. The gel was calibrated using DNA fragments of known molecular weight (PBR 322).

Statistics. Values in the text are means ± SEM. Student's t-tests were performed using Microsoft Excel for Windows XP (Microsoft Corporation). Differences were considered to be significant at $P < 0.05$.

4.4 Results

Maturing SW872 liposarcoma cells were stably transfected with a vector containing the human apoC-I gene or with vector alone. Several transfected cell lines were successfully propagated. Four lines of apoC-I over-expressing cells (C2, C6, C15 and C20) and two lines of vector-only cells (P2 and P9) were chosen on the basis of their apoC-I secretion into the medium. As shown in Figure 4-1, mRNA of the neomycin resistance gene was measured to provide evidence that the cells were indeed transfected with the vector. The control SW872 cells did not have a neomycin resistance gene and therefore did not have any measurable neomycin mRNA. The various amounts of neomycin mRNA synthesized by the transfected cells is a reflection of the transfection efficiency and mirrored the amounts of apoC-I mRNA found in the cells (Figure 4-2).

Overexpression of apoC-I in the selected cell lines ranged from 2-fold in the C6 cell line to 19-fold in the C20 line (Figure 4-2). The average overexpression for all four apoC-I cell lines was 8.3 ± 7.3-fold. P2 had a 40% increase ($P < 0.05$) in apoC-I expression compared to SW872 controls and P9 had a 50% increase ($P > 0.05$). The slight increases in vector-only apoC-I mRNA were very comparable to SW872 control cells and were considered acceptable. Transfected cells were all similar in morphology when initially seeded, but it was apparent that not all cell lines proliferated similarly. SW872 control cells reached 100% confluence after 3 days in culture and became growth arrested. The P2 and P9 cells matured similarly to the control cells, but there were fewer cells (~30% decrease, $P < 0.01$) with similar cellular protein (14% increase, $P > 0.05$)

after the 3-day maturation period (Figure 4-3). The apoC-I overexpressing cells continued to proliferate in spite of cellular contact. All of these cell lines had more cells than controls and at the end of the 3-day period there was a 1.6-, 1.1-, 2.8-, 1.3-fold increase in the C2, C6, C15, C20 cell lines, respectively. There were also increases of cellular protein for all 4 cell lines, ranging from 1.9 mg to 5.4 mg cell protein per well.

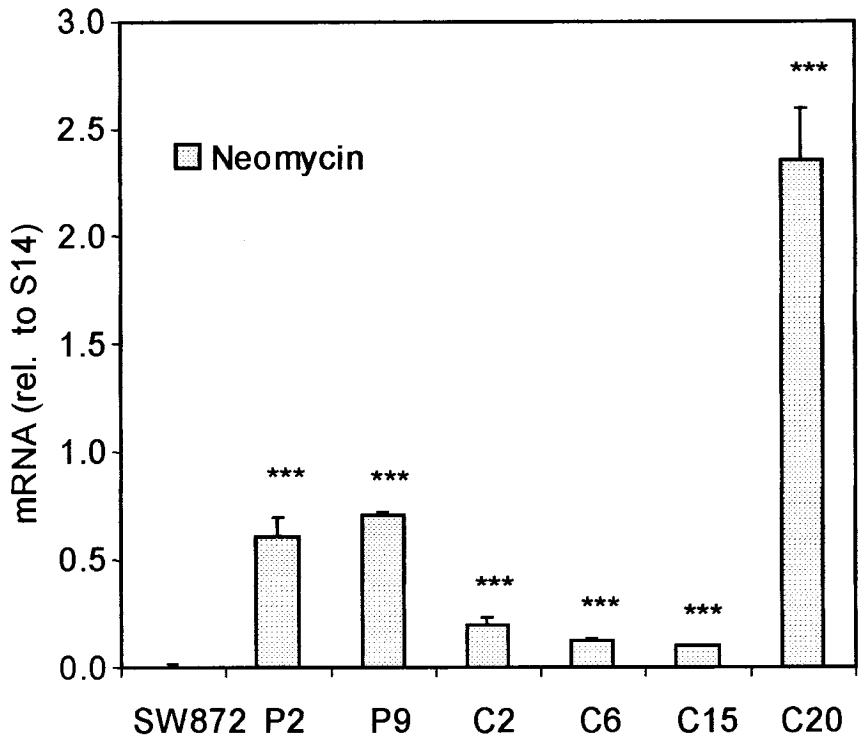

Figure 4-1. Neomycin mRNA quantification of transfected SW872 liposarcoma cells. Neomycin mRNA was quantitated by real-time RT-PCR and was expressed relative to S14 mRNA. At day 0, SW872 cells, apoC-I transfected cells (C2, C6, C15, C20) and vector-only transfected cells (P2, P9) were plated into six-well tissue culture plates (0.5 x 10^6 cells / well). Cells were allowed to grow in the presence of DMEM/F-12 (3:1,v/v) and 10% FCS. Cells reached confluence after 3 days in culture at which time cells were collected for mRNA measurements. As expected, SW872 cells had no detectable neomycin mRNA, whereas all transfected cell lines had measurable quantities of neomycin mRNA. Data represent means ± SE (n = 4). Significantly different from SW872 cells by t-test: *** $P < 0.001$.

