Apolipoprotein A5 is an inflammatory responsive gene down-regulated by Tumor Necrosis Factor α and Interleukin-1

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ABSTRACT

Several epidemiological studies have established that elevated plasma triglyceride concentrations constitute an independent risk factor for cardiovascular diseases. In addition, systemic inflammation is associated with severe hypertriglyceridemia and previous studies have demonstrated that cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1) can elevate plasma triglyceride levels. Recently, we identified a new apolipoprotein, APOA5, selectively expressed in the liver and showed that this gene is a crucial determinant of plasma triglyceride levels. In this study, we sought to determine whether inflammatory cytokines regulate APOA5 and consequently influence plasma triglyceride levels. We found initially that treatment of human hepatocytes with TNFα or IL-1 reduced the expression of APOA5 mRNA. Subsequent, we demonstrated through transient transfection experiments that both TNFα and IL-1 down-regulate human APOA5 at the transcriptional level. Further deletion analyses of the APOA5 promoter and binding assays revealed the presence of a promoter sequence, containing a PPARα Response Element, responsive to cytokine stimulation. *In vivo*, treatment of hAPOA5 transgenic mice with TNFα down-regulated the hAPOA5 gene expression in hepatocytes. In patients displaying systemic inflammation, plasma concentrations of triglycerides and ApoAV were inversely correlated. These findings demonstrate that APOA5 is an inflammatory responsive gene and constitutes a link between inflammation and triglyceride-associated cardiovascular risk.

INTRODUCTION

Coronary heart disease (CHD) continues to be a major cause of morbidity and mortality worldwide. Several epidemiological studies established that, in addition to elevated low density lipoprotein and reduced high density lipoprotein level, elevated triglycerides (TG) constitute an independent risk factor for CHD (1,2). In addition, hypertriglyceridemia is often associated with the metabolic syndrome that characterizes diabetes and obesity (3,4). Therefore, the identification of factors or genes affecting triglyceride metabolism is of significant medical importance for the correction of hypertriglyceridemia and associated-CHD.

Apolipoproteins play a determinant role in lipoprotein metabolism and in lipid homeostasis. More specifically, the *APOA1/C3/A4* apolipoprotein gene cluster is tightly linked to plasma lipid profiles. Indeed, mutations in members of this cluster have been shown to cause severe dyslipidemia and heightened atherosclerosis susceptibility (5-8). A comparative genomic characterization of the *APOA1/C3/A4* gene cluster flanking regions led to the identification of a new apolipoprotein gene, apolipoprotein A5 (*APOA5*), present in both mice and humans (9). This gene appears to be predominantly expressed in the liver and its product resides on HDL and VLDL lipoprotein particles (9,10). APOA5 has been described as a crucial determinant of plasma triglyceride levels both in mice and humans (9).

Activation of the immune system during infection and inflammation is under control of cytokines and frequently associated with hyperlipidemia (11-16). Tumor Necrosis Factor alpha (TNFα) and interleukin-1 (IL-1) stimulate and modulate inflammatory response, an effect that is accompanied with hyperlipidemia (17,18). In hyperlipidemic patients, TNFα levels correlated significantly with the concentrations of very-low-density lipoprotein (VLDL) triglyceride and cholesterol and negatively with HDL cholesterol (19). However, the mechanism involved is not completely understood.

Given the determinant link between *APOA5* and plasma triglycerides, and the alteration of triglycerides metabolism induced during inflammation and infection, we investigated

whether pro-inflammatory cytokines such as interleukin-1 and TNFα may affect *APOA5* gene expression and consequently influence plasma triglyceride levels. Our studies with human and mouse hepatocytes revealed that both cytokines modulate *APOA5* expression. Coupled with *in vitro* promoter analysis and the demonstration of a functional cytokine response element, these data identify the new apolipoprotein *APOA5* as an inflammatory responsive gene and suggest a novel mechanism for how cytokines can induce hypertriglyceridemia. To address the relevant of these results in vivo, we performed additional experiments in hAPOA5 transgenic mice treated with TNFα and in patients displaying a nephrotic syndrome known to display high TNFα plasma levels (20-22).

