Evidence for effect of mutant PCSK9 on apolipoprotein B secretion as the cause of unusually severe dominant hypercholesterolaemia

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Typically, autosomal dominant familial hypercholesterolaemia (FH) is caused by mutations in the low density lipoprotein (LDL) receptor or apolipoprotein B genes that result in defective clearance of plasma LDL by the liver, but a third gene (PCSK9), encoding a putative proprotein convertase, has recently been implicated. Two independent microarray studies support a role for PCSK9 in sterol metabolism and adenoviral-mediated over-expression of PCSK9 in mouse liver depletes hepatic LDL-receptor protein, but the mechanism by which dominant mutations cause human FH is unclear. We have identified the D374Y mutant of PCSK9 in three FH families of English origin; all 12 affected individuals have unusually severe hypercholesterolaemia and require more stringent treatment than typical FH patients, who are heterozygous for defects in the LDL receptor. We have stably expressed wild-type (WT) and variant PCSK9 in McArdle-7777 rat hepatoma cells and shown by confocal microscopy that all forms of PCSK9 co-localize with protein disulphide isomerase in the ER whether or not they can be autocleaved. Expression of the proposed pathogenic variants, but not of WT, S386A or F216L PCSK9, increases secretion of apolipoprotein B100-containing lipoproteins from the cells by 2–4-fold probably by reducing the degradation of nascent protein; no differences in LDL-receptor content were observed in cells expressing WT, S386A or F216L PCSK9 and only a small reduction in cells expressing the D374Y or S127R mutants. This suggests that the variants of PCSK9 found in FH influence the secretion of apoB-containing lipoproteins, providing an explanation for the marked increase in circulating LDL in heterozygous carriers.

INTRODUCTION

Familial hypercholesterolaemia (FH) is an autosomal dominant disorder usually caused by mutations in the low density lipoprotein (LDL)-receptor gene (LDLR) that result in defective clearance of plasma LDL by the liver. Heterozygous FH occurs with a frequency of about 1/500 and is characterized by increased plasma LDL cholesterol that causes cholesterol deposition as tendon xanthomas and atheroma (1). As a result of this, FH patients are at markedly increased risk of premature arteriosclerosis and coronary heart disease (CHD), but this can be reduced by rigorous treatment of the disorder with cholesterol-lowering therapy (2). A similar phenotype is seen in patients with familial defective apolipoprotein B, where a mutation in the gene for apolipoprotein B (APOB), the ligand for the LDL receptor, impairs its uptake via LDL receptors. However, it has not been possible to find a mutation in the LDLR or APOB in all patients with a typical heterozygous FH phenotype (3) and, depending on the rigour of the diagnosis and the genetic analysis carried out, 15–40% of patients with a clinical phenotype of heterozygous FH do not appear to have a mutation in the LDLR or carry the APOB R3500Q mutation. In some cases, it has been possible to exclude co-segregation of an allele of the LDLR or APOB (3,4). Inactivating mutations in ARH, the gene for an adaptor protein required for internalization of the LDL receptor by cells, have recently been shown to be a third genetic cause of severe hypercholesterolaemia; this disorder is inherited as...
an autosomal recessive, rather than dominant trait, and is now named ARH (5,6). Although there is evidence of mild hypercholesterolaemia in a few heterozygous carriers, mutations in ARH are unlikely to be a common underlying cause of dominant hypercholesterolaemia (7).

Two independent linkage studies in single large families, one French (8) and the other from Utah (9), revealed a new locus for dominant hypercholesterolaemia on chromosome 1p32 and heterozygous missense variants in a candidate gene in this region have since been found, which co-segregate with hypercholesterolaemia in three out of 24 French families tested including the one in which linkage was first established (10). The gene, proprotein convertase subtilisin/kexin9 (PCSK9), encodes a putative protease that has also been named apoptosis-regulated convertase (NARC-1) (11); the variants found are predicted to result in single amino acid substitutions (S127R in two families and F216L in the third). Another variant (D374Y) of PCSK9 was subsequently found in the Utah family (12) and this same variant has also been found in three Norwegian FH families (13).