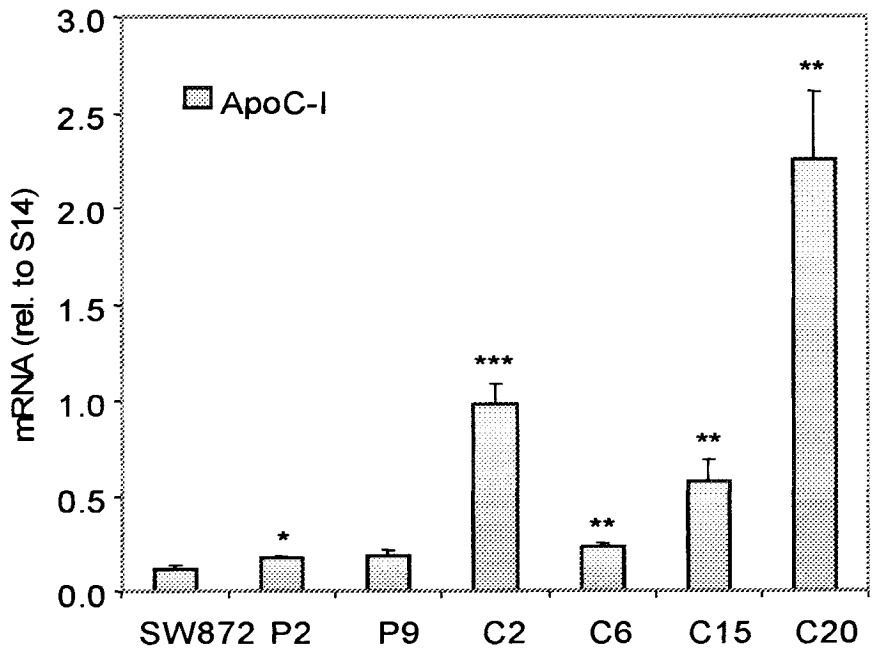

Figure 4-2. Apolipoprotein C-I mRNA quantification of transfected SW872 liposarcoma cells. ApoC-I mRNA were quantitated by real-time RT-PCR and were expressed relative to S14 mRNA. At day 0, SW872 cells, apoC-I transfected cells (C2, C6, C15, C20) and vector-only transfected cells (P2, P9) were plated into six-well tissue culture plates (0.5 x 10^6 cells / well). They were allowed to grow in the presence of DMEM/F-12 (3:1,v/v) and 10% FCS. Cells reached confluence after 3 days in culture at which time cells were collected for mRNA measurements. Data represent means ± SE (n = 4). Significantly different from SW872 cells by t-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

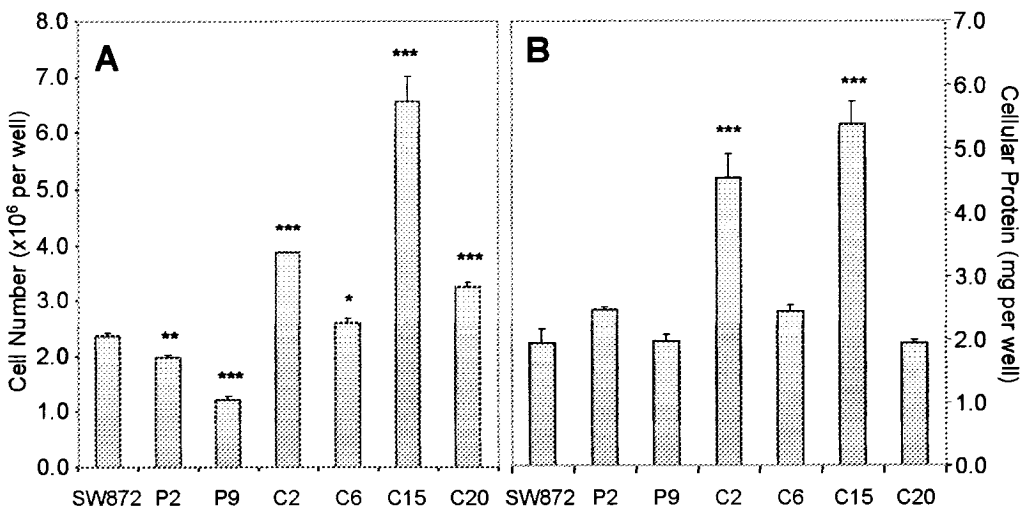

Figure 4-3. Effect of apolipoprotein C-I overexpression on cell number and cellular protein levels. Cells were plated into six-well tissue culture plates (0.5 x 10^6 cells / well) in the presence of DMEM/F-12 (3:1,v/v) containing 10% FCS. A) Cells were counted using a hemacytometer and trypan-blue staining after 3 days in culture. B) Cellular proteins were extracted with NaOH and quantitated by Lowry (17) after 3 days in culture. Results represent means ± SE for 6 wells. Significantly different from SW872 cells by t-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