METHODS

Reagents

Fenofibric acid was a kind gift of Dr A. Edgar (Laboratoires Fournier, Daix, France). Wy 14,643 was from Chemsyn, Lenexa, USA. Human recombinant IL-1 and TNFα were purchased from Tebu (Le Perray-en-Yvelines, France).

Cloning and Construction of Recombinant Plasmids

Human *APOA5* promoter fragments (-305/+62, -210/+62) were amplified by PCR using a *APOA5* genomic BAC clone (9,23) as template and cloned in pGL3 luciferase vector. The followed forward oligonucleotides 5'-TCTGTTGGGGCCAGCCAG-3', 5'GCTCCTGGGAAGCACTTCTCTACT-3' and the reverse oligonucleotide 5'-AATGCCCTCCCTTAGGACTGTGAC-3' primer were used for the PCR reaction. The human *APOA5*-cytokine response element (-284/-243) (5'-AGGTCAGTGGGAAGGTTAAAGGTCATGGGGTTTGGGAGAAAC-3') oligonucleotide was cloned in four copies into SV40-pGL3.

Cell culture and transfections

HepG2 cells, obtained from the European Collection of Animal cell Culture (Porton Down, Salisbury, UK) were cultured exactly as described previously (24). HepG2 cells were transiently transfected using the calcium phosphate precipitation method. The total amount of DNA was kept constant by complementation with corresponding empty vector mock DNA. After a 2-h incubation period, cells were washed with PBS and refed with DMEM supplemented with 0.2% FCS and Wy 14,643 or vehicle and TNFα or IL-1 as indicated in the figure legends. Cells were harvested after 24-h incubation and collected for the determination of the luciferase activity performed using a luciferase assay system (Promega Corp., Madison, WI, USA).

RNA analysis

Total RNA extraction was performed as described (25). RNA expression of APOA5 and GAPDH genes were analysed by real time quantitative PCR using SYBR Green technology on a MX4000 apparatus (Stratagene Europe, Amsterdam, The Netherlands). PCR was performed with oligonucleotides forward: 5' ACGCACGCATCCAGCAGAAC 3' and reverse 5' TCGGAGAGCATCTGGGGGTC 3' for human APOA5; forward: 5' CTCTGTCCCACAAACTCACACG 3' and reverse 5' AGGTAGGTGTCATGCCGAAAAG 3' for mouse APOA5; forward 5' TGATGACATCAAGAAGGTGGTGAAG 3' and reverse 5' TCCTTGGAGGCCATGTGGGCCAT 3' for GAPDH. Quantification of APOA5 mRNA levels were corrected for using GAPDH mRNA levels as a internal control.

Preparation of nuclear extracts

Nuclear extract of HepG2 cells were prepared in the presence of protease inhibitors (Rohe Diagnostic) and all steps were performed at 4°C. Cells were washed twice with cold PBS, scrapped off in PBS containing protease inhibitors and collected by centrifugation at 800g for 5 minutes. Cells were pelleted again and resuspended in 400 µl of cold hypotonic buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and protease inhibitors. Cells were allowed to swell 15 minutes on ice. 25 µl of 10% nonidet P-40 was added and tubes were vortexed. Nuclei were pelleted by centrifugation at 1000g for 10 minutes, the supernatant was removed and the pellet containing the nuclei was washed with cold PBS containing protease inhibitors. Pellets were resuspended in 20 mM HEPES, pH 7.9, 0.4 M KCl, 1 mM EDTA, 1mM EGTA, 1 mM DTT and protease inhibitors

and were centrifuged for 5 minutes at 10,000g. The supernatants (nuclear extracts) were collected and stored in aliquots at –80°C.

