

Complement regulation in murine and human hypercholesterolemia and role in the control of macrophage and smooth muscle cell proliferation

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ABSTRACT

OBJECTIVE: Mounting evidence suggests that activation of complement, an important constituent of innate immunity, contributes to atherosclerosis. Here we investigated the expression of complement components (CCs) in the setting of experimental and clinical hypercholesterolemia, a major risk factor for atherosclerosis, their effects on vascular smooth muscle cell (VSMC) and macrophage proliferation, and the underlying molecular mechanisms.

METHODS: For this study we analyzed the mRNA and protein expression of several CCs in plasma and aorta of hypercholesterolemic atherosclerosis-prone apolipoprotein E-null mice (apoE-KO) and in plasma of normocholesterolemic subjects and familial hypercholesterolemia (FH) patients. We also carried out in vitro molecular studies to assess the role of CCs on the control of macrophage and VSMC proliferation.

RESULTS: Fat-fed apoE-KO mice experiencing severe hypercholesterolemia (~400 mg/dL), but not fat-fed wild-type controls with plasma cholesterol level <110 mg/dL, displayed in aortic tissue upregulation of several CC mRNAs, including C3, C4, C1s, and C1q. In apoE-KO mice, induction of C3 mRNA was already apparent two days after fat feeding when hypercholesterolemia was manifest yet atherosclerotic lesions were absent or incipient. Rapid C3 and C4 protein upregulation was also observed in the plasma of fat-fed apoE-KO mice, and FH patients exhibited higher plasmatic C3a, C4 γ chain, C1s and C3c α chain protein levels than normocholesterolemic subjects. In vitro, C3 and C3a, but not C3a-desArg, C4 and C1q, promoted macrophage and VSMC proliferation through G_i protein-dependent activation of extracellular signal-regulated kinase 1/2 (ERK1/2). We also found that C3-enriched FH plasma evoked a stronger mitogenic response in macrophages than normocholesterolemic plasma, and treatment with anti-C3 antibodies eliminated this difference.

CONCLUSIONS: Both experimental and clinical hypercholesterolemia coincides with a concerted activation of several CCs. However, only C3 and C3a elicited a mitogenic response in cultured VSMCs and macrophages through G_i protein-dependent ERK1/2 activation. Thus, excess of C3/C3a in hypercholesterolemic apoE-KO mice and FH patients may contribute to atheroma growth by promoting neointimal cell proliferation.

INTRODUCTION

Atherosclerosis is a complex inflammatory disease triggered by several cardiovascular risk factors (e. g., hyperlipidemia, hypertension, obesity, diabetes, smoking), which promote endothelial damage and neointimal accumulation of various cell types (e.g. VSMCs, monocytes/macrophages, T cells and dendritic cells), and non-cellular material, such as modified lipids and extracellular matrix components [1,2]. Circulating monocytes adhere to the injured endothelium, undergo transendothelial migration and differentiate into macrophages that avidly absorb modified low-density lipoproteins (LDLs) and become lipid-laden foam cells. Activated neointimal macrophages and lymphocytes produce inflammatory mediators that induce their own proliferation, as well as VSMC hyperplasia and migration towards the growing atherosclerotic lesion [3].

The complement system, an important constituent of innate immunity, consists of a complex group of serum proteins and glycoproteins as well as soluble or membrane-bound receptors, which mediate critical functions in inflammation and in the body's defence against invading microorganisms [4,5]. A cascade of serine proteases can be activated by antibody/antigen complexes (classic complement pathway), by harmful substances such as zymosan (alternative pathway), or by mannose-binding lectin (lectin pathway). The three complement pathways converge in the activation of the C3-convertase that ultimately mediates the formation of the terminal membrane attack complex C5b-9 causing cell lysis and secretion of proinflammatory molecules. In addition to its role in the recognition of foreign antigens and pathogens, the complement system seems to play an important role in immunoregulatory functions, in the generation of tissue inflammatory reactions, and in the clean-up and repair of tissues during ischemic, autoimmune and inflammatory disorders (e.g., rheumatoid arthritis, asthma, lupus nephritis, and atherosclerosis) [4-8]. Deposits of C5b-9, a marker of in situ complement activation, have been found to colocalize with macrophages within human

atherosclerotic lesions [9,10]. Subsequent studies have demonstrated the local expression of various complement components (CCs) in endothelial cells, VSMCs and dendritic cells from advanced atherosclerotic plaques in humans [11-15] and experimental animals [16-21]. Moreover, plasmatic levels of CCs are elevated in coronary artery disease patients [22-25].

Whereas the aforementioned studies suggest a role to the complement system in atherosclerosis, it remains controversial whether the net effect of complement activation is pro or antiatherogenic [6]. Moreover, little is known regarding the expression of CCs at the onset of atherosclerosis, and their effects on VSMC and macrophage functions involved in the growth of early atherosclerotic lesions (e.g. cell proliferation). In this study we examined the expression of CCs in hypercholesterolemic atherosclerosis-prone apolipoprotein E-null (apoE-KO) mice [26], and in patients with familial hypercholesterolemia (FH), an autosomal dominant inherited disorder featuring elevated plasma LDL-cholesterol levels and premature coronary artery disease [27]. We also investigated the effects of CCs on macrophage and VSMC proliferation in culture, and the underlying signaling pathways.

METHODS

Animals and diets

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. Male wild-type and apoE-KO mice (C57BL/6J, Charles River) were maintained on a low-fat standard diet (LF-diet, 2.8% fat, Panlab, Barcelona, Spain). When indicated, 2-3-month-old mice received for up to 30 days a high-fat cholesterol-rich diet (HFC-diet, 12% fat, 1.25% cholesterol, 0.5% sodium cholate, S8492-S010, Ssniff). Blood was extracted through the retroorbital plexus and collected into

EDTA tubes to obtain plasma. Cholesterol was measured by spectrometry (Chol-H-L-Type, Wako), and C3 and C4 by turbidimetry (Alfa-Wassermann, Ref. P/N05760216).

Human studies

The investigation conforms with the principles outlined in the Declaration of Helsinki. The studies included control normolipidemic subjects recruited from family physicians lists and hospital staff, and patients with a genetic confirmation of FH (see supplementary material). All participants gave written informed consent to a protocol approved by the local review board. The exclusion criteria included acute infectious disease in the previous week, active chronic inflammatory disease, anti-inflammatory drugs consumption, including oral steroids and non-steroidal anti-inflammatory drug in the previous week, and serum C-reactive protein concentration >10 mg/L.

Plasmatic lipid levels were determined as follows: (a) Cholesterol and triglycerides by standard enzymatic methods, (b) HDL-cholesterol by precipitation with phosphotungstic acid and magnesium chloride [28], (c) LDL-cholesterol was estimated with the Friedewald equation [29], (d) apolipoprotein AI (apoAI) and B (apoB) using turbidimetric techniques, (e) C3a by ELISA (BD Biosciences, ref. 550449).