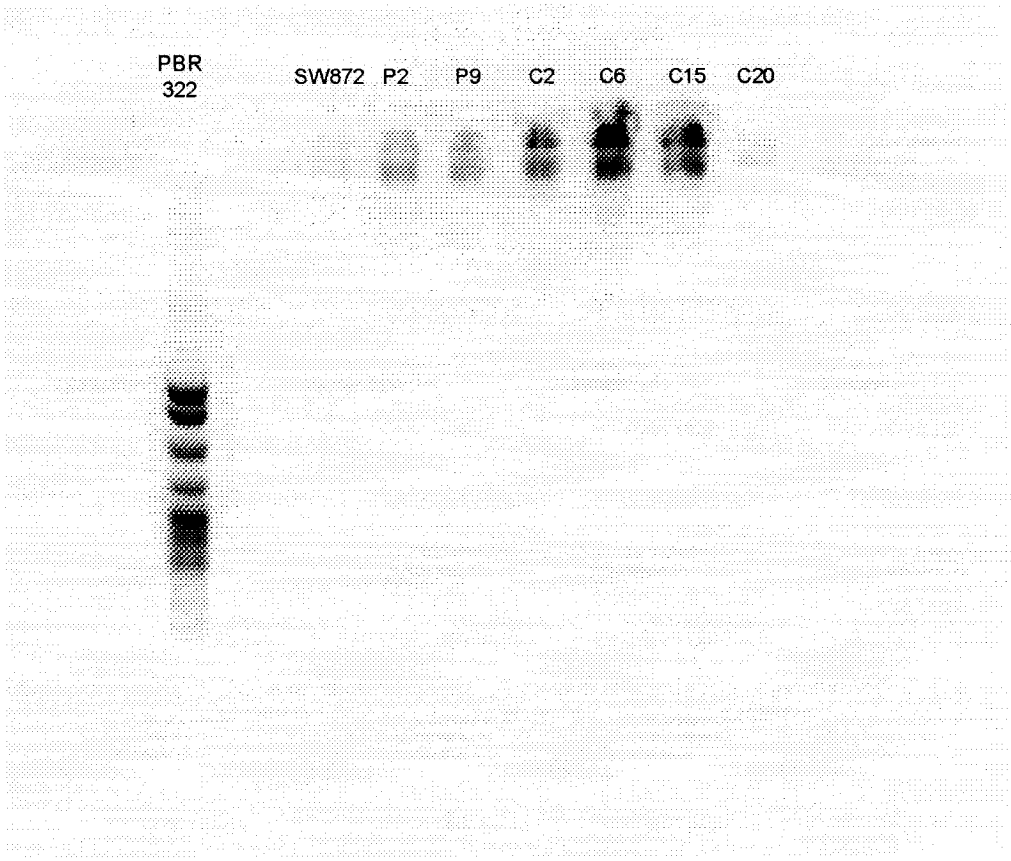

Figure 4-4. Effect of apolipoprotein C-I overexpression on DNA degradation in SW872 liposarcoma cells. Transfected cells were grown in DMEM/F-12 (3:1,v/v) containing 10% FCS for 3 days before. SW872 were non-transfected control cells; P2 and P9 were vector-only transfected cells; C2, C6, C15 and C20 were apoC-I transfected cells. Cellular DNA was isolated using Trizol© as per the manufacturer's instructions. DNA (5ml) was subjected to electrophoresis on a 2% agarose gel. Gels were stained with ethidium bromide and photographed after electrophoresis.

Increased apoC-I expression was not associated with cellular apoptosis as shown in Figure 4-4. In apoptotic cells, DNA fragmentation into 180 to 200 base-pair fragments occurs due to endonucleosomal cleavage. The DNA of the control cell line, vector-only transfected cell lines (P2 and P9) and the apoC-I transfected cell lines (C2, C6, C15 and C20) were isolated with Trizol© and run on a 2% agarose gel. There was no evidence of DNA fragmentation in any of the cell lines.