The PCSK9 gene product was first identified as a protein whose expression was increased in neuronal cells after injuries that induce apoptosis (14). It is a member of the subtilisin-like proprotein convertase family of enzymes that are largely involved in the processing of inactive precursor proteins to the active product (15); although its catalytic domain is similar to that of proteinase K, it appears to form a new family of such enzymes (11,16). Proprotein convertases are frequently synthesized as inactive precursors that catalyse autocleavage of an N-terminal prodomain to form an active enzyme, with the prodomain often functioning as an intracellular chaperone and/or inhibitor until the active enzyme reaches its site of action (17). In keeping with this, PCSK9 expressed in heterologous cells in vitro has the ability to autocleave its own propeptide, but the role of its prodomain and the physiological substrates for its proteolytic activity remain unknown (11,16). In the rat, PCSK9 mRNA is found in liver and small intestine, more so in the ileum than in the jejunum, and to a lesser extent in lung, spleen, kidney, testis and thymus (11).

Although the physiological function of PSCK9 is unknown, strong indirect support for its involvement in cholesterol homeostasis has come from two microarray analyses of gene expression in mice (18,19). These studies showed that PCSK9 mRNA levels in the liver were markedly reduced in mice fed with a cholesterol-rich diet and were increased several-fold in transgenic mice over-expressing sterol response element-binding proteins, transcription factors that regulate the expression of genes involved in cholesterol and fatty acid biosynthesis (20); it has also been shown that cholesterol depletion of cultured human hepatocytes and HepG2 cells increases PCSK9 mRNA (21). Adenovirus-mediated over-expression of wild-type (WT) PCSK9 in mice was reported to cause severe hypercholesterolaemia by reducing the amount of LDL-receptor protein, but not mRNA, in the liver (21). This has subsequently been confirmed in a second study, in which over-expression of two naturally occurring mutant forms of PCSK9 was also found to reduce LDL-receptor protein content of the liver; an engineered mutant with a substitution in one of the catalytic triad had no effect suggesting that the catalytic activity of PCSK9 was involved (22). From these observations, it was deduced that the protein may normally play a role in the regulation of the LDL receptor, but it is not easy to explain how or why dominant missense mutations in human subjects would produce the same effect as over-expression of the WT protein in mice. Furthermore, it has been suggested that over-expression of proprotein convertases may result in spurious activity and sheds little light on their true physiological function (23).

Thus, a different mechanism may underlie the hypercholesterolaemia in the patients. Indeed, measurement of lipoprotein turnover in two patients heterozygous for the S127R variant of PCSK9 has suggested that the disorder is associated mainly with overproduction of apolipoprotein B, especially of LDL (24). PCSK9 mRNA is highly expressed in liver and intestine, major sites of lipid and lipoprotein synthesis in vivo. As discussed in two recent editorials, clearly much remains to be explained about the function and malfunction of PCSK9 (25,26).

In this study, we describe three families, comprising 12 affected individuals, in whom the D374Y variant of PCSK9 co-segregates with an unusually severe form of hypercholesterolaemia that is difficult to treat and is associated with very early-onset premature CHD. We have investigated whether PCSK9 is expressed in Epstein–Barr virus (EBV)-transformed lymphocytes and whether expression and regulation of the LDL receptor are affected in cells from these patients. We have also studied the processing and intracellular localization of normal and variant human PCSK9 proteins and their effect on apoB secretion in McArdle-RH7777 (McA) cells, a rat hepatoma cell line that has been used extensively to study the synthesis and secretion of apoB-containing lipoproteins (27).