Quantitative real time reverse transcription-polymerase chain reaction (real-time RT-PCR)

Total RNA was extracted from frozen aortic tissue pooled from 2-6 mice using TRIzol reagent (Invitrogen). DNase-treated RNA (1-2 μ g) was reverse transcribed either with M-MLV Reverse Transcriptase RNase H minus and Oligo(dT)₁₅ as primers (Promega), or with High-Capacity cDNA reverse transcription kit with random hexamers (Applied Biosystems). cDNA was amplified using assays-on-demand kits containing primers, TaqMan probes and the TaqMan universal PCR mix (Applied Biosystems). Reaction mixtures without template served as negative

controls. For template input normalization, GAPDH or β -actin transcript levels were measured to calculate the threshold cycle number (Ct). Fold change values were calculated as the ratio of the Δ^{Ct} sample averages.

Cell culture

Murine RAW macrophages (RAW 264.7 TIB-71, American Type Culture Collection) were maintained in 10% FBS/DMEM. Rat primary VSMCs were obtained from aortic tissue as previously described [30] and maintained in 10% FBS/DMEM F12. Starvation media for cell proliferation studies contained 0.1% FBS.

When indicated, cells were treated with human CCs (C3, Ref.204885; C3a, Ref.204881; C3a-desArg, Ref.204884; C1q, Ref.204876; C1s, Ref. 204879; C4, Ref.204886; Calbiochem). Cell proliferation and Western blot assays are described in supplementary material.

Statistical analysis

All values are expressed as mean \pm SEM. Differences were evaluated using either 2-tail, unpaired *t*-test (experiments with 2 groups) or 1-way ANOVA and Bonferroni's post-hoc test (experiments with ≥ 3 groups). The F-test, which uses the sum of the squares and mean squares to calculate an F-statistic and an associated *p* value, was used to measure the significance of the regression analysis. Statistical significance was taken at $p < 0.05$.

RESULTS

Increased expression of CCs in aortic tissue and plasma of hypercholesterolemic apoE-KO mice

We first examined by real-time RT-PCR the expression of CCs in the aorta of apoE-KO mice fed with LF-diet and HFC-diet. C3 and C4 mRNA steady-state levels were significantly increased at 2 and 7 days of HFC-diet, respectively, and augmented expression of these CCs was sustained for up to 30 days of fat feeding (Fig.1A). Induction of C1s and C1q γ mRNA was only observed in the aorta of apoE-KO mice fed with HFC-diet for 30 days. We have previously shown in apoE-KO mice absence of aortic atheromas or presence of only incipient lesions after 2 and 7 days of HFC-diet, and formation of prominent lesions after 30 days of HFC-diet [31]. We also measured the levels of plasmatic cholesterol and C3 and C4 proteins in apoE-KO mice (Fig.1B). All parameters reached maximum levels by 3 days of HFC-diet and were maintained up to 30 days of fat feeding. Both C3 ($p < 0.04$) and C4 ($p < 0.0007$) protein levels directly correlated with the level of circulating cholesterol (Fig.1C).

In contrast to the findings in apoE-KO mice, CC mRNA expression was not elevated in the aorta of wild-type controls fed with HFC-diet for 7 and 30 days (Fig. 1A), which exhibited a level of plasmatic cholesterol much lower than apoE-KO mice fed with HFC-diet (< 110 mg/dL versus ~ 400 mg/dL, respectively) (Fig.1B).

Induction of complement components in the plasma of FH patients

Having demonstrated that severe hypercholesterolemia in apoE-KO mice is associated with CC upregulation, we sought to analyze plasma samples from normocholesterolemic subjects and FH patients. We found statistically significant increases in total cholesterol, LDL-cholesterol and apoB in FH patients compared with controls (Table). Plasmas from 4 normocholesterolemic

subjects and 8 FH patients were simultaneously subjected to 2D-PAGE to identify and quantify protein spots displaying differential expression, as assessed using the PDQuest software. Protein spots exhibiting reproducible differential expression were excised from the gels, digested with trypsin and analyzed by MS/MS (Fig.2A). These analyses identified in FH patients increased intensity of spots containing peptides from the C4 γ chain, C1s and the C-terminal fragment of the α chain of C3b. Taken into account both the apparent molecular weight and the pI of the respective spots in the 2D gel, we conclude that these peptides upregulated in FH plasma represent C4 γ chain, C1s and C3c α chain, respectively. Consistent with the notion that C3-convertase cleaves C3 into C3a and C3b, we also observed by ELISA a significant increase in C3a level in FH plasma compared with normocholesterolemic controls (514 ng/mL versus 327 ng/mL, respectively, $p < 0.01$; $n = 14$) (Fig.2B). As in the apoE-KO mouse model (Fig. 1C), we found a significant direct correlation between plasma cholesterol and C3a level in controls and FH patients, as revealed by regression analysis ($p < 0.05$) (Fig.2C).

C3 and C3a, but not C1q or C4, induce the proliferation of cultured VSMCs and macrophages via Gi protein-dependent ERK activation

We next performed [³H]-thymidine incorporation assays to examine the effect of CCs on the proliferation of murine RAW macrophages and primary rat VSMCs. Increasing doses of C3, C3a, C4 and C1q were added to starvation-synchronized cultures. C3 up to 1 μ M and C3a up to 0.1 μ M elicited a dose-dependent mitogenic response in VSMCs (Fig. 3A) and macrophages (Fig.3F). In contrast, acylation stimulating protein/C3a-desArg, a desarginated form of C3a which is unable to bind to the C3a receptor (C3aR) [32], failed to induce de novo DNA synthesis in these cell types (Fig.3B, G). Likewise, the members of the classic complement pathway C4 and C1q did not induce VSMC and macrophage proliferation at concentrations up to 10 μ M (Fig.3C, D, H, I). Using the cell growth MTT-based assay, we confirmed the growth promoting

activity of C3, but not of C1q, on VSMCs (Fig. 3E) and RAW macrophages (Fig. 3J). None of the CCs tested in the proliferation assays affected the uptake of acetylated LDLs by RAW macrophages (data not shown).

Induction of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway plays a pivotal role in transducing signals required for cell proliferation [33]. Treatment of serum-starved RAW macrophages (Fig.4A) and VSMCs (Fig.4B) with C3a induced a rapid activation of the ERK isoforms of 44 and 42 kDa (ERK1 and ERK2, respectively), as revealed by Western blot using an antibody specific for their phosphorylated (active) forms (p-ERK1/2). This increase in phosphorylated ERK1/2 occurred without concomitant changes in total ERK1/2 protein expression. Neither C3a-desArg (Fig.4C) nor C1q (Fig.4D) caused ERK1/2 phosphorylation.