Electrophoretic mobility shift assay (EMSA)

The sequences of the double-stranded oligonucleotide used for EMSAs were derived from the human APOA5 promoter (23). Complementary oligonucleotides were annealed and end-labelled with [g-32P] ATP using T4 polynucleotide kinase. Nuclear extract (4 µg protein/2 µl extract) was incubated with about 0.5 ng of 32P-labelled double stranded oligonucleotide probe in incubation buffer constituted of 16 mM HEPES, pH 7.9, 1 mM EDTA, 2.5 mM DTT, 0.25 % NP40, 1 µg of polydI-dC at 4°C for 10 minutes and another 20 minutes incubation at room temperature. DNA binding complexes were resolved by 5% native polyacrylamide gel electrophoresis (PAGE) in 0.25 X TBE at 10 V/cm. The gel was dried and exposed at –80°C to XOMAT-AR film (Kodak).

Treatment of human APOA5 transgenic mice

We used hAPOA5 transgenic mice previously generated in an inbred FVB genetic background (9). Two groups of 6 mice were treated with vehicle or recombinant TNFα (500 ng per injection). We performed one intraperitoneal injection per day for 3 consecutive days. After sacrificing the mice, liver RNA expression of hAPOA5 and GAPDH genes were analysed by real time quantitative PCR as previously described (You need to Reference where previously described). Identical experiments were performed with two groups of wildtype FVB strain control mice.

Cytokines measurements

For this study, 32 children, between 2 and 18 years old, displaying an idiopathic nephrotic syndrome were selected (Hôpital Jeanne De Flandre – CHRU Lille). Triglyceride serum concentration was determined with an Hitachi 912 analyser, and a 2 ml EDTA tube was collected for each patient in order to measure hApoAV and TNFα plasma levels. The protocol was approved by the local Ethical Committee. Plasma TNFα was determined using a commercially available kit (Quantikine human TNFα, R&D Systems, Minneapolis, MN).

Plasma ApoAV protein measurement

An enzyme-linked immunosorbent sandwich assay was used to measure ApoAV in sera. A pool of two monoclonal anti-human apoAV antibodies solution, raised in mice by using a protein recombinant, was used at 10 µg/mL in PBS 0.1M, pH 7.2 to coat the wells of the microtiter plates at room temperature overnight. The wells were washed twice with PBS 0.1M. The remaining sites for protein binding were saturated with 3% BSA/PBS for 1 hour at 37°C. The wells were washed twice with PBS. 90 µL of the antigen solution was added to the wells. For quantitation, a pool of human plasma was calibrated and titrated using apoAV recombinant protein as a primary standard. Next, the pool of human plasma was used to generate a calibration curve. All dilutions were done in blocking buffer (1% BSA/PBS) and the antigen solution was incubated for 2 hours at room temperature. The wells were washed four times with PBS. The horseradish peroxydase labeled second anti-apoAV polyclonal antibody, produced in rabbit using synthetic peptide, was diluted in the blocking buffer and added to the wells. After incubation for 2 hours at 37°C, the plates were washed with several changes of PBS. Prior to developing the enzyme label, 30 mg of o-phenylenediamine (ODP) was dissolved in 20 mL 0.1M citrate/phosphate buffer and 20 µL of 30% H2O2. Next, 100 µL

of the enzyme substrate solution were added to each microtiter well. After incubation for 30 min at room temperature in the dark, the reaction was terminated by adding 100 µL of HCl l M and the absorbance at 492 nm was measured using a microplate photometer (Dynex Technologies).

RESULTS

Human APOA5 mRNA levels ares lowered by TNFα and IL-1 in human hepatocytes

To determine whether pro-inflammatory cytokines can modulate *APOA5* gene expression in humans, we analyzed *APOA5* mRNA levels in human hepatocytes upon incubation with TNFα or IL-1. In either case, treatment with TNFα (25 ng/ml) or IL-1 significantly reduced *APOA5* mRNA level (Figure 1). These initial observations demonstrate that the pro-inflammatory cytokines TNFα and IL-1 result in lowered steady-state APOA5 mRNA levels and suggest *APOA5* is an inflammatory responsive gene.

