Inactivation of Apoe and Apoc1 by two consecutive rounds of gene targeting: effects on mRNA expression levels of gene cluster members

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The genes encoding apolipoprotein (apo) E and apoC1 are, together with the gene for apoC2, located in a conserved gene cluster on human chromosome 19q12-13.2 and mouse chromosome 7. Although the significance of apoE as a ligand for receptor-mediated uptake of lipoprotein remnant particles is undisputed, the in vivo function of apoC1 and the possible interaction between apoE and apoC1 in the modulation of plasma cholesterol and triglyceride levels is far from understood. Our strategy to unravel the metabolic relationship between apoE and apoC1 in vivo is to first generate mice deficient in both apolipoproteins, enabling future production of transgenic mice with variable ratios of normal and mutant apoE and apoC1 on a null background. Here we report the creation and characterization of mice deficient in both apoE and apoC1. As these genes are tightly genetically linked, double-deficient mice were obtained by two consecutive rounds of gene targeting in mouse embryonic stem cells. Surprisingly, double inactivation of the Apoe and Apoc1 gene loci as well as single inactivations at either one of these loci were found to affect also the RNA expression levels of the other gene members in the Apoe-c1-c2 cluster. This indicates that targeted insertions are not necessarily neutral for the expression of nearby gene members in a given gene cluster. Homozygous Apoe-c1 knockout mice are hypercholesterolemic, with serum cholesterol levels of 12.5 ± 4.3 mM compared with 2.9 ± 0.5 mM in control mice, resembling mice solely deficient in apoE.

INTRODUCTION

The genes encoding apolipoprotein (apo) E and C1, together with the pseudo APOC1* gene and the gene for apoC2, are located within a 48 kb gene cluster on human chromosome 19q12-19q13.2 (1). This entire linkage group is syntenic with a linkage group localized on mouse chromosome 7 (2). Also the gene organization of individual genes within the cluster is highly conserved, with the exception that the mouse gene cluster is shorter and does not contain a pseudo Apoc1* gene (3). ApoE is a major structural component of various plasma lipoproteins, including chylomicrons, very low density lipoproteins (VLDL) and their remnants. It is synthesized primarily in the liver, although most tissues produce apoE to various extents. The major physiological role of apoE in lipoprotein metabolism is that it serves as a ligand for the receptor-mediated clearance of lipoprotein remnants by the liver (4). Mutations in the APOE gene can lead to type III hyperlipoproteinaemia, a disease associated with premature atherosclerosis (5). Mice deficient in apoE have been generated in our and other laboratories (6-8). These animals develop severe hypercholesterolaemia and atherosclerosis, with atherosclerotic lesions similar to those observed in humans (9,10).

ApoC1 principally resides on chylomicrons, VLDL and high-density lipoproteins (HDL), and is also mainly produced by the liver (11). In contrast to apoE, the in vivo function of apoC1 is not well understood. We have reported previously that apoC1-deficient mice show an impaired receptor-mediated clearance of remnant lipoproteins (12). Contrary, in vitro work demonstrated that apoC1 can block the apoE-mediated uptake of β-VLDL by hepatic receptors (13,14), suggesting that lack of apoC1 would lead to an enhanced uptake of lipoprotein remnants by the liver. Studies in transgenic mice overexpressing overlapping human APOE and APOC1 containing genomic fragments identified several elements controlling the tissue-specific expression of these genes (15). While the overexpression of APOC1 resulted in elevated levels of plasma cholesterol and triglyceride, the overexpression of both APOE and APOC1 under control of the same regulatory complex had no effect on plasma lipid levels (15). This suggests that apoE and apoC1 may interact coordinately to modulate the amount of plasma cholesterol and triglyceride (16).