We next investigated whether ERK1/2 activation was required for C3/C3a-dependent cell proliferation. Preincubation of RAW macrophages with the MEK inhibitor PD98059 abrogated C3a-dependent ERK1/2 phosphorylation coinciding with a significant reduction in ³H-thymidine incorporation (Fig.4E, F). Similar results were obtained when these cultures were pretreated with PTX, an inhibitor of signaling mediated by heterotrimeric G proteins of the Gi subfamily (Fig.4E, F). PD98059 also blunted ERK1/2 phosphorylation and proliferation in starvation-synchronized VSMCs treated with 1 μ M C3 (data not shown), and the MEK inhibitor U0126 reduced ³H-thymidine incorporation in VSMCs exposed to C3 and C3a (Fig.4G). Collectively, these results suggest that ERK1/2 activation via Gi protein-dependent signaling is necessary for full C3/C3a mitogenic activity in macrophages and VSMCs.

C3 contributes to the increased mitogenic activity of plasma from FH patients

We sought to compare the mitogenic activity of control and FH plasma. Starvation-synchronized RAW macrophages were incubated for 14 hours with DMEM containing 3% control or FH

plasma. Treatment with either control or FH plasma significantly increased proliferation compared to untreated cells ($p < 0.001$, $n = 10$), although the mitogenic effect of FH plasma was higher ($p < 0.001$ versus control plasma) (Fig.5A). To evaluate whether C3 might contribute to the observed differential response, we immunoprecipitated control and FH plasma with anti-C3 IgG or control IgG and employed the resulting supernatant to carry out [3 H] thymidine incorporation assays. In agreement with the results in Fig.5A, both control and FH plasmas immunoprecipitated with control IgG induced cell proliferation compared with untreated cells ($p < 0.001$), and this response was stronger for FH plasma ($p < 0.01$ versus control plasma) (Fig.5B). Notably, whereas anti-C3 IgG did not affect the mitogenic activity of control plasma, cell proliferation induced by anti-C3-treated FH plasma was significantly reduced (Fig.5B).

Discussion

Whilst studies in animals and humans have demonstrated the upregulation of various CCs within advanced atherosclerotic lesions (see Introduction), studies in complement-deficient animals have yielded confusing results regarding the possible role on atherosclerosis of these compounds. In rabbits, C6 deficiency protected against diet-induced atherosclerosis without affecting plasma cholesterol levels and lipoprotein profiles [34]. However, atherosclerosis was similar in C5-deficient and C5-competent apoE-KO mice [35]. Likewise, serum lipid content and lesion size were comparable in LDLR-KO mice lacking C3, although immunohistological studies suggested that intact C3 may be necessary for atheroma maturation beyond the foam cell-rich stage [19]. It is noteworthy that C3 absence increased diet-induced hyperlipemia and atherosclerosis in the more atherogenic apoE/LDLR doubly deficient mouse model [17]. Very recently, Bhatia et al. [36] reported augmented atherosclerosis in C1qa/LDLR doubly deficient mice compared with

LDLR-KO mice, suggesting a protective role of C1qa against atherosclerosis. These seemingly conflicting results may reflect the complexity of the complement system, which comprises approximately 30 factors that may play partially redundant and/or opposite roles on cellular processes involved in atherosclerosis [6]. Since previous animal and human studies have focused on the analysis of advanced atheromas, the present study was undertaken to investigate the expression of CCs in atherosclerosis-prone apoE-KO mice fed with LF-diet and receiving HFC-diet for short (2-7 days) and long (30 days) periods, which caused comparable hypercholesterolemia. Using the same animal model, we previously found prominent aortic atheromas at 30 days of HFC-diet, but lesions were absent or just incipient after 2-7 days under this dietary regimen [31]. At all time points examined here (3-30 days HFC-diet), we found augmented C3 and C4 protein expression in the plasma of apoE-KO mice experiencing severe hypercholesterolemia (~400 mg/dL). Likewise, aortic expression of C3, C4, C1q and C1s mRNA expression was elevated at 30 days of HFC-diet. Notably, aortic upregulation of C3 and C4 mRNA steady-state level was already evident after 2 and 7 days of HFC-diet, respectively. In contrast, aortic CC mRNA expression was not elevated in fat-fed wild-type mice, which maintained a plasma cholesterol level <110 mg/dL even after 30 days of HFC-diet. Thus, only when associated with hypercholesterolemia, fat feeding produces in murine plasma and aorta a rapid upregulation of CCs which precedes atheroma formation and is sustained at later disease stages featuring prominent atherosclerotic lesions.

Consistent with our findings in hypercholesterolemic apoE-KO mice, we found increased plasmatic levels of human C3a, C4 γ chain, C1s and C3c α chain proteins in FH patients compared with normocholesterolemic subjects. High plasmatic C3 and C4 levels have been reported in individuals with primary hypercholesterolemia [37], and increased circulating level of C5b-9, a clear indicator of complement activation, is associated with human dyslipidemias, including hypercholesterolemia, hypertriglyceridemia and low HDL-cholesterol level [38]. These

findings are consistent with the observation that cholesterol, modified LDLs, and chylomicrons represent complement-activating lipids [6,7,39,40].

We found that C3 and C3a, but not C3a-desArg, C4 and C1q, induced in vitro macrophage and VSMC proliferation, a key event in early stages of atheroma development [3]. The C3aR is a member of the G-protein-coupled receptor family [41]. Many biological responses of C3a can be blocked by PTX, suggesting that C3aR is coupled to Gi proteins [42,43]. However, C3aR is coupled to both PTX-sensitive $G_{i\alpha}$ and PTX-insensitive $G_{\alpha 16}$ in leukocytes [44], and C3aR activation in endothelial cell is sensitive to Rho inhibitors, implying that this receptor can signal through distinct G proteins in different cell types [45]. Recently it was reported that C3aR-dependent signaling leads to PTEN activation in both intrinsic renal cells and extrinsic inflammatory cells [46]. Our studies in macrophages and VSMCs demonstrate C3/C3a-dependent activation of ERK1/2, a signaling pathway required for cell proliferation induced by numerous mitogens [33]. Moreover, blockade of ERK1/2 activation by treatment with PTX, PD98059 or U0126 blunted de novo DNA synthesis in cells exposed to C3/C3a. Previous studies in human vein endothelial cells have shown ERK1/2 activation by C3a and C5a [42], and C5b-9-dependent proliferation of human aortic endothelial and smooth muscle cells [47,48]. Moreover, C3 may contribute to the exaggerated growth of VSMCs from spontaneously hypertensive rats at an age preceding significant increases in blood pressure [49]. Thus, rapid complement activation within the artery wall may contribute, at least in part through Gi protein-dependent ERK1/2 activation, to abnormal cell proliferation at the onset of atherosclerosis and hypertension. Our observation that, compared with normocholesterolemic plasma, C3-enriched FH plasma elicited a stronger mitogenic response in macrophages and that this difference was abolished upon addition of anti-C3 antibodies to the plasma, suggests that high circulating C3 level in FH patients may contribute to atherosclerosis by facilitating neointimal cell proliferation. However, additional studies are required to test this possibility. C3a and C5a evoke chemoattraction of

leukocytes [50-52], suggesting an additional mechanism by which CCs may promote atheroma growth.