RESULTS
Identification of genetic variants in PCSK9

DNA from a group of 25 patients attending the Hammersmith Hospital Lipid Clinic, with a clinical diagnosis of definite heterozygous FH based on the Simon Broome Register criteria (28), was examined for variants of PCSK9 and ARH. These patients were selected solely on the basis that we had been unable to find a mutation in the LDLR and none of them carried the common APOB mutations. They comprised seven women and 18 men with a mean age of 31.9 ± 11.7, all apparently unrelated, with a mean plasma cholesterol value of 10.7 ± 12.9; 15/25 had CHD and 16/25 had tendon xanthomas. Thus, these patients exhibited the usual range of phenotypic variation found in heterozygous FH in the UK (28). The entire coding regions of PCSK9 and ARH together with at least 50 bp of intronic sequence flanking each exon were amplified from genomic DNA and sequenced. No potentially functional variants of ARH were observed, but the same single base pair substitution in PCSK9 (G1120T) was found in two patients, which was predicted to change Asp374 to Tyr (D374Y). Analysis of family members of each patient showed that the base change, which introduces a new site for the restriction enzyme AluI, co-segregated with severe hypercholesterolaemia. Affected individuals in a third family
in which the proband attended a Lipid Clinic in Liverpool were also found to be heterozygous for the same variant (Fig. 1A–C); the index patient in this family had been referred to us because he was ‘difficult to treat’ and had very severe phenotype. The D374Y variant was not found in 86 control subjects from the general UK population, and both the aspartate residue that is predicted to be substituted and the region in which it lies are highly conserved among human, rat and mouse PCSK9.

**Screening of additional FH patients**

Because the D374Y variant has now been detected in Norway and the UK, as well as in a Utah family that is also likely to be of northern European origin, we wondered if it might be relatively common in the UK. However, screening of a further 110 heterozygous FH patients whose genotype had not previously been analysed in detail failed to reveal any further carriers. We also failed to detect any carriers of the S127R or F216L variant, which have been observed in French families.

**Clinical characteristics of D374Y carriers**

The clinical characteristics of the 12 carriers of the D374Y variant of PCSK9 are summarized in Table 1. In several respects, the clinical phenotype in these three families appears to be different from that of typical heterozygous FH patients attending the Hammersmith Hospital Lipid Clinic. For example, despite their relatively young age (mean age 21.2, range 2–57 years), the untreated mean plasma total cholesterol in the 12 carriers was significantly higher than that in 40 of our most severely affected FH patients with known LDLR mutations (mean age 51 ± 9), selected for having either coronary artery disease (28) or ‘refractory’ hypercholesterolaemia (29) (13.8 ± 2.8 versus 10.8 ± 1.6 mmol/l, \( P < 0.002 \) in Mann–Whitney test). However, it should be noted that this comparison is based on a small number of individuals with this rare genetic disorder. In addition, in our experience, long-term follow-up of the patients with the D374Y variant of PCSK9 over 12–29 years showed that improved control of their hypercholesterolaemia only became possible with the advent of the potent statins (e.g. atorvastatin or rosuvastatin), given at high doses and in combination with ezetimibe, a cholesterol absorption inhibitor (Table 1). Perhaps as a result of this, the affected individuals appear to be rather susceptible to premature coronary disease; for example, in two of the three families shown in Figure 1, affected women died of premature CAD aged 31 (II,2 in family 2) and 30 (II,2 in family 3). In a previous study, analysis of mortality in ‘typical’ FH in the UK revealed only two deaths in 1190 person years in British women aged 20–39 (2). Although most of our patients were APOE3/4, which could explain some of their increased risk of CAD, one equally severely affected individual was APOE3/3 (Table 1).

**Regulation of LDL-receptor expression in lymphocytes from PCSK9 patients**

Analysis of total RNA from EBV-transformed lymphocytes by RT–PCR revealed that PCSK9 mRNA was present, but at a much lower level than that in rat hepatoma cells (McA) relative to 18S rRNA (Fig. 2A). There was no apparent difference in the level of PCSK9 mRNA expression in cells from normolipaemic controls and in cells from two different hypercholesterolaemic carriers of the D374Y variant in PCSK9.

We next examined the regulation of LDL-receptor expression by immunoblotting of extracts of EBV lymphocytes with specific antibodies. In extracts of cells incubated with 10% serum, the level of LDL-receptor protein relative to the housekeeping protein \( \gamma \)-tubulin was very low, but appeared to be similar in cells from normolipaemic controls and from patients who were heterozygous for D374Y in PCSK9; in contrast, cells from heterozygous FH patients with null mutations in the LDLR contained significantly less LDL-receptor protein (Fig. 2B). Pre-incubation of the cells with lipoprotein-deficient serum (LPDS) and compactin up-regulated LDL-receptor expression equally well (~5-fold) in control cells and in cells from D374Y carriers (Fig. 2C), suggesting that sterol-mediated SREBP-dependent regulation is intact in these cells. Thus, we have no evidence to suggest that expression or regulation of LDLR mRNA or protein in EBV lymphocytes from patients is influenced by the presence of the PCSK9 D374Y variant. However, the level of expression of PCSK9 in these cells is low and does not, of course, exclude that the variants may influence LDL-receptor expression in the liver.
Activity of human PCSK9 protein expressed in rat liver cells