To investigate the metabolic relationship between apoE and apoC1 in vivo, we plan to use transgenic mice with graded expression levels of the human APOE and APOC1 genes. To avoid interference of the endogenous mouse genes, the transgenic mice overexpressing the human genes should be bred with Apoe-c1 double-knockout animals. We have previously generated mice lacking either apoE (6) or apoC1 (12). However, double-deficient mice cannot be obtained by cross-breeding Apoe and Apoc1 null mutant mice, because these genes are
closely genetically linked (4 kb apart from each other, see ref. 3). One possibility to generate double-mutant mice would be to design a large deletion targeting vector, which upon homologous recombination would delete the \textit{Apoe} and \textit{Apoc1} genes simultaneously. A similar approach has been used by Mombaerts and co-workers (17), who deleted a 15 kb genomic segment of the T-cell antigen receptor \( \beta \)-subunit locus. A disadvantage of this strategy, however, is that regulatory elements could be deleted as well. Another approach would be to target the \textit{Apoe} and \textit{Apoc1} genes sequentially. Because a heterozygous apoE-deficient embryonic stem (ES) cell line and a targeting vector for \textit{Apoc1} were already available, we chose the latter option.

The following questions were addressed. Is it possible to disrupt closely adjacent genes by consecutive rounds of gene targeting, and if yes, how do single or multiple insertions affect the mRNA expression of other nearby apolipoprotein gene members in the \textit{Apoe-c1-c2} gene cluster? Furthermore, we were interested to know whether lack of apoC1 in addition to lack of apoE would influence the severe hypercholesterolaemia observed in apoE-deficient mice (6–8).

Here we report that double-mutant mice are viable. Like apoE-deficient mice, homozygous \textit{Apoe-c1} null mutants display hypercholesterolaemia, which becomes more severe on a high fat/cholesterol diet. Strikingly, the RNA expression levels of genes in the \textit{Apoe-c1-c2} gene cluster are affected by the targeted disruption of single (\textit{Apoe} or \textit{Apoc1}) or multiple (\textit{Apoe} plus \textit{Apoc1}) genes, indicating that targeted insertions are not necessarily neutral for the expression of nearby gene members in a given gene cluster.

**RESULTS**

**Generation of apoE-C1-deficient mice**

A replacement-type targeting vector, as used previously to disrupt the \textit{Apoc1} gene (12), was transfected into a heterozygous apoE-deficient E14 ES cell line (7). This ES cell line was already resistant to G418, because a neomycin resistance (neo\(^{\prime}\)) gene had replaced exons 1 and 2 of the \textit{Apoe} gene. To select for targeted clones, the \textit{Apoc1} targeting construct contained a hygromycin B resistance (hygroB\(^{\prime}\)) cassette and a herpes simplex virus thymidine kinase (HSV-tk) gene. The selection strategy is outlined in Figure 1. In case the targeting of the \textit{Apoc1} gene takes place in the wild-type chromosome, the endogenous 8.0 kb band will disappear upon Southern blot analysis of \textit{HindIII} digested DNA with probe A. A 9.3 kb band for the targeted \textit{Apoc1} gene will become visible, next to

![Figure 1](image-url)
a 10.2 kb band already present for the disrupted Apoe gene on the other chromosome. However, if the same chromosome is targeted twice, then an 11.5 kb band for the double-targeted locus will be present together with an 8.0 kb band for the endogenous locus. Figure 2A shows that both types of targeting events occurred. Of 212 clones screened, 63 were targeted in the Apoc1 gene, which is a targeting frequency of 1 in 3-4.

In 29 of these clones, both the Apoe and the Apoc1 genes were disrupted on the same chromosome. This means that both alleles were targeted with equally high efficiency. To verify further the genetic organization of the targeted alleles, two additional probes (probes B and C, Fig. 1) and an additional digestion with EcoRI were used (not shown). No abnormal targeting events were detected.