TNFα and IL-1 down-regulate APOA5 at the transcriptional level

To determine whether TNFα and IL-1 directly repressed transcriptionof APOA5, we performed functional analysis of the *APOA5* promoter in response of cytokine stimulation. HepG2 cells were transiently transfected with a Luciferase reporter vector driven by a human *APOA5* promoter fragment (-305/+62) (Figure 2). Transcriptional activity of the *APOA5* reporter construct was decreased in a dose dependant manner by the incubation of IL-1 (Figure 3). Treatment with TNFα also led to a dose dependent lowering of APOA5 gene promoter activity. Next, we sought to define the cytokine responsive element within the APOA5 promoter by deletion analysis. HepG2 cells were transiently transfected with Luciferase reporter vectors driven by two human *APOA5* promoter fragments (-305/+62) or (-210/+62). Compared to construct (-305/+62), construct (-210/+62)t was unresponsive to TNFα or IL-1 treatment (Figure 4). These results support that the gene regulation of *APOA5* by TNFα and IL-1 occurs at the transcriptional level and that the responsive region is located in the –305/-210 APOA5 gene regulatory region.

The APOA5 gene promoter contains a sequence that confers responsiveness to TNFα and IL-1

Transcriptional activation of the *APOA5* gene by cytokines suggests the presence of a functional sequence in the *APOA5* promoter. Sequence analysis did not reveal the presence of a highly conserved putative response element for TNFα or IL-1 activated transcription factors such as NF-kB or AP-1. However, we assessed the binding capacities of the –305/-210 APOA5 gene regulatory region in response to TNFα and IL-1 by electromobility shift assay. Different double-stranded oligonucleotides covering the –305/-210 APOA5 gene regulatory region were radio-labelled and incubated with nuclear extracts of HepG2 cells incubated with either TNFα or IL-1 or vehicle. Whereas no signal was observed with oligonucleotide spanning the –305/-262 promoter region, stimulation of the cells with either TNFα or IL-1 led to the binding of a protein complex on the oligonucleotide spanning the -284/-243 promoter region (Figure 5). To further support that the putative *APOA5*-cytokine response element is functional, we cloned it in four tandem copies in front of a heterologous promoter and examined it's response following cytokine treatment in HepG2 cells {Which cytokine did you use in this case? Both of them? Only one?}. We found that this site could transmit TNFα repressive effect (Figure 6). Taken together these results demonstrate that the *APOA5* promoter contains a functional response element that confers TNFα and IL-1 responsiveness.

TNFα and IL-1 prevent induction of the APOA5 gene by PPARα agonists

Recently, we reported that the *APOA5* gene is a strongly induced by agonists of the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα), providing a possible explanation for how fibrates reduce plasma triglycerides in humans (23). A response element for PPARα (PPRE) was identify at position [-272/-260]. Since, this PPRE site at [-272/-260] is found within the -284/-243 cytokine repression fragment, we wondered whether

activation of the APOA5 gene by PPARα agonists could be prevented by cytokine action. HepG2 cells were transiently transfected with a Luciferase reporter vector driven by the human *APOA5* promoter fragment (-305/+62). As previously reported (23), transcriptional activity was induced when cells were incubated with Wy 14,643, a prototype PPARα agonist (Figure 7). Interestingly, co-incubation with cytokines TNFα or IL-1 abolished the response of the APOA5 promoter to Wy 14,643 {When I look at the figures 7 and 8 I don't see complete abolishment. In fact it looks like there is still sizable induction. Can we give quantitiative #'s for this effect. And Statistics?Figure 9 looks much more like there is no effect}. We obtained similar results using the region containing the putative APOA5-cytokine response element (-284/-243) (Figure 8) Next, we checked whether this effect consequently affected APOA5 expression at the mRNA level. HepG2 cells were treated with Wy 14,643 or fenofibric acid at a physiological concentration (100 µM) (similar to that reached in plasma from treated patients) and subjected to real-time PCR analysis of the APOA5 gene expression (Figure 9). Increased *APOA5* mRNA levels occurred for both Wy 14,643 and fibric acid confirming our previously reported findings (Reference). This induction was prevented when cells were stimulated by TNFα or IL-1. We observed a similar effect with Wy 14,643 treatment. These data indicate that opposite regulation of APOA5 by PPARα agonist and cytokines TNFα and IL-1 may occur in an exclusive manner {Can we conclude which one is more dominant? Figure 9 implies that cytokines can completely block PPAR agonist effects, but the other figures seem to show the PPAR agonist still work despite the presence of cytokines. This ambiguity will probably raise a flag for reviewers.}.