To investigate whether known human variants of PCSK9 (S127R, F216L and D374Y) had autocalytic activity, we expressed them in rat hepatoma cells in culture; in addition, we expressed a construct in which the serine residue in the

Figure 2. Relative expression of PCSK9 mRNA and LDL-receptor protein in EBV lymphocytes from patients. (A) McA cells and EBV lymphocytes from three control subjects (CON 1–3) and from two different D374Y carriers (D374Y-1, individual II,2 in family 1; D374Y-2, individual II,2 in family 2), as indicated below each lane, were pre-incubated for 16 h in medium containing 10% LPDS. Total mRNA was isolated and analysed by semi-quantitative RT–PCR with primers specific for PCSK9 (top) and 18S rRNA (below); PCR products were fractionated on 2% agarose gels stained with ethidium bromide. (B) EBV lymphoblasts from a normolipemic control subject, PCSK9 patients or heterozygous FH patients with known LDLR mutations were pre-incubated for 18 h in medium containing 10% (v/v) LPDS and total cell extracts (20 μg of protein/lane) were analysed by non-reduced SDS–PAGE and immunoblotting with rabbit anti-LDL receptor (LDLR) and mouse anti-γ-tubulin. Bound antibody was detected with peroxidase-labelled anti-rabbit or anti-mouse IgG and chemiluminescence; exposure was for ~45 s. A typical gel is shown in the left panel. The bands were quantitated by scanning, and the amount of LDL-receptor protein relative to γ-tubulin in mutant cells was expressed relative to the value in control cells (100%). The right panel shows the mean of values obtained in at least three separate experiments with cells from the two different PCSK9 patients shown in (A), two different control subjects and two different heterozygous LDLR FH patients (Y167X and C660S). (C) EBV lymphocytes from the same subjects as in (A) were pre-incubated for 18 h in medium containing 10% (v/v) fetal calf serum (left) or 10% (v/v) LPDS (right). Total cell extracts (~30 μg of protein/lane for serum-treated cells and 20 μg/lane for LPDS treated cells) were analysed by immunoblotting as described earlier.

Table 1. Clinical characteristics of carriers of the D374Y variant of PCSK9

<table>
<thead>
<tr>
<th>Individual no.</th>
<th>Gender</th>
<th>Age at diagnosis (years)</th>
<th>BMI at diagnosis (kg/m²)</th>
<th>Pre-treatment (or highest) total cholesterol (mmol/l)</th>
<th>ApoE phenotype</th>
<th>Clinical signs</th>
<th>Current age (years)</th>
<th>CAD (age at diagnosis)</th>
<th>Current treatment</th>
<th>Current total cholesterol (mmol/l)</th>
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<tbody>
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<tr>
<td>II,2</td>
<td>M</td>
<td>40</td>
<td>21.1</td>
<td>13.9 (19.2)</td>
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<td>69</td>
<td>39</td>
<td>Tx, A</td>
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<td>F</td>
<td>11 years</td>
<td>22.1</td>
<td>6.0</td>
<td>3/4</td>
<td>X, X</td>
<td>57</td>
<td>39</td>
<td>Tx, X</td>
<td>5.0</td>
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<td></td>
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<tr>
<td>II,2</td>
<td>F</td>
<td>30 years</td>
<td>26.2</td>
<td>15.0 (15.5)</td>
<td>3/4</td>
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<td>46</td>
<td>39</td>
<td>Tx, X</td>
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<td>13.4 (16.4)</td>
<td>3/4</td>
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<td>48</td>
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<td>5.3</td>
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<tr>
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<td>F</td>
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<td>22.1</td>
<td>13.4 (16.4)</td>
<td>3/4</td>
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<td>24.1</td>
<td>13.5 (16.0)</td>
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<td>11.0</td>
<td>3/4</td>
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<tr>
<td>II,2</td>
<td>M</td>
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<td>6.0</td>
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<td>30</td>
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<td>14 years</td>
<td>23.1</td>
<td>12.9</td>
<td>3/3</td>
<td>X, X, X</td>
<td>37</td>
<td>30</td>
<td>No, X</td>
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<tr>
<td>II,1</td>
<td>M</td>
<td>18 years</td>
<td>23.4</td>
<td>19.0</td>
<td>3/4</td>
<td>X, X, X</td>
<td>46</td>
<td>46</td>
<td>X</td>
<td>5.3</td>
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<td>21.2</td>
<td>10.6</td>
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<td>48</td>
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<tr>
<td>I,3</td>
<td>F</td>
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<td>20.0</td>
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<td>67</td>
<td>67</td>
<td>X</td>
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<td>8 years</td>
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<td>11.0</td>
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<td>III,3</td>
<td>F</td>
<td>6 years</td>
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A, corneal arcus; Tx, tendon xanthoma; X, xanthelasma; CAD, coronary artery disease; LDL-apheresis, low density lipoprotein apheresis.
proposed catalytic triad in the active site was substituted with alanine (S386A), which has been shown to abolish autocleavage when expressed in heterologous cells (16).