A total of four double-targeted ES cell clones were injected into C57BL/6 host blastocysts, and embryos were reimplanted into foster mothers. Chimeric males were bred to C57BL/6 females, and males derived from one clone were found to transmit the mutation through the germline. Heterozygous mutants were interbred to generate mice deficient in both apoE and apoC1 with a mixed C57BL/6 and 129/Ola background. Figure 2B shows an example of the genotype of these animals, obtained by Southern blot analysis.

Expression of the Apoe-c1-c2 gene cluster

To study whether targeting of Apoe and/or Apoc1 would influence the expression level of the other apolipoprotein genes in the cluster, a Northern blot was prepared from RNA from mouse livers of heterozygous and homozygous apoE-deficient (6), apoC1-deficient (12) and apoC1-deficient mice as well as of controls. In the homozygous deficient animals, there is obviously no expression of the respective mutated gene(s), confirming that these mice are true null mutants (Table 1). In the heterozygotes, expression levels of the mutated genes are reduced.

The effect of a targeted null mutation of a gene on its neighbouring gene in the cluster is striking. Complete lack of apoE results in reduced Apoc1 mRNA levels (68%), and absence of apoC1 leads to a decreased level of Apoe mRNA (57%). The influence of the single and double Apoe and Apoc1 null mutations on the Apoe2 gene, which is located approximately 15 kb downstream from Apoe in the cluster (3), is more complex. Apoc2 expression levels are clearly downregulated in the homozygous double-mutants and to a somewhat lesser extent in the apoC1-deficient mice, whereas this level is not affected in the apoE-deficient homozygotes. Remarkably, an overexpression of Apoc2 is observed in heterozygous Apoe knockout mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RNA expression level as percentage of control</th>
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<tbody>
<tr>
<td>Apoe</td>
<td>Apoc1</td>
</tr>
<tr>
<td>wt/wt</td>
<td>100 ± 17</td>
</tr>
<tr>
<td>wt/E1-C1</td>
<td>66 ± 23</td>
</tr>
<tr>
<td>E1-C1/E1-C1</td>
<td>60 ± 26</td>
</tr>
<tr>
<td>wt/E</td>
<td>68 ± 16</td>
</tr>
<tr>
<td>E/c1-C1</td>
<td>62 ± 12</td>
</tr>
<tr>
<td>C1/c1-C1</td>
<td>83 ± 18</td>
</tr>
</tbody>
</table>

RNA was isolated from liver and prepared as described in Materials and Methods. Each value represents the mean ± SD of three to four livers, on four individual Northern blots. wt, wild-type allele; E1-C1, apoE-C1-deficient allele; E1, apoE-deficient allele; and C1, apoC1-deficient allele.

Table 1. RNA expression level of the Apoe, Apoc1 and Apoc2 genes in apoE-, apoC1- and apoE-C1-deficient mice

*Significant difference as compared with wt/wt liver using non-parametric Mann-Whitney test (P<0.01 and P<0.05, respectively).
Effect of apoE-C1-deficiency on apolipoprotein composition

VLDL (d < 1.006 g/ml) and HDL (d = 1.063–1.21 g/ml) were isolated from fasted wild-type, heterozygous and homozygous apoE-C1-deficient animals by density ultracentrifugation, and subjected to SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and protein staining (Fig. 3A). In VLDL, apoE is clearly absent in the homozygous double-mutant mice and reduced in the heterozygous animals. Instead, apoA1 appears in the VLDL of homozygotes, whereas apoA4 is present in homozygous and heterozygous apoE-C1-deficient mice. Most protein in HDL is apoA1. ApoB48 is normally not present in HDL, but because of high levels of VLDL/LDL-sized particles (see also Fig. 4) residual amounts of these particles are also present in the 1.063–1.21 g/ml density fraction.

![Figure 4](image_url)

**Figure 4.** Lipoprotein profiles of apoE-C1-deficient and control mice on chow and a mild high fat/cholesterol diet. Sera from at least nine fasted controls, heterozygous and homozygous apoE-C1-deficient mice were pooled and size separated by FPLC. Mice had been fed a chow diet (top) or a mild high fat/cholesterol diet (HFC) containing 15% fat and 0.25% cholesterol (bottom). Fractions 13–23 represent VLDL+LDL and fractions 24–34 represent HDL (based on the elution profile of human serum lipoproteins).