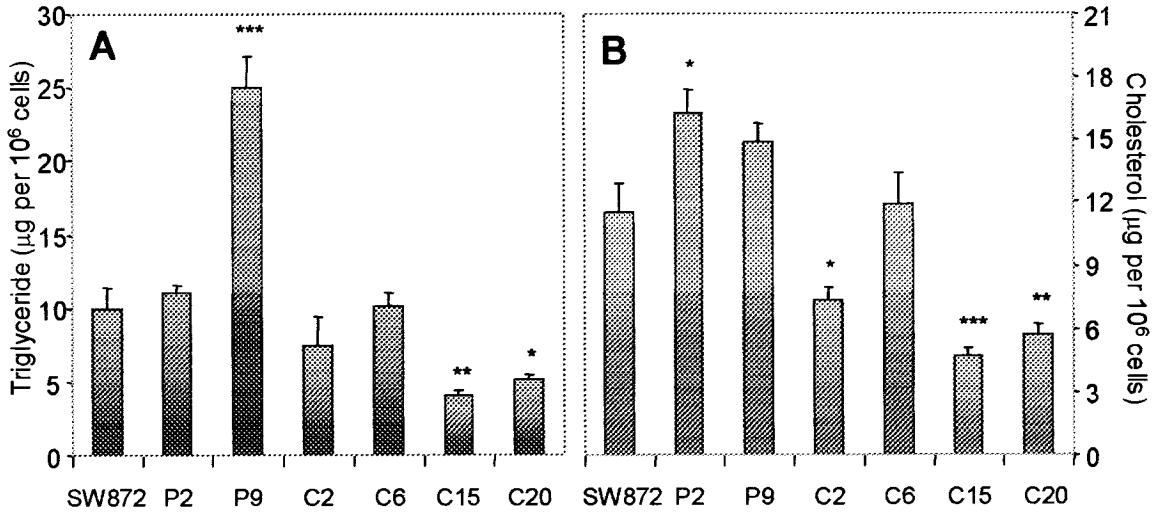

Figure 4-5. Effect of apolipoprotein C-I transfection on cellular lipids. Cells were plated into six-well tissue culture plates (0.5 x 10^6 cells / well) in the presence of DMEM/F-12 (3:1,v/v) and 10% FCS. At 100% confluence (day 3), cells were washed with PBS and cellular lipids were extracted using hexane/isopropanol (3:2, vol/vol). Solvent extracts were analyzed for triglyceride (A) or cholesterol (B) using commercial reagents. Results represent means ± SE for 6 wells. Significantly different from SW872 cells by t-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4-5 illustrates the amount of triglyceride and cholesterol measured in cells after 3 days in culture. Vector-only transfection led to an average 83% increase of triglyceride and 35% increase of cholesterol. In the C2, C6, C15, C20 cells, there was an average 32 ± 27% decrease of triglyceride and 36 ± 28% decrease of cholesterol in the cell. In the apoC-I overexpressing cells, it was evident that only the C6 cell line did not have significantly lower triglyceride (10.2 ± 0.8 vs. 9.9 ± 1.5 mg/10^6 cells, $P > 0.05$) or cholesterol (11.9 ± 1.5 vs. 11.5 ± 1.4 mg/10^6 cells, $P > 0.05$) as compared to the SW872 controls.

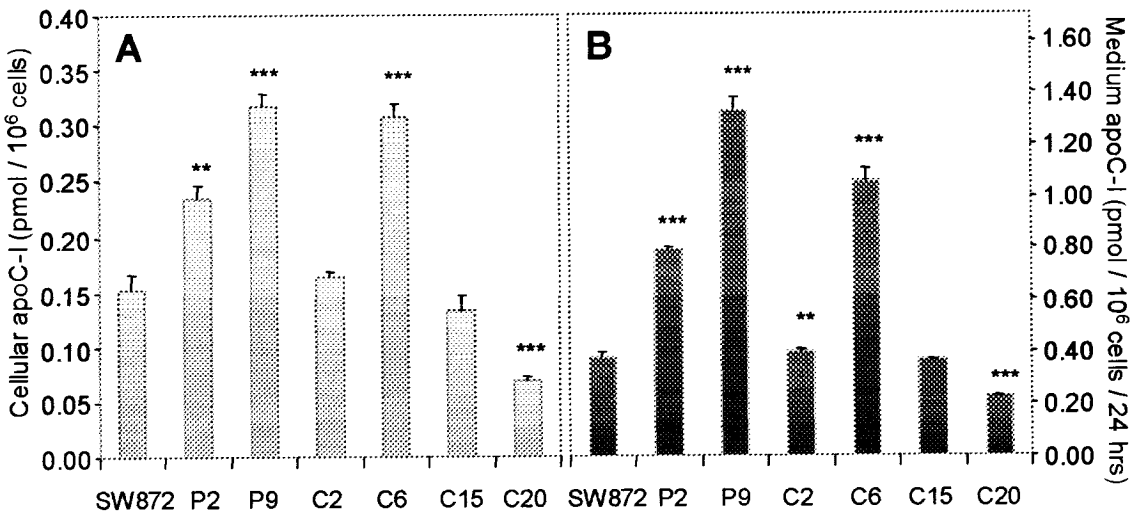

Figure 4-6. Effect of apolipoprotein C-I transfection on cellular and medium apoC-I levels. Cells were grown for 3 days in DMEM/F-12 (3:1,v/v) medium supplemented with 10% FCS. Apolipoproteins were measured by ELISA (15, 16) in cells dissolved in lysis buffer or in medium recovered from cells after 24h of incubation. Cellular apolipoprotein levels (A) were expressed in units of pmol per 10^6 cells and medium apolipoprotein levels (B) were expressed in pmol per 10^6 cells per 24h. Results represent means ± SE of 5 wells. Significantly different from SW872 cells by t-test: ** $P < 0.01$, *** $P < 0.001$.

Cellular and medium apoC-I levels are shown in Figure 4-6. Overexpression of the apoC-I gene was not associated with a similar increase of apoC-I protein. Within the cell, there was an average increase of apoC-I protein of 10% as compared to the SW872 control cells. Only the C6 cell line had a 2-fold increase. In the medium, there was a similar average apoC-I protein increase (36%) and it again was the C6 cell line which produced the most protein (~ 3-fold increase). Significant increases of apoC-I protein were also measured in the vector-only cell lines as compared to the control cells. There were 1.8- and 2.8-fold increases of apoC-I protein in the cell and in the medium, respectively. Because of this discrepancy, all comparisons were performed against SW872 control cells since the vector-only cells did not prove to be suitable controls.

4.5 Discussion

Results of the present experiments have shown that apoC-I overexpression was successfully achieved in SW872 liposarcoma cells. All transfected cell lines had quantifiable amounts of neomycin-resistance gene mRNA (Figure 4-1), present in the plasmid with which the cells were transfected. ApoC-I overexpression had a significant effect on cellular proliferation resulting in a significant increase in the number of cells over a three day maturation period. ApoC-I overexpression also resulted in significant effects on cellular lipid accumulation. ApoC-I transfected cells accumulated less triglyceride and cholesterol per cell compared to SW872 control cells however, there was no dose-effect in relation to apoC-I mRNA.