TNFα down-regulates human APOA5 gene expression in hAPOA5 transgenic mouse hepatocytes {I think we have to be careful how strongly we say what we are finding throughout the manuscript. For many of these studies we are really only looking at APOA5 steady state mRNA levels and not gene expression directly. So the title of this section should be TNFα causes decreased human APOA5 mRNA levels in hAPOA5 transgenic mouse] We need to have this tone for most of these experiments.s

In order to determine whether cytokines down-regulate APOA5 gene expression in vivo, we treated hAPOA5 transgenic mice for three consecutive days with 500 ng of recombinant TNFα and determined APOA5 mRNA levelsby real time quantitative PCR in transgenic mouse hepatocytes. We observed a decrease of APOA5 mRNA levels in transgenics treated with TNFα in comparison with untreated transgenic mice (Figure 10). These observation demonstrate that the pro-inflammatory cytokine TNFα decreaseAPOA5 mRNA levels not only in vitro but also in vivo.

At the protein level, we detected no significant decrease of APOAV concentration, but this may be solely due to clear cross reactivity between our antibody and both human and mouse versions of this protein.

Did you test what IL-1 does in transgenic mice?

Did we test what TNF does in wildtype mice? It seems like we present half the story for each cytokine

IL-1 up-regulates mouse APOA5 gene expression in wild-type mouse hepatocytes
More accurately:
IL-1 increases mouse APOA5 mRNA levels in wild-type mouse hepatocytes

To determine the in vivo effect of IL-1 on APOA5 expression, we treated wild-type mice for three consecutive days with 500 ng of recombinant IL-1 and determined mouse APOA5 mRNA levelsby real time quantitative PCR in mouse hepatocytes. Whereas pro-inflammatory cytokines (TNF) decreased human APOA5 mRNA levels in hAPOA5 transgenic mice, we observed a significant increase of mouse APOA5 mRNA levels in wild-type control

mice treated with IL-1 (Figure 11). These observation demonstrate that there is an opposite regulation of APOA5 gene expression by IL-1 in human versus this rodent species (Again we don't formally show opposite regulation, only opposite amounts of resulting mRNA levels). Is this opposite regulation also found for TNF?

Low APOAV protein concentration is associated with high TNFα plasma level in patients displaying a nephrotic syndrome

Patients displaying Nephrotic Syndrome and high TNFα plasma levels have low ApoAV protein concentrations

Patients with an idiopathic nephrotic syndrome display an increased risk of cardiovascular heart disease (26-28) associated with severe hypertriglyceridemia. Hypertriglyceridemia in these patients is strongly correlated with high plasma level of TNFα. To determine whether a decrease of APOAV expression can explain in part the observed increased triglycerides associated with nephritic syndrome, we determined TNFα and APOAV protein levels by ELISA methods. Of the 32 children selected for this study, 11 were in clinical relapse (proteinuria higher than 3 g/L) and the other 21 children were in remission. The 21 children in remission were used as controls for this study. First, as previously described, we observed increased TNFα plasma levels in the control versus affected groups, directly correlated with the severity of the renal disease. TNFα levels were X% higher in Y versus Z(7,32 ± 2,98 versus 1,84 ± 1,46 pg/mL, $p<0.001$) (Figure 12a). In addition, we found APOAV concentrations were significantly ($p<0.001$) decreased in patients in relapse (7,25 ± 0,96 ng/mL) versus patients in remission (14,40 ± 5,27 ng/mL) (Figure 12b). Moreover, we demonstrated that this decrease of APOAV protein level is strongly correlated with an increase of plasma triglyceride level (2,11 ± 1,14 g/L in patients in relapse versus 0,78 ± 0,40 g/L in patients in remission, $p<0.001$) (Figure 12c). These findings suggest that pro-inflammatory cytokine TNFα, especially increased in nephrotic syndrome, strongly down-regulates APOAV protein levels in vivo. Combined, these in vivo data obtained in transgenic mice and in patients displaying nephrotic syndrome suggest an important role for APOAV decrease in hytpertriglyceridemia associated with inflammatory diseases in human.