Because a 4–20% gradient gel cannot distinguish between the different apoC proteins and apoA2, Western blotting was required to demonstrate that apoC1 is absent in the homozygous and reduced in the heterozygous mutant mice (Fig. 3B). When the filters were incubated with antibodies against apoE and apoB, similar results were obtained as with the gel stained for protein. The amount of apoC3 is comparable for all mice (Fig. 3B).

**Hypercholesterolaemia in apoE-C1-deficient mice**

As shown in Table 2, apoE-C1-deficient mice are severely hypercholesterolaemic on a chow diet, with average serum cholesterol levels of 12.5 ± 4.3 mM compared with 2.9 ± 0.5 mM in controls. Heterozygous double-deficient animals display normal serum lipid levels. There is no significant difference between male and female serum cholesterol levels. To determine the distribution of cholesterol among the lipoprotein fractions, fast protein liquid chromatography (FPLC) analysis was performed on pooled fasted mouse sera (Fig. 4, upper panel). In control and heterozygous mice, the main cholesterol carrier in serum is HDL, while at most 10% of total serum cholesterol is contained in the VLDL/LDL-sized fractions. In null mutants, however, most of the cholesterol (71%) is confined to the VLDL/LDL-sized fractions.

Upon feeding a mild high-fat/cholesterol (HFC) diet, containing 15% fat and 0.25% cholesterol, serum cholesterol is increased eightfold in homozygous apoE-C1-deficient mice, as compared with controls fed the HFC diet (31 ± 10.1 mM and 3.8 ± 0.9 mM, respectively). The increase in total cholesterol is mainly found in the VLDL/LDL-sized fractions, as is depicted in the lower panel of Figure 4.

**DISCUSSION**

This paper describes the generation of mice deficient in both apoE and apoC1 by two consecutive rounds of gene targeting in ES cells. Because the Apoe and Apoc1 genes are physically very close, and because the recombination distances across the pertinent area of mouse chromosome 7 are extremely small (2), double-deficient mice could not be obtained by cross-breeding apoE- and apoC1-deficient mice. Instead, two selection markers (neo' and hygro') were subsequently introduced into the same locus. The inactivation of the Apoc1 gene in the second round of gene targeting was very efficient (1 in 3–4).

**Table 2.** Serum cholesterol and triglyceride levels in apoE-C1-deficient mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Diet</th>
<th>Cholesterol (mM)</th>
<th>Triglyceride (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>d+?</td>
<td>d</td>
</tr>
<tr>
<td>+/-</td>
<td>Chow</td>
<td>2.9 ± 0.5</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>+/-</td>
<td>Chow</td>
<td>2.4 ± 0.5</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>+/-</td>
<td>HFC</td>
<td>12.6 ± 4.3</td>
<td>13.4 ± 4.5</td>
</tr>
<tr>
<td>+/-</td>
<td>HFC</td>
<td>3.8 ± 0.9</td>
<td>4.4 ± 0.8</td>
</tr>
<tr>
<td>+/-</td>
<td>HFC</td>
<td>3.6 ± 0.9</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>+/-</td>
<td>HFC</td>
<td>31.0 ± 10.1</td>
<td>30.6 ± 10.5</td>
</tr>
</tbody>
</table>

Serum triglycerides and serum cholesterol levels are given as mean ± SD. Each group contained 10 male and/or 10 female age-matched animals. +/-, control mice; +/-, heterozygous apoE-C1-deficient mice; +/-, homozygous apoE-C1-deficient mice. HFC, mild high fat/cholesterol diet with 15% fat and 0.25% cholesterol.