In conclusion, the present study demonstrates that severe hypercholesterolemia is associated with a concerted upregulation of CCs in the plasma and aorta of fat-fed apoE-KO mice and in the plasma of FH patients. Our in vitro findings suggest, however, that different CCs play distinct roles on cellular processes involved in atheroma formation, since C3 and C3a, but not C1q and C4, promoted VSMC and macrophage proliferation via Gi protein-dependent ERK activation. We found that C3 induction in aorta and plasma of fat-fed apoE-KO mice occurs at early stages of hypercholesterolemia preceding atheroma formation. Thus, given the evidences presented here suggesting that C3 contributes to the mitogenic activity of FH plasma, we propose that chronic elevation of plasma C3/C3a level in dyslipidemic subjects may contribute to atheroma development by inducing neointimal cell proliferation.

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FIGURE LEGENDS**Fig.1. Increased expression of CCs in aortic tissue and plasma of fat-fed apoE-KO mice.**

Wild-type and apoE-KO mice fed with LF-diet or HFC-diet for the indicated times were sacrificed to obtain aortic tissue and plasma. **(A)** CC mRNA levels in aortic tissue were determined by real-time RT-PCR. For each gene, results are represented relative to the RNA level of mice receiving LF-diet (set as 1) (n=4-6 RNA samples obtained from aortic tissue pooled from 2-6 mice). **(B)** Plasmatic levels of C3, C4 and total cholesterol (n=8-10). **(C)** Simple regression analysis of the data presented in panel B for discerning relationships between plasmatic levels of cholesterol and CCs in apoE-KO mice. The F-test was used to measure the significance of the regression analysis.

Fig.2. Increased levels of CCs in plasma of FH patients. **(A)** Left upper panel, 2D-electrophoretic separation of FH patient plasma indicating the positions of CC spots whose volume was found to be increased in comparison to controls. Displayed are the MS/MS-derived amino acid sequences of the tryptic peptide ions that served to identify the proteins listed at the right. Panels C1s, C3, and C4 show close-up views of equally-labeled regions in the 2D-gel and illustrate the differential expression levels of CC bands in controls and FH patients from both sexes. The graphs represent the fold induction in FH patients (4 females/4 males) relative to average control levels (2 females/2 males) (set as 1). **(B)** Plasmatic C3a levels determined by ELISA in control subjects (14 females) and FH patients (8 females/6 males). **(C)** Simple regression analysis of the data presented in panel B for discerning relationships between plasmatic levels of cholesterol and C3a. The F-test was used to measure the significance of the regression analysis.

Fig.3. Effect of CCs on the proliferation of primary VSMCs (A-E) and RAW macrophages (F-J). (A-D, F-I) Confluent cells were serum-starved for 48 hours and then stimulated for 24 hours with CCs. In B and G, the amount of C3a and C3a-desArg was 0.01 μ M. De novo DNA synthesis was assessed as 3 H-thymidine incorporation (n=3-4 experiments). Results are shown relative to unstimulated cells (set as 100%). Comparisons versus unstimulated cells: *, p<0.05; **, p<0.01; ***, p<0.001. Comparisons versus C3a-desArg: †, p<0.01; ††, p<0.001. (E, J) MTT assay-based growth curves of cells incubated for different times in DMEM F12 media supplemented with 1 μ M C3 (black squares) or 1 μ M C1q (white squares). The values obtained in control cells incubated with PBS were subtracted from those of CC-treated cells. Comparisons versus t=0 (same treatment): *, p<0.0004; **, p<0.0001. Comparisons C3 versus C1q (same time point): †, p<0.0025; ††, p<0.0001. The discontinuous lines show the p value for other relevant comparisons.

Fig.4. ERK1/2 activation is required for C3a-induced RAW and VSMC proliferation. When indicated, CCs were added to culture plates at 0.01 μ M (C3, C3a, C3a-desArg) and 1 μ M (C1q) and cells were preincubated with PD98059 (5 μ mol/L, 1 hour), PTX (0.5 μ g/mL, overnight) or U0126 (10 μ mol/L, 1 hour) prior to addition of CCs. Western blots are representative of 2-3 experiments. The results of 3 H-thymidine incorporation assays are shown relative to controls (=100%) and represent the mean \pm SEM of 3-4 experiments. Confluent RAW macrophages (A, C) and VSMCs (B, D) were serum-starved for 48 hours and left untreated (control) or incubated with CCs (2, 20 and 60 minutes in A and B; 20 minutes in C, D). Cell lysates were analyzed by Western blot with antibodies against total and phosphorylated (active) ERK1/2 (ERK1/2 and p-ERK1/2, respectively). (E, F) 3 H-thymidine incorporation and Western blot of serum-starved confluent RAW macrophages stimulated for 24 hours (E) or 20 minutes (F) with C3a, with or

without PTX or PD98059. (G) Effect of U0126 on ^3H -thymidine incorporation in starvation-synchronized VSMCs stimulated for 24 hours with C3 or C3a.

Fig.5. C3 contributes to the increased mitogenic activity of plasma from FH patients. RAW macrophages were serum starved for 48 hours and then restimulated for 20 hours with 3% human plasma. Cells were pulse-labeled with ^3H -thymidine for the last 4 hours and harvested to estimate de novo DNA synthesis. Results are represented relative to ^3H -thymidine incorporation in untreated cells (set as 100%). (A) Mitogenic activity was assayed in untreated cells (n=10) and in cells incubated with plasma from 10 controls and 10 FH patients. (B) Mitogenic activity was assayed in untreated cells (n=4) and in cells incubated with the supernatant of control or FH plasma obtained after immunoprecipitation with either anti-C3 IgG or control IgG (n=4 independent pools obtained by mixing plasma from either 5 control subjects or 5 FH patients).