DISCUSSION

In humans, inflammation is often associated with hypertriglyceridemia through stimulation by cytokines (29). Numerous studies have shown that the TNFα and IL-1 class of cytokines increases serum triglycerides by two different mechanisms. First, pro-inflammatory cytokines stimulate lipid synthesis via a direct effect on the hepatocyte (30,31). Secondly, cytokines can inhibit the catabolism of triglyceride-rich particles by decreasing lipoprotein lipase and hepatic lipase activities (32,33). The elevation of plasma triglycerides by these pro-inflammatory cytokines is linked to an increase in very low density lipoprotein production (19). In this report we found that TNFα and IL-1 down-regulate human APOA5 gene expression, an apolipoprotein that behaves as an important determinant of plasma triglyceride levels (9). The present data describe the existence of a new link between inflammation and lipid metabolism via gene modulation of an apolipoprotein. These data suggest a new mechanism by which inflammation may lead to atherosclerosis and cardiovascular heart disease in humans.

Interestingly, we observed a significant increase of mouse APOA5 in wild-type mice treated with pro-inflammatory cytokine IL-1, suggesting an opposite regulation of APOA5 gene expression in human versus this rodent species. Further studies are necessary to understand the involved mechanism of regulation in mice, but our results suggest that mouse APOA5 may be a positive acute phase protein.{you might leave this paragraph out, I'm not sure what it adds}

Whereas the cytokine-mediated increase in lipoprotein plasma levels contribute to host defense (34), prolonged exposure to high level of circulating pro-inflammatory cytokines such as TNFα and IL-1 can lead to chronic inflammation thought to be a risk factor for cardiovascular diseases (35-37). The repressive effect of cytokines on apolipoproteins has already been reported by several authors (Reference). Cytokines have been described able to decrease APOA1 and APOB accumulation, a mechanism thought to be responsible of acquired hypercholesterolemia (38). The most numerous studies are for APOC3. This apolipoprotein which participate in the control of triglyceridemia, is considered as a negative acute phase protein. TNFα and IL-1 decrease APOC3 mRNA level via a transcriptional activity control (39,40). The trans-repression mechanism implies different signalling pathways, of which primarily NF-κB activation {I'm not sure what this means? ApoC3 is downregulated just like ApoA5, why need it be a different signalling pathway? } (41,42). This already represents a link between inflammation and dyslipidemia involving regulation of apolipoproteins. Our data suggest that constant lowering of APOA5 expression by TNFα and IL-1 would therefore constitutes risk factors predisposing to hypertriglyceridemia-associated vascular diseases in humans.{I think the point of the last two sentences should be: Since ApoC3 levels are decreased in response to TNF and IL-1, and lower ApoC3 levels functionally results in lower triglycerides (opposite that found for ApoA5), the hypertriglyceridemia associated with increased cytokines can not be explain by alterations in ApoC3 levels. In contrast, decreased ApoA5 does cause increased Tgs, and thus ApoA5 is consistent with explaining the effect of cytokines causing hypertriglyceridemias.}