The peripheral effects of human apoC-I overexpression mice is characterised by cutaneous abnormalities, including hair loss, epidermal hyperplasia and atrophic sebaceous glands (133) and reduced subcutaneous fat pad mass and smaller adipocytes (35). In light of these observations, we originally hypothesised that apoC-I overexpression in SW872 cells would lead to a significant decrease in cell number and cell protein concentration. Surprisingly, we observed a significant increase in both cell number and total cell protein in cells overexpressing apoC-I (Figure 4-3), independent of apoC-I mRNA (Figure 4-2). We hypothesized that growth arrest was either totally or partially inhibited in these cells. The reason why apoC-I transfected cells continued to proliferate in spite of cellular contact, is presently unknown. It has previously been reported that the growth arrest of 3T3-L1 pre-adipocytes is key to post-confluent mitosis,

which allows the cells to activate genes required for subsequent cellular maturation (94,146,147). During growth arrest, Notch-1 activates the transcription factors CAATT enhancer-binding protein-α (C/EBP-α) and PPARγ, which regulate cellular differentiation (148-151). SW872 are differentiated cells and we have shown in previous work that these cells become growth arrested before accumulating lipids (97, Chapter III). It is possible that Notch-1, C/EBP-α and PPARγ , individually or together, play critical roles in cellular maturation and that either apoC-I transfection or apoC-I synthesis interferes with their regulation. Examining the expression of these genes would provide a better understanding of why apoC-I transfected cells did not undergo growth arrest.

Earlier studies have shown that mice overexpressing human apoC-I are hypercholesterolemic and hypertriglyceridemic, due to decreased uptake of VLDL by the liver (35,129,131,133-136). It was also shown that these mice had reduced uptake of free fatty acids by their adipocytes (35). In our apoC-I overexpressing cells, we observed an average decrease of cholesterol and triglyceride accumulation in cells greater than 30%, yet the average ratio of triglyceride to cholesterol in the cell remains virtually unchanged (0.85 in SW872 cells and 0.91 in apoC-I overexpressing cells). Although this observation is not a direct measurement of lipid uptake, it does support the hypothesis that apoC-I inhibits peripheral lipid uptake. The mechanism through which apoC-I affects lipid uptake and cellular proliferation is still unclear. Jong *et. al.* (132) have reported that apoC-I strongly inhibits lipoprotein binding to the VLDLr. It is possible that overexpression of apoC-I in our cells affected similar or

alternatively, other lipid transport proteins such as CD36 and/or LRP. Because the total lipid per well does not vary significantly between SW872 cells and apoC-I overexpressing cells, it is likely that there was an accumulation of immature cells as established by the decreased quantity of lipids per cell and increased cell number. Examining the expression of lipid transport genes would more clearly explain our observations. In addition, it has been previously demonstrated that apoC-I synthesis is dependant on cellular lipid accumulation (97, Chapter III). To determine if either cholesterol and/or triglyceride is responsible for apoC-I synthesis and to what extent, the inhibition of endogenous lipid production could be studied. HMG-CoA reductase inhibitors, commonly known as statins, inhibit cholesterol synthesis (152). Utilizing HMG-CoA reductase inhibitors could elucidate if cholesterol is responsible for apoC-I synthesis in adipocytes. Also, the addition of exogenous apoC-I to cells could be done to further examine the effects of apoC-I in adipose tissue lipid metabolism. Because apoC-I is a secreted protein, its local effects on adipocytes could be extra-cellular. Exogenous apoC-I addition to SW872 cells could help elucidate the effects of apoC-I on cellular lipid and/or fatty acid uptake.

Surprisingly, overexpression of the apoC-I gene was inversely proportional to the amount of apoC-I produced by the cells. For example, mRNA analysis of apoC-I transfected cells showed that the C20 cell line contained the most apoC-I mRNA, yet it produced the least cellular and medium apoC-I (Figures 4-2 and 4-6). Similarly, the C6 cell line contained the least amount of apoC-I mRNA of the transfected cell lines, yet had the highest levels of apoC-I protein in the cell and

in the medium. Interestingly, it was determined that the vector-only cell lines acted in a slightly different manner from the SW872 control cells. The P9 cell line contained similar amounts of apoC-I message as did the P2 cell line yet, the P9 cell line expressed more protein in the cell and in the medium than did the P2 cell line. Both vector-only cell lines expressed much more protein, with an average increase of 1.8-fold in the cell and 2.8-fold in the medium, as compared to the control cell line. Although cell transfections were successful, as evidenced by the neomycin mRNA data, we were unable to obtain evidence for increased amounts of apoC-I either in the transfected SW872 cells or in the medium of transfected cells. Sequencing of the gene after its introduction into the pcDNA3 vector removed the possibility of a frame-shift error which would give us our observed phenotype. Therefore, it is possible that regulatory mechanisms acting at the post-transcriptional level could have regulated the amounts of proteins produced by the cells. For example, Le Lay *et. al.* (153) reported that during 3T3-L1 differentiation, ATP binding cassette transporter A1 (ABCA1) mRNA increased 36-fold, whereas ABCA1 protein only increased 2- to 3-fold. The discrepancy was not attributed to protease action and it was suggested that other post-transcriptional mechanisms regulate ABCA1 expression. Protease inhibitor experiments would be useful to understand if the low apoC-I synthesis observed in our experiments was due to protein stability or if other post-transcriptional mechanisms were responsible for the control of protein expression. Also, Western blot analysis would be worthwhile in order to validate the results obtained by ELISA.