Table: Characteristics of control and FH patients

	n	Age (years)	Weight (Kg)	Total cholesterol (mg/dL)	HDL- cholesterol (mg/dL)	LDL- cholesterol (mg/dL)	Triglycerides (mg/dL)	apoB (mg/dL)	apoAI (mg/dL)
Control	19	29.8±3.0	69.5±2.9	188.4±6.0	44.1±2.5	129,3±5.8	74.7±7.6	76.5±3.8	136.5±5.2
Females	6	25.3±2.0	58.8±2.5	190.2±7.4	57.2±1.0	119,8±6.2	66.0±11.9	67.2±5.5	156.3±9.0
Males	13	31.9±4.3	74.4±3.3	187.5±8.2	38.1±1.8	133,7±7.7	78.8±9.8	80.8±4.7	127.3±4.6
FH patients	14	36.4±2.1	69.5±3.3	328.8±15.4	47.6±4.4	261,3±15,7	99.1±13.2	178.8±9.1	132.6±8.6
Females	8	34.8±3.3	64.8±5.1	312.5±20.1	56.5±5.4	240,8±18,3	76.1±11.2	164.3±7.3	152.3±7.7
Males	6	38.7±2.5	75.8±2.2	350.5±22.9	35.8±3.3	288,7±24.5	129.8±22.1	198.2±16.6	106.5±9.7
t-test (Control vs FH)		NS	NS	p<1x10⁻⁹	NS	p <6x10⁻¹⁰	NS	p<1x10⁻¹¹	NS

NS: not significant (p>0.05)