The repression of *APOA5* expression by cytokines was due to a direct effect on hepatocytes and occurs via a decrease in transcriptional activity of the APOA5 promoter. Interestingly, binding and functional studies indicates that this regulation may be attributed to a short sequence located in the proximal promoter of the APOA5 gene (-284/-243) on which a cytokine-stimulated transcription factor bind. Remarkably, this short region contains a Peroxisome Proliferator-Activated Receptor (PPAR) response element (PPRE) that confers a strong PPARα-mediated transactivation of the APOA5 promoter (23). It is therefore conceivable that PPARα is competed out for the binding to its PPRE by the binding of other transcription factors stimulated by TNFα or IL-1. Our data argue in favour of this hypothesis.

Indeed, the transcriptional induction of the APOA5 gene by PPARα activation was diminished (I think this is the right tone, it was diminished but not abolished. You might say in the results that normal fibrates cause an X% increase in ApoA5 gene expression, but when you co-incubate with cytokines this increase is on Y%. so clear there is some competition or cross talk that causes this effect) when cells were incubated with either TNFα or IL-1. This effect was also observed at the APOA5 mRNA expression level. It was previously reported that PPARα physically interacts with NF-kB, a transcription factor complex activated by TNFα/IL-1 class of cytokines, leading to a reciprocal inhibition of their respective target genes (43). Our findings represent an additional mechanism of interference between PPARα and cytokine signalling pathways via a competition for accessibility to their own response element {I confused as to whether you are saying that we have a new pathway because in the sentence before you say that PPAR and cytokine cross talk has been previously described. So I see this as a similar finding to ours.}.

To assess the physiological relevance of these in vitro studies,, we determined whether proinflammatory cytokines also modulate APOA5 *in vivo* by examining ApoAV protein levels. We measured APOAV protein plasma level in patients displaying idiopathic nephrotic syndrome. Several studies have demonstrated that the nephrotic syndrome is associated with an increased TNFα plasma level directly correlated to the severity of the renal disease. Our study confirmed the increased levels of TNFα in patients in relapse and demonstrated that APOAV protein level is inversely correlated with TNFα level. Previous studies have demonstrated that APOA5 may be defined as a major determinant of triglyceride levels through studies in genetically engineered mice as well as human genetic association studes(9). Our data are consistent withAPOA5 having a directly role in hypertriglyceridemia associated with the increase of TNFα level in patients displaying a nephrotic syndrome, and may explain their increased cardiovascular risk. The understanding of this mechanism may lead to a new therapeutic approach of the severe hypertriglyceridemia described in this disease. Modulation of APOA5 via an anti-inflammatory, or more specifically an anti-TNFα, pathway may offer a new strategy for correcting hypertriglyceridemia and limiting TG-associated cardiovascular risk in these patients.

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FIGURE LEGENDS

Fig. 1. Effect of TNFα and IL-1 on human *APOA5* expression. Human hepatocyte (HepG2) cells were treated 24 h with TNFα (25 ng/ml), IL-1(25 ng/ml). Total RNA (1 μg) was subjected to quantitative RT-PCR. Results are mean +/- SD.

Fig. 2. Sequence upstream of the putative APOA5 promoter (-305/+62) used for the transient transfections. Exon 1 is depicted by a box with the start site of transcription indicated by +1. The PPRE-DR1 site (-272/-260) is bold and the region containing the sequence responsive to pro-inflammatory cytokines TNFα and IL-1 (-284/-243) is underlined.

Fig. 3. Effect of TNFα and IL-1 on human APOA5 promoter activity. HepG2 cells were transfected with human *APOA5* promoter fragment reporter plasmids (-305/+62) cloned into Luciferase reporter vector. Cells were incubated with TNFα (10, 25, and 50 ng/ml), IL-1(10 and 25 ng/ml), and TNFα (25 ng/mL) + IL-1 (25 ng/mL) as indicated for 24h. Luciferase activity is expressed as means ± SD. Luc: luciferase. {The fact that both cytokines together don't give more of an effect than either alone suggests that they might be using the same exact mechanism. It might be worth explicitly stating this in the results}

Fig. 4. Delineation of a cytokine response region in the human *APOA5* promoter. HepG2 cells were transfected with different human *APOA5* promoter fragment reporter plasmids (-305/+62), (-210/+62) cloned into a Luciferase reporter vector. Cells were incubated with TNFα (25 ng/ml) and IL-1(25 ng/ml) as indicated for 24h. Luciferase activity is expressed as means ± SD. Luc: luciferase.