Stable transfectants of McA cells expressing WT or mutant human PCSK9-myc were analysed for PCSK9 protein expression and for cleavage of the propeptide by immunoblotting with antibodies to myc. As shown in Figure 3B, bands of \(~73\) and \(60\) kDa were observed in cells expressing WT PCSK9-myc; the larger band corresponds to the precursor form of PCSK9-myc formed after signal peptide cleavage (calculated MW \(= 73.4\) kDa), and the smaller band to processed PCSK9-myc (calculated MW \(= 59.7\) kDa). As expected, cells expressing S386A PCSK9-myc produced only the larger band, confirming that this protein failed to be processed. Of the three naturally occurring variants tested, only S127R showed reduced processing, in that only a trace of the smaller protein was visible on the blot; cells expressing F216L or D374Y PCSK9-myc contained the same proportion of 60 and 73 kDa proteins as cells expressing WT PCSK9. Thus, these mutations did not appear to interfere with normal processing. The total amount of PCSK9-myc was similar in extracts of cells expressing each variant, including WT, with the exception that cells expressing S127R contained slightly less; no bands were detected in cells transfected with vector alone. Similar results were obtained with independent clones of cells transfected with the same plasmids and with cells expressing FLAG-tagged rather than myc-tagged PCSK9.

Incubation of cells with \(^{35}\)S-methionine confirmed previous observations (30) that some newly synthesized and newly cleaved PCSK9 was secreted into the medium (data not shown), but this was insufficient to be detected by immunoblotting (Fig. 3C). Surprisingly, cells expressing the S386A mutant secreted full-length uncleaved protein into the medium in amounts that could be detected by immunoblotting of cell extracts (Fig. 3C).

Confocal microscopy of McA cells transiently expressing WT PCSK9-myc and stained by immunofluorescence showed that the protein was found almost exclusively in the endoplasmic reticulum (ER) (Fig. 3D), as judged by its co-localization with protein disulphide isomerase (PDI), a resident ER protein (31). Little, if any, WT PCSK9 appeared to be present in the Golgi, as PCSK9-myc was not co-localized with GM130, a cis-Golgi matrix protein (32). When the variant forms of PCSK9-myc were expressed, no differences were observed in their localization compared with the WT protein (data not shown). Because at least 50% of the PCSK9 protein in cells expressing WT PCSK9-myc had been cleaved, it is apparent that cleavage of the prodomain does not necessarily result in transport of PCSK9 to the Golgi network. It also suggests that PCSK9 may function in the ER, although we cannot exclude that PCSK9 functions in another compartment under physiological conditions because over-expression may mask trace amounts of PCSK9 in those compartments.