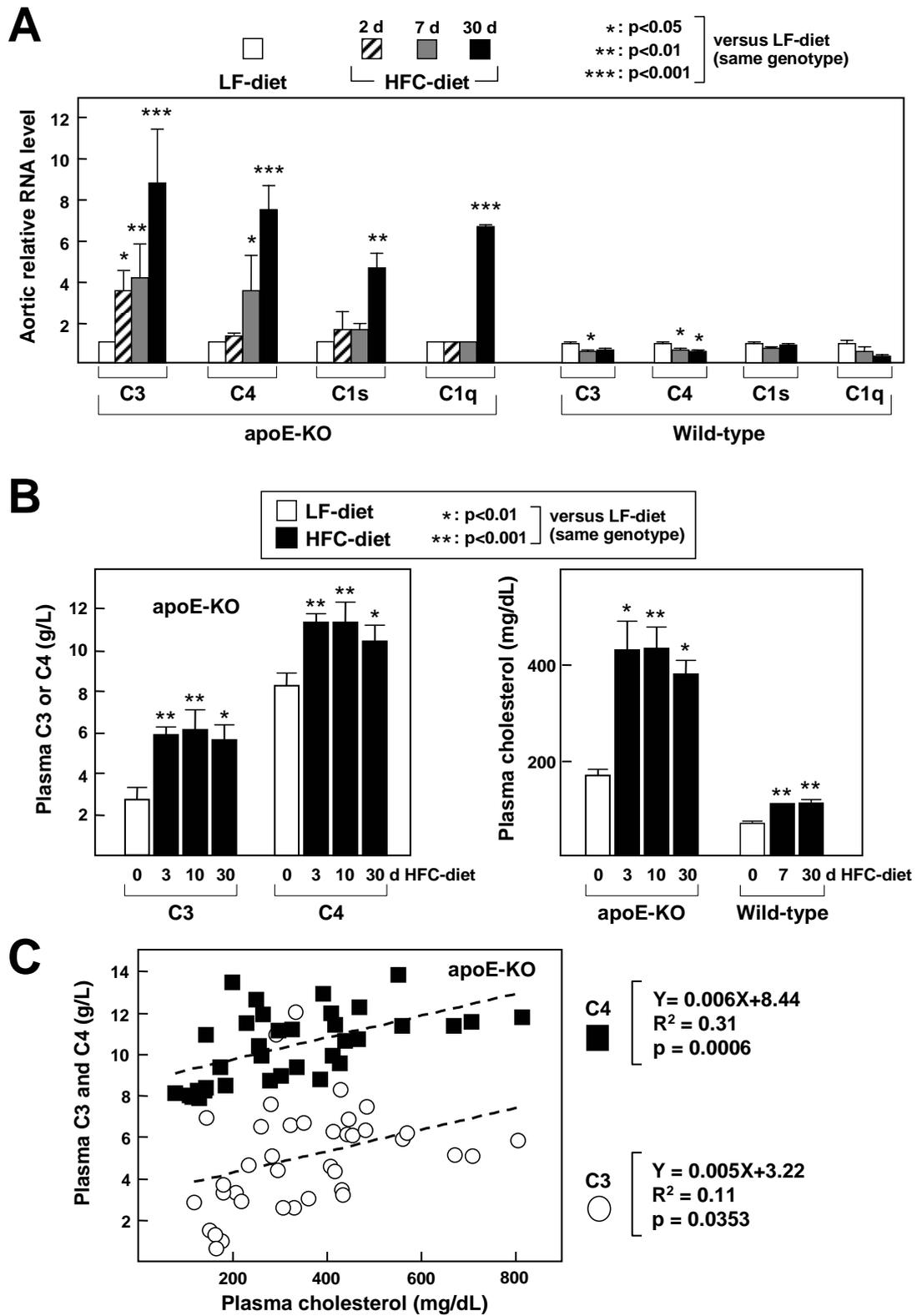
FIGURE 1

FIGURE 2

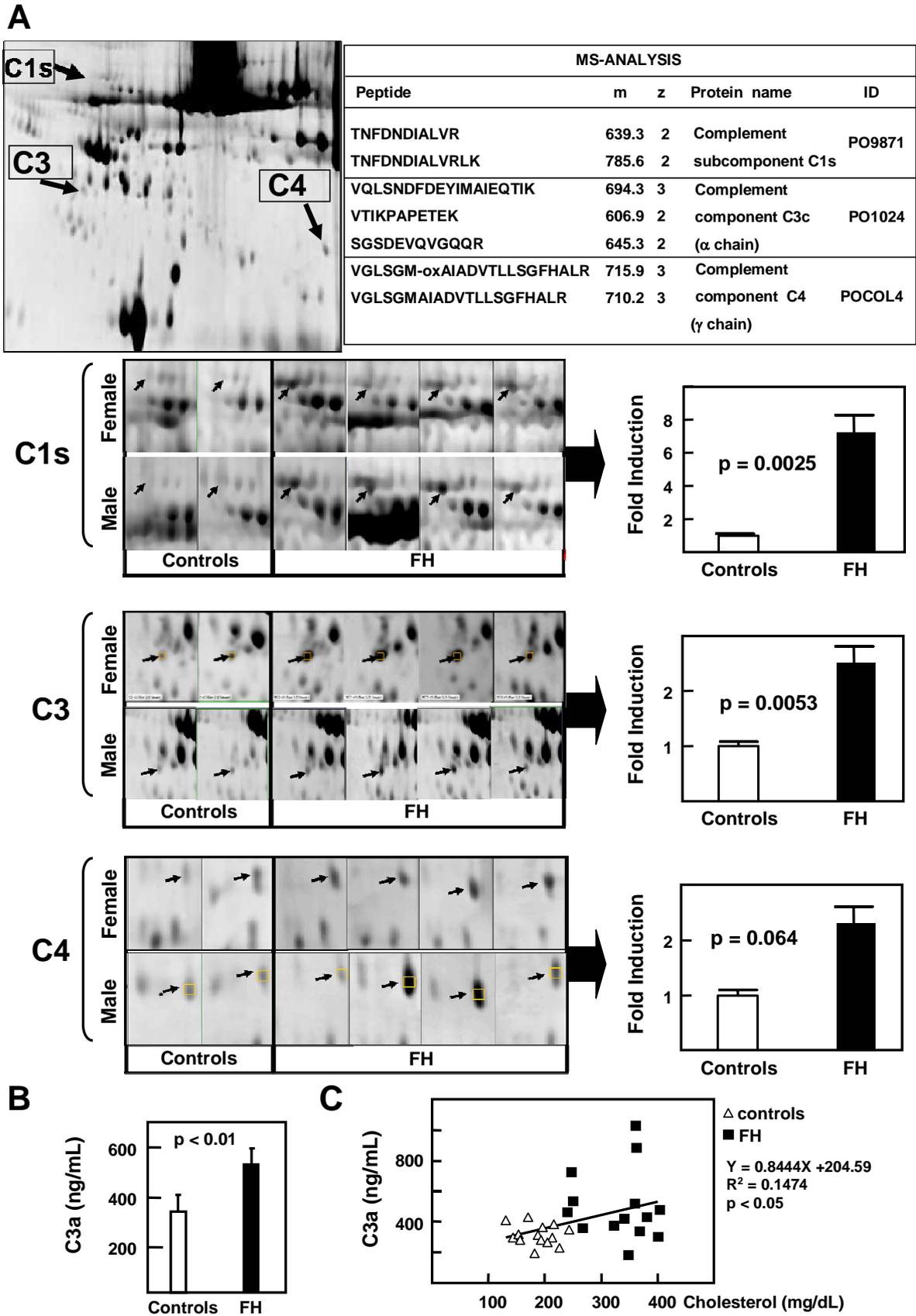


FIGURE 3

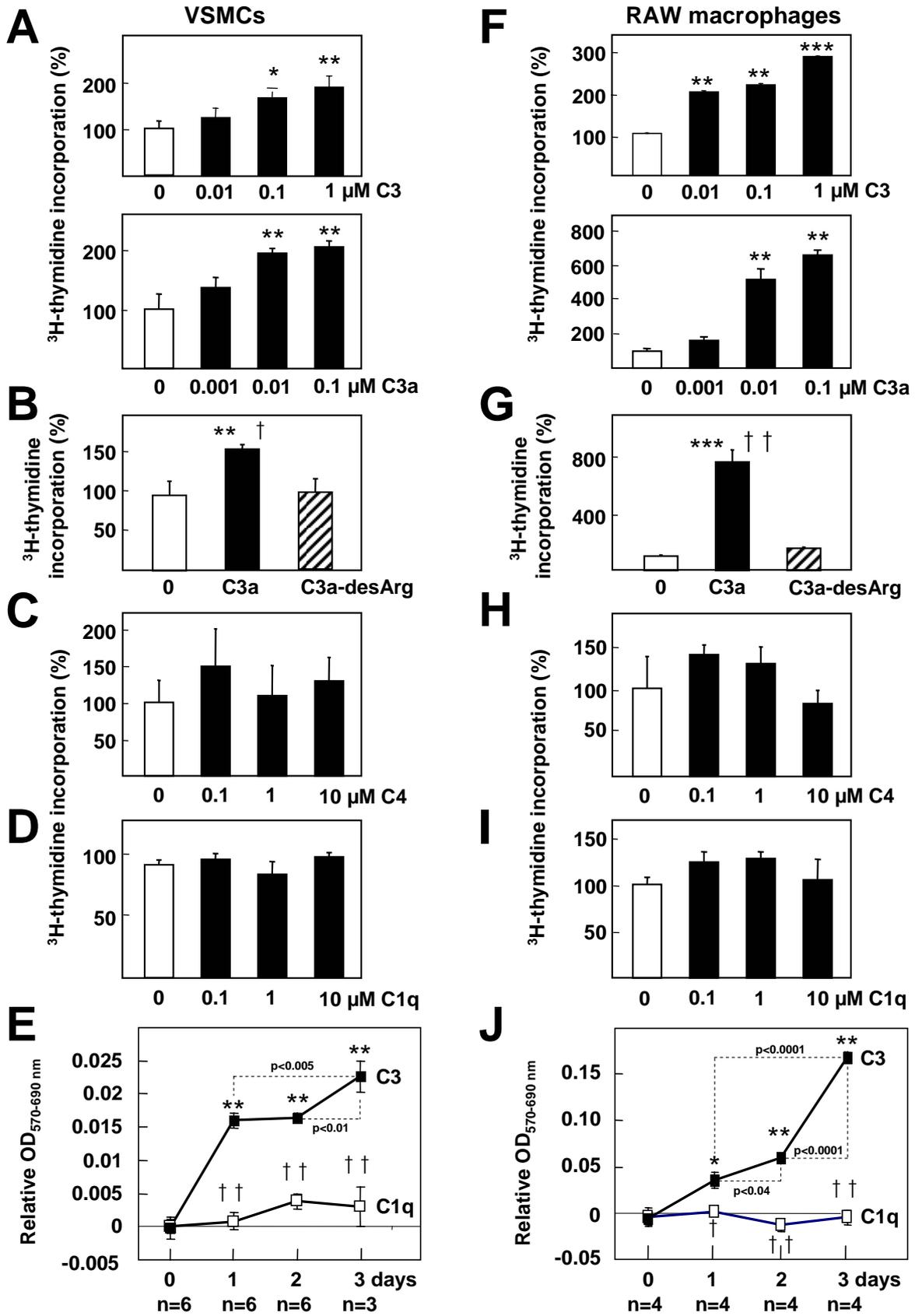


FIGURE 4

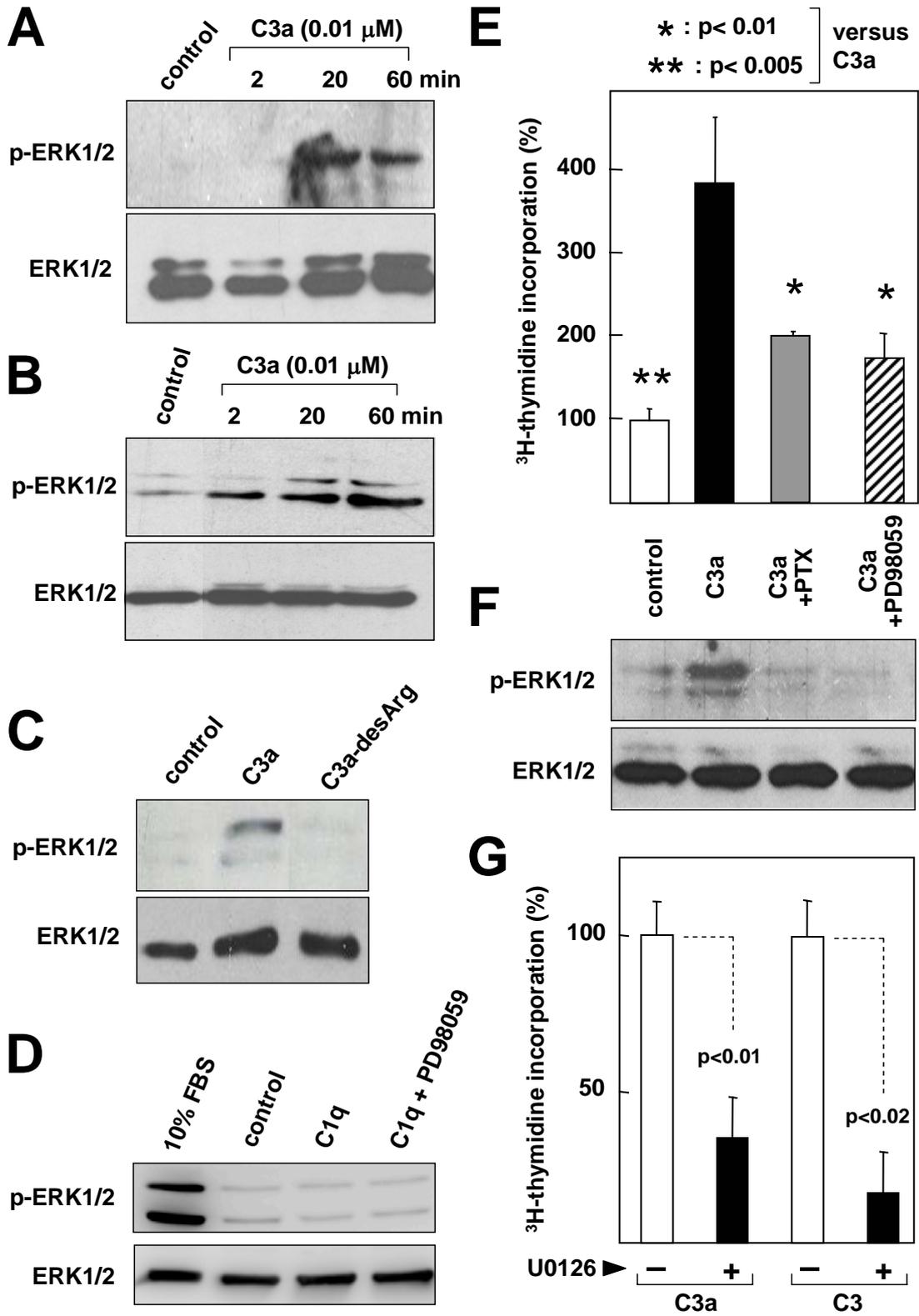
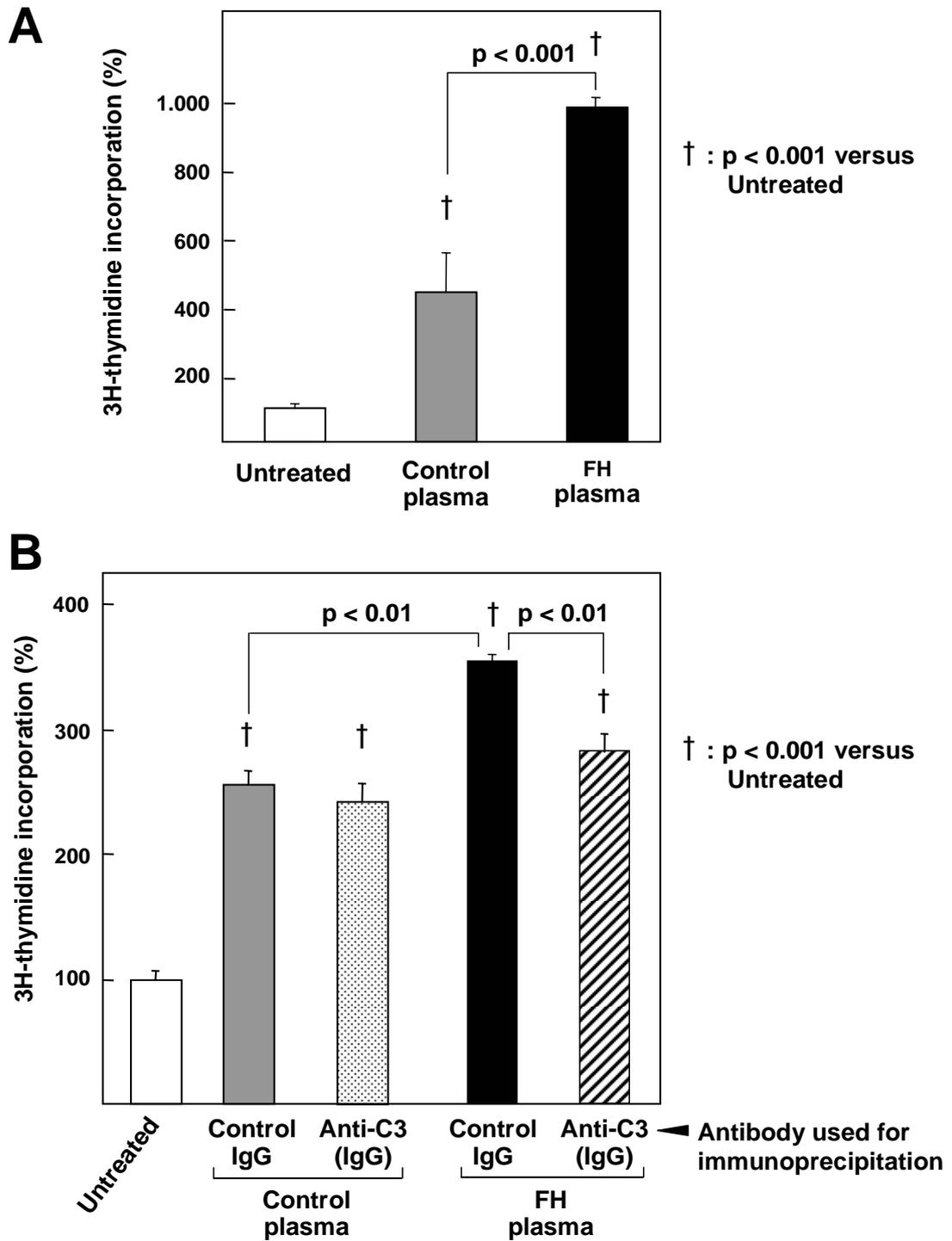


FIGURE 5



SUPPLEMENTARY MATERIAL

METHODS

Genotyping of FH patients

Genomic DNA from individuals with a clinical diagnosis of FH was screened for the presence of mutations in the LDL receptor gene (*LDLR*) using the Lipochip® microarray [1]. When no mutations were detected using this microarray, the promoter region and all the 18 exons and exon-intron boundaries of the *LDLR* gene were sequenced to search for additional mutations [2]. Large rearrangements in the *LDLR* gene were analyzed using a method based on quantitative fluorescent multiplex PCR [3]. The presence of mutations within the putative LDLR-binding region of the *APOB* gene was screened as previously described [4]. Twelve different *LDLR* mutations were identified among the FH subjects included in this study: 7 missense mutations (S156L, V408M, D630N, C255G, D157N, C297F and G314V), 3 splicing mutations (1358+1G>C, 1706-10G>A and 2140+5G>A), 1 nonsense mutation (Q427X), and 1 deletion including exons 3 to 5. Moreover, one FH subject carried a R3500Q mutation in the *APOB* gene.