aSignificant difference (P<0.05) as compared with +/- and +/+, respectively, on the same diet using non-parametric Mann–Whitney test.
bSignificant difference (P<0.05) as compared with female mice on the same diet using non-parametric Mann–Whitney test.
cSignificant difference (P<0.05) as compared with mice of same genotype on chow diet using non-parametric Mann–Whitney test.
Apoal and proteiins may interact coordinately to modulate plasma lipid genes in the Apod venting the normal interaction between the LCR and the p-
located p-globin gene (21). The inactivation was suggested to whether these elements also regulate the expression of deficient mice.
sequence has been proposed by Maeda promoter (19,20). A similar hepatic control element was revealed that expression in liver of the human APOE and work in transgenic mice already suggested that these two interaction between apoE and apoCl is quite feasible, because enhancer/promoter introduced by the hygror cassette, pre­of these genes. A targeted insertion of a hygror cassette into moters. Consequently, this would lead to reduced expression for this m-acting regulatory domain with apolipoprotein pro­and gene(s) (the hygroBr and neor genes, both driven off of comm.). It is conceivable that the newly introduced promoter(s)
cluster in the various knockout mice, as we have previously shown that serum cholesterol in apoE-deficient mice is age-dependent (6), but might also be due to absence of apoC1 in addition to lack of apoE. It will be interesting to see whether these mice develop atherosclerosis to a similar extent as mice solely deficient in apoE (7–10), and if heterozygous Apoe-cl knockout mice fed a severe high-fat diet would also develop gender-dependent atherosclerosis, as we have observed in heterozygous Apoe null mutants (6).
When animals carrying a human transgene are studied, a null background of the mouse counterpart of that gene would be the ideal genetic environment of the study. Especially if the interaction between two transgenes is investigated, it is of great importance that there is no endogenous expression of these genes, so that there will be no compensatory up- or downregulation of the endogenous mouse genes. This can be reached by two rounds of crossbreeding of mice carrying a transgene with mice deficient in the endogenous protein of that gene. In this respect, the apoE-C1-deficient mice are extremely valuable for future use in studies on the metabolic relationship between apoE and apoC1 in vivo.

MATERIALS AND METHODS
Cell culture and transfection
An E14 ES cell line with one functional and one disrupted Apoe allele (7, kindly provided by Dr A.S.Plimp, Rockefeller University, New York) was used as the source of ES cells. The Apoe1 gene was targeted with a replacement-type vector designed to disrupt solely the Apoe1 gene, as described previously (12).
Lipid and lipoprotein analyses

After an overnight fasting period (16–17 h), approximately 150 μl of whole blood was obtained from each individual mouse through tail bleeding. Levels of total serum cholesterol and triglyceride (without measuring free glycerol) were determined using Boehringer Mannheim enzymatic assay kit #236901 and Sigma GPO-Trinder kit #837T, respectively.

Diets

Mice were given free access to water and food. A regular breeding chow diet (RMH-B) containing 6.2% fat and a semisynthetic mild HFC diet containing 15% cocoa butter, 0.25% cholesterol, 40.5% sucrose, 10% cornstarch, 1% corn oil, and 6% cellulose (all percentages are by weight) were used. The latter diet was composed essentially according to Nishina and co-workers (28). Diets were purchased from Hope Farms, Woerden, the Netherlands.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

Apo, apolipoprotein; VLDL, very low density lipoprotein(s); HDL, high-density lipoprotein(s); k/b, kilobase pairs; ES, embryonic stem; neoR, neomycin resistance; hygroB, hygromycin B resistance; HSV-A, herpes simplex virus thymidine kinase; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; LDL, low-density lipoprotein(s); FPLC, fast protein liquid chromatography; HFC, mild high fat/cholesterol diet; LCR, locus control region; FIAU, l-[2-deoxy, 2-fluoro-B-D-arabinofuranosyl]-5-iodouracil; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

REFERENCES

gene are linked closely to the apolipoprotein E gene. J. Biol. Chem., 263, 7277-7286.