Expression of the LDL receptor in rat liver cells expressing PCSK9

To investigate whether expression of normal or mutant forms of PCSK9 affected the expression of the LDL receptor in liver

Figure 3. Expression of WT and variant PCSK9 in transfected cells. (A). Diagram of PCSK9 protein showing the autocleavage site. (B). Cleavage of PCSK9 variants in McArdleRH-7777 (McA) cells. Extracts of untransfected cells (McA) and stable transfectants expressing WT PCSK9-myc or mutant PCSK9-myc with different single amino acid substitutions, as indicated below the gel, were fractionated on 11% SDS–PAGE gels (30 \(\mu\)g protein/lane), transferred to a nitrocellulose membrane and immunoblotted with rabbit anti-myc antibody. Bound antibody was detected with peroxidase-conjugated anti-rabbit IgG followed by chemiluminescence. Exposure was for \(~1\) min. (C). Immunoprecipitation of PCSK9 from the medium. Medium from the cells mentioned earlier was precipitated with anti-myc and Protein A Sepharose beads, and the solubilized immunoprecipitate was fractionated as described earlier; the amounts of immunoprecipitate and cell lysate analysed derived from approximately equivalent quantities of cells. (D). Subcellular localization of PCSK9. Transiently transfected McArdle RH-7777 cells expressing WT PCSK9-myc were grown on coverslips to 90% confluence, then fixed, permeabilized and stained with anti-myc (left, upper and lower panels), together with either anti-PDI as an ER marker (centre, upper panel) or anti-GM-130 as a Golgi marker (centre, lower panel), as indicated on the figure; nuclei were stained with DAPI (blue). Overlays are shown on the right, with co-localization of PCSK9 and PDI (upper panel) or GM-130 (lower panel) appearing yellow.
cells, total RNA was isolated from McA cells stably expressing the different variants of PCSK9-myc. No differences were observed between the amounts of LDLR mRNA in cells expressing any of the forms of PCSK9-myc (data not shown), but semi-quantitative immunoblotting of cells revealed that cells expressing the D374Y and S127R variants contained slightly less LDL-receptor protein than untransfected cells or cells expressing either the F216L or the S386A variant (Fig. 4A and B).

**Secretion of apoB-containing lipoproteins from rat liver cells expressing PCSK9**

McA cells stably expressing approximately equal amounts of WT or mutant PCSK9-myc were pre-incubated for 18 h in medium containing 0.8 mM oleate complexed with bovine serum albumin to stimulate lipoprotein synthesis and secretion (27). The cells were then labelled for 30 min with a mixture of 35S-methionine and 35S-cysteine (pulse), followed by 3 h incubation in complete medium containing 10 mM cold methionine + cysteine (chase); apoB-containing lipoproteins in the medium were then immunoprecipitated and subjected to SDS–gel electrophoresis (Fig. 4C). Untransfected control cells and cells expressing WT PCSK9-myc secreted similar amounts of total apoB, mostly as apoB48, as did cells expressing the catalytically inactive mutant S386A. However, cells expressing D374Y PCSK9-myc secreted much more apoB, mostly due to increased secretion of apoB100, than control cells or cells expressing WT PCSK9. Cells expressing S127R PCSK9 also secreted increased amounts of apoB100, but total apoB secretion was not significantly greater than that from cells expressing WT PCSK9. ApoB secretion from cells expressing F216L PCSK9 was not different from cells expressing WT PCSK9 or untransfected cells. In three separate experiments with different clones of stable transfectants, the mean amount of 35S-apoB100 secreted from cells, relative to total cellular protein, was significantly greater than that from cells expressing S127R or D374Y mutation (Fig. 4D). No significant differences in the amount of apoB48 secretion were observed and incorporation of 35S into total cellular protein was similar in all cell lines.

**DISCUSSION**

In this paper, we have described three unrelated families of English origin in which all 12 hypercholesterolaemic individuals carry one copy of the D374Y variant of PCSK9; the phenotype in these carriers appears to be severe in comparison with heterozygous FH patients attending the same Lipid Clinic whose disorder is caused by known LDLR mutations. Although this has not been reported previously, we note that two Norwegian female subjects aged 19 and 41 with the D374Y variant of PCSK9 gene also had very high total serum cholesterol levels (13). In contrast, the hypercholesterolaemia in the French families carrying the F216L or D374Y variant does not appear to be as severe (10).