The vector-only transfected cells used in these experiments do not properly reflect the effect of this manipulation on cellular function because of their significant differences with SW872 control cells. It is likely that transfecting cells has some effect on cellular homeostasis, yet the large effects on cellular lipid accumulation and cellular protein synthesis appear suspect. Re-transfecting the cells or choosing other vector-only cells should be done to better control for the effects of transfection.

In conclusion, the present data have shown that there is a definite effect of apoC-I overexpression on SW872 cells. ApoC-I has been shown, in these experiments, to increase cellular proliferation and inhibit cellular lipid accumulation. It has also been shown that there no dose relationship between the amount of apoC-I mRNA and protein synthesized, nor is there a dose dependent relationship between apoC-I expression and cellular proliferation or cellular lipid accumulation. Although the mechanisms through which this protein affects cellular function and lipid accumulation are not completely understood, these experiments have come closer to elucidating role(s) for apoC-I in adipocytes. Further studies are needed to more clearly understand the possible roles and mechanisms associated with this protein in human adipocytes.

V - Discussion and conclusions

Apolipoprotein C-I is a plasma glycoprotein that plays an important role in lipid metabolism. As explained in detail in Chapter II, apoC-I activates LCAT (120), inhibits CETP (121), LPL (122) and the apoE mediated binding of β-VLDL to the LDL receptor (123). It is found clustered on chromosome 19 alongside apoE, pseudo-apoC-I, apoC-IV and apoC-II (36). ApoE has been identified in human adipose tissue biopsies and adipocytes cell lines (137,138). Although apoC-I is only 5.5kb downstream of apoE and under the control of the same multi-enhancer regions in adipose tissue and macrophages, its presence in adipose tissue or adipocyte cell lines has never been demonstrated (116,137). Mice over-expressing human apoC-I have decreased fatty acid uptake by their adipose tissue (35). With this information as background, the aim of the present project was to characterize the synthesis and secretion of apoC-I by human adipocytes, study the factors which might affect its regulation, and try to determine the role of apoC-I in adipose tissue lipid metabolism.

Through a collaboration with Dr. Katherine Cianflone at the MUHC, we were able to obtain mRNA from human subcutaneous and omental adipose tissue. PCR analysis revealed that apoC-I mRNA was present in both adipose tissue sites and was expressed in similar quantities to HepG2 hepatosarcoma cells and to differentiated and undifferentiated THP-1 macrophage cells (Figure 1-1). This evidence prompted us to study the synthesis and possible secretion of apoC-I by human adipocytes. Human SW872 liposarcoma cells were used as a cellular model. The synthesis and secretion of apoE by SW872 cells was also

investigated, due to its proximity and shared control regions with the gene for apoC-I.

RT-PCR analysis of time course experiments determined that apoC-I mRNA was present in SW872 cells and that the amount of apoC-I mRNA did not increase, as was the case for apoE (Figure 3-2B). Surprisingly, ELISA assays revealed that there were increases in apoC-I protein within the cell and in the culture medium (Figure 3-3A). Similar increases were observed in apoE (Figure 3-3B) yet the molar ratio of cellular apoE to apoC-I was approximately 22 to 1 and approximately 13 to 1, respectively, in the medium. Similar changes were observed when cells were incubated in medium devoid of lipoproteins (Figure 3-4) and when cells were incubated in a medium containing an adipogenic cocktail (Figure 3-5). We therefore concluded that although both genes were under the control of ME-1 and ME-2 in adipocytes, the transcriptional regulation of these genes was independent. In addition, the synthesis and secretion of both proteins was correlated with cellular accumulation of cholesterol and triglyceride. The apoE results are similar to what has been demonstrated in 3T3-L1 preadipocytes (138). In support of the conclusion that apoC-I and apoE were differentially regulated in human adipocytes, we found that there was an inverse relationship between insulin concentration and apoC-I mRNA and no significant change in apoE mRNA with increasing insulin concentrations (Figure 3-6).

To further examine the role of apoC-I in adipose lipid metabolism and more clearly understand the relationship between apoC-I expression and lipid accumulation, it would be practical to carry out additional experiments using

statins. Statins are a class of HMG-CoA reductase inhibitors which significantly reduce plasma cholesterol and can also reduce plasma triglyceride levels (152,154). In hepatocytes, cholesterol loading significantly increased apoC-I in the medium without affecting apoC-I mRNA. Treatment of hepatocytes with statins significantly lowered cholesterol accumulation and reduced apoC-I and apoE in the medium (155). It would be interesting to carry out similar experiments in SW872 cells. If apoC-I synthesis is dependent on cellular lipid accumulation, then statin treatment would inhibit lipid accumulation and possibly decrease apoC-I synthesis as was shown in HepG2 cells. Conversely, it is also possible that the expected result would differ from what was observed in HepG2 cells because in hepatocytes, apoC-I is under the transcriptional regulation of two hepatic control regions, HCR-1 and HCR-2 (156).