Proliferation assays

For proliferation assays using the MTT-based Cell Growth Determination Kit (Sigma), RAW macrophages and rat VSMCs were seeded in 24-well plates at 1.5×10^4 cells/well and starved for 48 hours in DMEM F12 containing 0.5 % FCS. Cells were stimulated with CCs or PBS. Fresh media with CCs was added every 24 hours. At the indicated times, cells were incubated for 3.5 hours with 10 % MTT solution in DMEM medium without phenol red. After washing with PBS, the MTT formazan was dissolved in MTT solvent and absorbance at 570 nm was measured. The background absorbance at 690 nm was subtracted from the 570nm absorbance values.

For cell proliferation assays based on [³H]-thymidine incorporation, 2×10^4 cells/well were seeded in 24-well plates. Cultures were serum-starved for 48 hours when they reached ~80% confluence, and then were preincubated with pertussis toxin (PTX, 0.5 µg/mL, overnight, Calbiochem), PD98059 (5 µM, 1 hour, Tocris) or U0126 (10 µM, 1 hour, Promega) prior to 24 hours of treatment with human CCs (C3, Ref.204885; C3a, Ref.204881; C3a-desArg, Ref.204884; C1q, Ref.204876; C1s, Ref. 204879; C4,

Ref.204886; Calbiochem) in the presence of 1 mCi/L [³H]-thymidine (Amersham Biosciences) during the last 4 hours. After washing with PBS, DNA was precipitated with 10% trichloroacetic acid and solubilized with 0.2M NaOH/0.1% SDS. Radioactivity incorporated into DNA was measured in a scintillation counter (Wallac).

The mitogenic activity of human plasma on RAW macrophages was determined by [³H]-thymidine incorporation. Four samples of human plasma were prepared by pooling plasma from five controls or FH patients. When indicated, samples were incubated at 4 °C for 6 hours either with a 1:25 dilution of mouse monoclonal anti-C3 antibody (sc-28294, Santa Cruz Biotechnology) or control isotype IgG (Calbiochem), followed by incubation with protein A/G PLUS-Agarose (sc-2003, Santa Cruz Biotechnology) under gentle agitation for 40 minutes at room temperature. Immunoconjugates were precipitated by centrifugation (5 minutes, 1000g, 4 °C) and the supernatant was used for proliferation assays.

Western blot

Primary rat VSMCs and murine RAW macrophages were grown in 10 mm dishes: When reaching ~90% confluence, cells were serum-starved for 48 hours, then stimulated with CCs or 10% FBS for various times as indicated. When PTX or PD98059 were used, they were applied prior to the addition of CCs (see above). Cells were lysed by three consecutive cycles of vortex/freezing/thawing at 37 °C. Lysis buffer contained 50 mM HEPES (pH=7.5), 150 mM NaCl, 2.5 mM EGTA, 10 mM glycerophosphate, 10% glycerol, 0.1% Tween 20, 1mM DTT, 0.1 mM NaOV₃, and protease inhibitor CompleteTM Mini-Mixture (Roche Molecular Biochemicals). Total protein (40-60 µg) was electrophoresed on 12% SDS-PAGE to perform immunoblot using the following primary antibodies from Santa Cruz Biotechnologies: anti-pERK1/2 (1:100, reactive with phosphorylated ERK1 and ERK2; ref. sc-7383), and anti-ERK2 (1:300, reactive with ERK2 and to a lesser extent with ERK1; ref. sc-154). After washes and incubation with species-specific secondary antibodies, immunocomplexes were visualized with the ECL Plus Kit (Amersham).

Proteomic studies

Plasma from controls and FH patients was precipitated with three volumes of cold acetone (-20 °C overnight) and resuspended in 7M urea/2M thiourea/2% CHAPS/0.2% ampholytes/100mM DTT/10mM iodoacetamide with protease inhibitors (Amersham). Total protein (400-500 µg) was rehydrated in IPG strips (pH4-7, ref. 17-6001-10, Amersham) (no

voltage overnight, 1.5 hours at 30V and 1.5 hours at 50V) and separated by isoelectric focusing using an Invitrogen Mini Cell (250V - 20 min, 450V - 20 min, 750V - 20 min, and 2000V - 60 min). For the second dimension, strips were subjected to 12% SDS-PAGE. Gels stained with 10% Coomassie Brilliant Blue (1 hour) were scanned (ImageScanner, Amersham) and analyzed with the PDQuest software (BioRad). Selected spots were excised from the gels and submitted to automated reduction (10 mM DTT) and carbamidomethylation (100 mM iodoacetamide), followed by digestion with sequencing grade bovine pancreatic trypsin (Roche) using a ProGest digester (Genomic Solutions). Tryptic peptide mixtures were dried in a Speed-Vac (Savant) and redissolved in 5 μ l of 70% acetonitrile/0.1% trifluoroacetic acid (TFA). Digests (0.65 μ l) were spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of α -cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile containing 0.1% TFA, air-dried, and analyzed with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer operated in delayed extraction and reflector modes. A tryptic peptide mixture of *Cratylia floribunda* seed lectin (SwissProt accession code P81517) prepared and previously characterized in our laboratory was used as mass calibration standard (mass range, 450-3300 Da). For peptide sequencing, the protein digest mixture was loaded in a nanospray capillary column and subjected to electrospray ionization mass spectrometric analysis using a QTrap mass spectrometer (Applied Biosystems) equipped with a nanospray source (Protana). Doubly- or triply-charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were analyzed in Enhanced Resolution MS mode and the monoisotopic ions were fragmented using the Enhanced Product Ion tool with Q_0 trapping. Enhanced Resolution was performed at 250 amu/s across the entire mass range. Settings for tandem mass spectrometry (MS/MS) experiments were as follows: Q1-unit resolution; Q1-to-Q2 collision energy-30-40 eV; Q3 entry barrier-8 V; LIT (linear ion trap) Q3 fill time-250 ms; and Q3 scan rate-1000 amu/s. CID spectra were interpreted manually or using the on-line form of the MASCOT program (<http://www.matrixscience.com>).

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