Our results on the expression of variants of PCSK9 in rat liver cells suggest that dominant mutations in PCSK9 can increase secretion of apoB-containing lipoproteins, thereby
offering an explanation for at least some of the excess lipoproteins found in the circulation of heterozygous carriers. Interestingly, we found that F216L PCSK9, in two members of a single French family (10), had no effect on apoB secretion, raising the possibility that this rare variant that involves the conservative substitution of one bulky hydrophobic residue for another may not in fact be pathogenic. The differences observed were not due to the insertion site of the construct as the same results were obtained with at least three independent clones.

Despite the fact that adenovirus-mediated over-expression of WT or mutant PCSK9 in mice has been shown to reduce hepatic LDL-receptor protein content to almost undetectable levels, we found no evidence for a reduction in LDL-receptor content of our stable cell lines expressing WT PCSK9, and only a slight reduction in cells expressing D374Y or S127R. This small reduction was unlikely to have accounted for the marked increase in apoB secretion from cells expressing mutant PCSK9, and this is supported by the observation of Benjannet et al. (30) who showed that the expression of PCSK9 in LDLR−/− mice increased their plasma cholesterol by the same amount as in mice in which the LDLR was intact. It is well known that apoB100 secretion can be regulated at multiple levels, including transcription, post-transcriptional mRNA editing, translation and translocation (reviewed in 33), but the rate of secretion is determined largely by intracellular degradation in which ubiquitin-dependent proteosomal degradation plays a major role (34). Thus, it is possible that PCSK9 is normally involved in the apoB100 degradation pathway and that mutant forms somehow interfere with this pathway.

As yet, the mechanism by which D374Y and S127R PCSK9 influence apoB secretion is not clear. From this study, it does not appear to be related to lack of protease activity, at least as assessed by the ability of the proteins to undergo autocatalytic cleavage, because in our hands the propeptide of D374Y PCSK9 is cleaved to the same extent as WT PCSK9 both in McA cells (Fig. 3) and in HepG2 cells (data not shown), although S127R PCSK9 showed reduced cleavage. Others have reported that the D374Y mutant does not undergo autocleavage when expressed in HEK293 cells, but we have no explanation for this apparent discrepancy (30).

Even though prodomain cleavage of D374Y and S127R mutants occurs, suggesting that they have retained catalytic activity, it is possible that the cleaved prodomain in WT PCSK9 inhibits inappropriate enzyme activity, as with other proprotein convertases (35). If the mutant proteins are not protected in this way, they could have gained activity in an inappropriate site or against an inappropriate substrate protein or may have interfered with the activity of normal PCSK9 or another unrelated protease. Another possibility is that mature PCSK9 does not itself function as a protease, but has some other function, for example, as a chaperone protein through interaction with other proteins in the ER. In this study, we have shown that although it has no ER retention signal, both mature and processed PCSK9 are localized in the ER in rat hepatoma cells, suggesting that PCSK9 binds to other proteins. Finally, because PCSK9 is a serine protease related to yeast kexin and bacterial subtilisin, it is possible that PCSK9 is directly involved in the degradation of apoB100 in a ‘non-proteosomal serine protease pathway in the ER’ (36). However, we have failed to show a direct interaction between apoB and PCSK9 by co-immunoprecipitation. It is very unlikely that the D374Y and S127R mutants of PCSK9 increase apoB100 secretion by affecting apoB mRNA editing, because there was little or no effect on secretion of apoB48, whereas apoB100 secretion increased several-fold; furthermore, we could detect no differences in the steady state amount of apoB mRNA in cells expressing WT or variant PCSK9 compared with untransfected cells (data not shown), suggesting that the increase in apoB secretion is not due to increased apoB gene transcription. The mechanism by which PCSK9 increases apoB secretion is currently under investigation.

In conclusion, we have characterized three families with an unusually severe phenotype of dominant hypercholesterolaemia who are heterozygous for the D374Y mutant of PCSK9. Heterologous expression of this mutant form of PCSK9, but not WT PCSK9, resulted in increased secretion of apoB-containing lipoproteins from rat hepatoma cells. These preliminary results provide an insight into a possible role for PCSK9 in regulating intracellular apoB-containing lipoprotein production and an explanation for the marked increase in LDL found in the circulation of heterozygous carriers of some PCSK9 variants. However, it is clear that the physiological role of PCSK9 is unlikely to be explained fully on the basis of studies on its over-expression, either in vivo or in cultured cells, and will require further investigation, perhaps using conditional knockout/transgenic animal models. However, we hope that this study will shed some light on the function of PCSK9 and on the mechanism of how mutant PCSK9 causes hypercholesterolaemia.