Fig. 5. Cytokine regulation of the APOA5 gene occur in a DNA-binding dependent manner. Electrophorectic mobility shift assays were performed with radio-labeled oligonucleotides in the presence of nuclear proteins extracted from HepG2 cells treated with either TNFα or IL-1 (25 ng/ml) for 24h.

Fig. 6. Functionality of the putative APOA5-cytokine response element. HepG2 cells were transfected with the putative APOA5-cytokine response element (-284/-243) (x 4) reporter plasmid cloned into a Luciferase reporter vector. Cells were incubated with TNFα (25 ng/mL) for 24h. Luciferase activity is expressed as means ± SD. Luc: luciferase.

Fig. 7. Effect of TNFα and IL-1 on PPARα agonist-induced APOA5 promoter activity. HepG2 cells were transfected with human APOA5 promoter fragment reporter plasmids (-305/+62). Cells were incubated with Wy 14,643 (1 μM) or vehicle (DMSO) and stimulated or not with TNFα (25 ng/ml) or IL-1 (25 ng/ml) as indicated for 24h. Luciferase activity is expressed as means ± SD. Luc: luciferase.

Fig. 8. Effect of TNFα on PPARα agonist-induced APOA5 promoter activity, using the promoter fragment (-284/-243) in four copies. (**a**) Sequence (-284/-243) used in the transfection assay. Boxed and bold sequence represents the PPRE-DR1. (**b**) HepG2 cells were transfected with the putative APOA5-cytokine response element (-284/-243) (x 4) reporter plasmid cloned into a Luciferase reporter vector. Cells were incubated with Wy 14,643 (1 μM) or vehicle (DMSO) and stimulated or not with TNFα (25 ng/ml). Luciferase activity is expressed as means ± SD. Luc: luciferase.

Fig. 9. Effect of TNFα and IL-1 on PPARα agonist-stimulated APOA5 mRNA expression. HepG2 cells were treated 24 h with fenofibric acid (100 μM), Wy 14,643 (100 μM) or vehicle (DMSO, dimethylsulfoxide) and co-incubated with TNFα (25 ng/ml), IL-1 (25 ng/ml) as indicated. Total RNA (1 μg) was subjected to quantitative RT-PCR. Results are mean +/- SD.

Fig. 10. Effect of TNFα on human *APOA5* expression in hAPOA5 transgenic FVB mice. Transgenic mice were treated with recombinant TNFα (3 times 500 ng). Total RNA (1 μg) from transgenic mice liver was subjected to quantitative RT-PCR. Results are mean +/- SD.

Fig. 11. Effect of IL-1 on mouse APOA5 expression in wild-type FVB mice. Wild-type mice were treated with recombinant IL-1 (3 times 500 ng). Total RNA (1 μg) from mice liver was subjected to quantitative RT-PCR. Results are mean +/- SD. {I think I would completely leave out all the wild type mouse response to cytokines. It doesn't add anything and just confuses the big picture.}

Fig. 12. Clinical observation in children displaying idiopathic nephrotic syndrome in relapse. We divided selected patients into two different groups: patients in relapse and patients in remission (controls). (**a**) TNFα plasma level. TNFα plasma concentration was determined with a commercial ELISA kit. (**b**) APOAV protein plasma level. APOAV protein level was determined with by ELISA. (**c**) Triglyceride plasma level. Triglyceride level was determined with an Hitachi 912 analyser. Results are mean +/- SD. Statistical analysis was performed with the Student's t-test ($p<0.001$).