Following the characterization of apoC-I in human SW872 liposarcoma cells under different growth conditions, we attempted to overexpress apoC-I in this cell line to further characterize its role in adipocyte lipid metabolism. Overexpression of apoC-I in mice causes elevated plasma cholesterol and triglyceride concentrations, impaired hepatic uptake of VLDL particles, reduced whole body NEFA uptake, impaired uptake of fatty acids by adipose tissue as well as cutaneous abnormalities including hair loss, epidermal hyperplasia and atrophic sebaceous glands and reduced subcutaneous fat pad mass and smaller adipocytes (129,133,134,137). As shown in Chapter IV, transfected cells had increased levels of mRNA for the neomycin-resistance gene and apoC-I (Figures 4-1 & 4-2). There was no significant increase in apoC-I protein in the cell or in

the medium (Figure 4-6). We did however notice a significant effect on cellular proliferation resulting in rapid increase in the number of cells over a three day maturation period (Figure 4-3). Consequently, there was a strong reduction in the amount of triglyceride and cholesterol per cell, as compared to the SW872 control cells (Figure 4-5). We have hypothesized that the effects of apoC-I overexpression on cellular lipid accumulation were not caused by apoC-I's inhibition of lipoprotein uptake by TRL receptors since previous experiments with cells incubated with LPDS did not drastically impair cellular lipid accumulation. It is however possible that apoC-I impairs fatty acid accumulation as shown in the transgenic mice or that it impairs glucose transporters such as Glut-1 or Glut-4. On the other hand, the lack of an increase of apoC-I protein would exclude direct interaction between apoC-I and transport proteins. Therefore, it is suggested that although apoC-I is probably degraded rapidly after its synthesis, the increased transcription of apoC-I message impedes cellular maturation through either positive or negative interference of transcriptional machinery which could affect the transcription of certain genes involved in adipocyte maturation such as PPARγ and C/EBPα. Further studies are needed to determine why apoC-I protein was not increased in these transfected cell. One possibility would be to, re-transfect cells with a new vector to ensure that the results obtained were not due to methodological error. Secondly, if the apoC-I protein is again not significantly increased (as measured by ELISA), Western blot analysis should be performed to substantiate this result. Thirdly, protease inhibitors could be used to determine if there is increased proteosomal degradation of apoC-I by the cell.

Finally, experiments in which SW872 cells are incubated with exogenous apoC-I would be interesting to see if intracellular apoC-I levels can be increased and what effect this has on cellular maturation.

Experiments could also be carried out in which cells could be made deficient in apoC-I. The effect of apoC-I deficiency is humans has not been described because it is extremely rare. This has slowed our understanding of the importance of apoC-I in lipid metabolism. There is only one case in which apoC-I deficiency was observed in man, although it was also coupled with apoC-II deficiency which was believed to be the reason for the observed familial chylomicronemia (125). Much like mice over-expressing apoC-I, mice deficient in apoC-I had impaired hepatic uptake of VLDL by the LDL receptor, although their serum lipids were normal when kept on a regular chow diet (130,157). The specific effects of apoC-I deficiency in adipocytes has yet to be determined. To generate apoC-I deficient SW872 cells, small interfering RNA (siRNA) could be used to silence apoC-I. In this technique siRNA duplexes are introduced into the cell by transfection. The double stranded RNA is complimentary to a small area of the gene to be silenced. It activates nuclease-containing protein complexes which degrade the chosen sequence (158). The maturation of apoC-I deficient cells could be studied and the effects on cellular lipid accumulation could be measured. Another possibility to study apoC-I deficiency in adipocytes would be to generate adipose tissue specific apoC-I knockout in mice using the Cre/Lox system. In this system, mice would be generated from stem cells in which lox sites flank the apoC-I gene. These mice would be crossed with mice whose

stem cells were transfected with cre recombinase under the control of an adipose-specific promoter. These mice would be crossed and the cre recombinase would excise the apoC-I gene flanked by the lox sites generating mice with apoC-I-deficient adipose tissue (159).

In conclusion, we have demonstrated that apoC-I is synthesized and secreted by human adipocytes and that it can be regulated independently of apoE. Our results suggest that apoC-I and apoE play a role in the maturation of human adipocytes and may have an important role in mediating or regulating cell lipid accumulation. Further studies are nevertheless needed to more clearly understand the mechanisms by which apoC-I affects adipocyte lipid accumulation and what other roles it could have in adipocytes. It is possible that the increase of apoC-I by genetic manipulation or pharmaceutical agents could mimic the results seen in these studies thereby reducing human adiposity and preventing the development of type 2 diabetes and/or CAD.

VI - References

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Publications and presentations

Publications

1) **Wassef H**, Davignon J, Bernier L, Cohn JS. Synthesis and secretion of apoE and apoC-I by human SW872 liposarcoma cells. (Accepted Journal of Nutrition)

Abstracts

1) **Wassef H**, Cianflone K, Davignon J, Bernier L, Cohn JS. Synthèse et sécrétion de l'apoC-I par les adipocytes humains. *Medecine/Science* 2003 :19(3) :xxiii.