MATERIALS AND METHODS

Patients

The probands in this study were referred to the Hammersmith Hospital Lipid Clinic. Local Ethical Research Committee approval was obtained and all individuals gave informed written consent.

Sequence analysis of genomic DNA

Genomic DNA was isolated from whole blood and each exon of ARH and PCSK9 was amplified by PCR with specific primers (5,10) and subjected to automated nucleotide sequencing.

Cell culture

EBV-transformed lymphocyte cell lines were produced and maintained in culture as described (5). McArdle RH7777 (McA) cells (ATCC designation CRL 1601) were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) Glutamax medium supplemented with 10% (v/v) fetal calf serum and 10% horse serum (Invitrogen, Paisley, UK).
**Cloning and expression of PCSK9**

Human PCSK9 cDNA was amplified by RT–PCR from HepG2 cell mRNA with the 3’ primer encoding FLAG or myc fused to the C-terminal end of the PCSK9 coding sequence. The PCR product was cloned into pCDNA3 and its sequence verified by comparison with the published sequence (GenBank accession no. AX207686). Mutations were introduced by oligonucleotide-dependent mutagenesis (see Supplementary Material for details). Stable McA lines expressing PCSK9 variants were established by transfection with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions and selection with G418 (0.6 mg/ml).

**Analysis of gene and protein expression**

Total cellular RNA was isolated with RNA-Bee (Biogenesis Ltd, Poole, UK) and cDNA synthesized with AMV reverse transcriptase (Roche, Lewes, UK). For semi-quantitative RT–PCR, PCSK9 cDNA (for primer sequences see Supplementary Material) and 18S cDNA (primers from Applied Biosystems, Warrington, UK) fragments were amplified by PCR.

The LDL-receptor protein content of cells was determined by semi-quantitative immunoblotting (3). The cleavage and secretion of PCSK9 were determined by immunoblotting and/or immunoprecipitation of proteins from lysates and medium of McA cells transfected with plasmids containing PSCK9 cDNA, essentially as described by Naureckiene et al. (16). The following dilutions of antibodies were used: rabbit anti-human LDL receptor (Insight Biotechnology Ltd, Wembley, UK), 1:3000; rabbit anti-rat LDLR (kindly provided by Dr G. Ness, University of South Florida, USA), 1:3000; mouse anti-γ-tubulin (Sigma), 1:10 000; mouse/rabbit anti-myc (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), 1:3000; rabbit anti-mouse-HRP and goat anti-rabbit-HRP (DAKO Ltd, Ely, UK), 1:5000.

**Measurement of apoB secretion**

Cells were incubated in DMEM medium (Invitrogen) supplemented with 0.8 mM oleic acid conjugated to bovine serum albumin (Sigma) for 18 h. After pre-incubation in methionine- and cysteine-free DMEM (Invitrogen) supplemented with 0.6 mM oleic acid for 30 min, cells were then pulsed with 80 μCi/ml of [35S]-methionine/cysteine (530 Mbq/ml, Amersham BioSciences, Bucks, UK) for 30 min and chased for 3 h in fresh DMEM supplemented with 10 μM each of unlabelled methionine and cysteine and 0.6 mM oleic acid. Secreted apoB was immunoprecipitated from the medium with goat anti-apoB antibody (Chemicon International Ltd, Harrow, UK) as described (37).

**Confocal microscopy**

Cells were labelled with fluorescent antibodies and visualized by confocal microscopy essentially as described previously (38). In brief, cells on coverslips were fixed and permeabilized in cold methanol for 3 min, blocked in phosphate-buffered saline (PBS) containing 2% (w/v) fish skin gelatin (Sigma) and then incubated sequentially with rabbit anti-myc, anti-PDI or goat anti-GM130 antibodies (Santa Cruz Biotechnology Inc.), and then with Alexa 488/568 conjugated donkey anti-rabbit or goat secondary antibodies (Molecular Probes Europe BV, Leiden, The Netherlands).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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