2) **Wassef H**, Davignon J, Bernier L, Cohn JS. Facteurs affectants la synthèse et sécrétion de l'apoC-I par les liposarcomes humains SW872. *Medecine/Science* 2004 : 20(3) :xx

Presentations

1) **Wassef H**, Cianflone K, Davignon J, Bernier L, Cohn JS. Synthèse et sécrétion de l'apoC-I par les adipocytes humains. (Poster)
Société québécoise de lipidologie, de nutrition et de métabolisme
Sainte-Foy, Québec
April 2003

2) **Wassef H**, Cianflone K, Davignon J, Bernier L, Cohn JS. Synthèse et sécrétion de l'apoC-I par les adipocytes humains. (Poster)
Journée de la recherche des étudiants
Institut de recherches cliniques de Montréal
Montréal, Quebec
May 2003

3) **Wassef H**, Cianflone K, Davignon J, Bernier L, Cohn JS. Synthesis and secretion of apoC-I by human SW872 liposarcoma cells. (Oral Presentation)
Canadian Lipoprotein Conference
Muskoka, Ontario
October 2003

4) **Wassef H**, Davignon J, Bernier L, Cohn JS. Facteurs affectants la synthèse et sécrétion de l'apoC-I par les liposarcomes humains SW872. (Poster)
Société québécoise de lipidologie, de nutrition et de métabolisme
Sainte-Foy, Québec
April 2004

Awards

1) Trainee Oral Presentation Award (Graduate Student)
Canadian Lipoprotein Conference
Muskoka, Ontario
October 2003

Acknowledgements

I would like to express my deep gratitude and great appreciation for Dr. Jeffrey S. Cohn, research scientist of the hyperlipidemia and atherosclerosis research unit at the Clinical Research Institute of Montreal, for taking me on as his graduate student and providing patience, guidance, knowledge and financial support throughout my stay.

I would also like to thank Dr. Lise Bernier who guided me through my first year in the lab, and many other times since then. Her knowledge, patience and open door were invaluable attributes to my success.

This would not have been possible had Dr. Jean Davignon, director of the hyperlipidemia and atherosclerosis research unit at the Clinical Research Institute of Montreal, not opened the door and welcomed me with such enthusiasm. His brilliance and passion were exemplary.

The incredible technicians ; Claudia Rodriguez, Lucie Boulet, Michel Tremblay, Hélène Jacques, and Lise St-Germain not only taught me, but supported me and helped guide me through the project.

Catherine (IB) Bouchard provided much needed help and support. Her competitive nature and inquisitive mind forced me to strive for greater things. Her friendship and humor were invaluable to me. Jean-François Carmel helped tremendously with cellular transfections and I am extremely grateful for his help and laughter. Geneviève Dubuc helped me obtain my first publication and I am thankful for her friendship.

I am also very recognizant to my committee members, Dr. Peter Jones and Dr. Katherine Cianflone, who provided much support and encouragement during some very tumultuous times.

Appendix

A. Faculty of Agricultural and Environmental Sciences REB letter

B. Royal Victoria Ethics Review Board Letter

Faculty of Agricultural and Environmental Sciences

Academic Administration
McGill University, Macdonald Campus
21,111 Lakeshore Road
Ste-Anne-de-Bellevue, Québec, Canada H9X 3V9

October 12, 2004

Faculty of Graduate Studies and Research
McGill University

To Whom It May Concern:

RE: Use of Human Material by Hanny Wassef

The use of two human materials is approved for thesis research of Mr. Hanny Wassef, M.Sc. candidate:

1. Human mRNA donated by Dr, Katherine Cianflone (MUCH), for which there is approval from the Royal Victoria Hospital Ethics Review Board. The certificate is attached

2. Human SW872 liposarcoma cells that are commercially available and immortalized. http://www.atcc.org/SearchCatalogs/longview.cfm?view=ce,3848974,HTB-92&rexr=liposarcoma&max=20.

Harriet V. Kuhnlein, Ph.D.
Professor of Nutrition
Acting Chair, Macdonald Campus Research Ethics Board
McGill University

Centre universitaire de santé McGill
McGill University Health Centre

Bureau d'éthique de la recherche
Office of Research Ethics

January 28, 2004

Dr. Katherine Cianflone
Cardiology Division
H7.14
RVH

REB NO. MED-A 96-246

RE: Hormonal Regulation and Structure: Function of C5L2 Receptor in Adipose Tissue (CIHR); Acylation Stimulating Protein (ASP);: Regulation of Adipocyte Secretion (H&SFC); Mechanism of ASP Action in Human Adipocytes (MRC); Protease Inhibitor Lipodystrophy Mechanism in Human Adipose Tissue.

Dear Dr. Cianflone:

Thank you for your letter of January 16, 2004 in reply to the REB's letter of January 9, 2004 regarding the committee's comments following the review of the Amendment dated December 15, 2003 to the above referenced study for review by the Research Ethics Board of the Royal Victoria Hospital.

Dr. R. Palfree, Acting Chairman of the Medicine-A Committee reviewed your response and found it satisfactory. Therefore, Dr. Palfree approved the above mentioned amendment which is for the purpose of drawing a blood sample only. In addition, he reviewed and approved the consent form dated January 21, 2004.

Please take note that all research involving human subjects requires review at a regular interval and it is the responsibility of the principal investigator to submit an Application for Continuing Review before the expiration of the study approval. Should any revision to the research or other unanticipated development occur prior to the next required review, please advise the REB promptly and prior to initiating any revision.

Sincerely,

Lillian Fatee
Ethics Review Coordinator
RVH Research Ethics Board