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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 very 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 *Apoe* and *Apod* 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 β-subunit locus. A disadvantage of this strategy, however, is that regulatory elements could be deleted as well. Another approach would be to target the *Apoe* and *Apod* genes sequentially. Because a heterozygous apoe-deficient embryonic stem (ES) cell line and a targeting vector for *Apod* 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 *Apoe-cl-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 *Apoc1* null mutants display hypercholesterolaemia, which becomes more severe on a high fat/cholesterol diet. Strikingly, the RNA expression levels of genes in the *Apoe-cl-c2* gene cluster are affected by the targeted disruption of single (apoE or *Apoc1*) or multiple (*Apoe* plus *Apod*) 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 *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') gene had replaced exons 1 and 2 of the *Apoe* gene. To select for targeted clones, the *Apoc1* targeting construct contained a hygromycin B resistance (hygrob') 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 *Apoc1* gene takes place in the wild-type chromosome, the endogenous 8.0 kb band will disappear upon Southern blot analysis of *HindIII* digested DNA with probe A. A 9.3 kb band for the targeted *Apoc1* gene will become visible, next to

![Diagram](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 APOE 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 apoE-C1-deficient mice as well as of controls. In the homozygous deficient animals, there is obviously no expression of the respective mutated genes(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 (65%), 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 APOC2 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 1. RNA expression level of the APOE, APOC1 and APOC2 genes in apoE-, apoC1- and apoE-C1-deficient mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>APOE expression level as percentage of control</th>
<th>APOC1 expression level as percentage of control</th>
<th>APOC2 expression level as percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt/wt</td>
<td>100 ± 14</td>
<td>100 ± 14</td>
<td>100 ± 14</td>
</tr>
<tr>
<td>wt/E-C1</td>
<td>66 ± 23</td>
<td>62 ± 17</td>
<td>100 ± 14</td>
</tr>
<tr>
<td>E-C1/E-C1</td>
<td>60 ± 25</td>
<td>62 ± 17</td>
<td>100 ± 14</td>
</tr>
<tr>
<td>wt/E</td>
<td>88 ± 10</td>
<td>62 ± 17</td>
<td>100 ± 14</td>
</tr>
<tr>
<td>C1/C1</td>
<td>57 ± 7</td>
<td>4 ± 2</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; E-C1, apoE-C1-deficient allele; E, apoE-deficient allele. C1, apoC1-deficient allele.

**Table 1:** RNA expression level of the APOE, APOC1 and APOC2 genes in apoE-, apoC1- and apoE-C1-deficient mice.

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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.

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 + q</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>Chow</td>
<td>12.5 ± 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 ± 10.9</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.

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).

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).

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 Apocl 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 crossing apoE- and apoC1-deficient mice. Instead, two selection markers (neo and hygroB) were subsequently introduced into the same locus. The inactivation of the Apocl gene in the second round of gene targeting was very efficient (1 in 3–4),
and comparable with the targeting frequency previously found in wild-type ES cells (12). Both wild-type and already targeted alleles recombined with equal efficiency, indicating that the proximity of the neo' gene in the apoE-deficient locus did not affect the frequency of homologous recombination. Moreover, the presence of both of these resistance genes and the two rounds of transfection and selection of the ES cells did not interfere with the germine potency of the cell line.

While studying the mRNA levels of genes in the Apoe-cl-c2 gene cluster in apoE-, apoC- and double-deficient mice, some conspicuous observations were made. Not only is the expression of the targeted gene reduced by the respective mutation, also other genes in the cluster are influenced by the insertion of a targeting vector in the locus. There are several ways how this could occur. First, it is possible that a reduction of apoE protein would lead to a down- or upregulation of the Apoc1 or Apoc2 gene, or both, via an indirect metabolic effect in the lipoprotein metabolism. In that case, the amount of protein of the targeted gene would be critical for the effect on the expression of the other genes in the cluster. This type of metabolic interaction is seen in transgenic mice containing the gene for human cholesteryl ester transfer protein, in which the mRNA for the LDL receptor is down-regulated (18). Second, the effect might be due to changes in genomic structure of the entire apolipoprotein cluster by the homologous integration of the targeting vectors. The insertion of one or two selection cassettes that include promoter and enhancer elements may deregulate the transcription of neighbouring genes in a distance- and position-dependent manner. Work in transgenic mice revealed that expression in liver of the human APOE and APOC1 genes requires a common cis-acting regulatory domain, which is located about 9 kb downstream of the APOC1 promoter (19,20). A similar hepatic control element was identified in mice between Apoc1 and Apoc2 (J. Smith, pers. comm.). It is conceivable that the newly introduced promoter(s) and gene(s) (the hygroB' and neo' genes, both driven off of the tk-promoter and a mutant polyoma enhancer) will compete for this cis-acting regulatory domain with apolipoprotein promoters. Consequently, this would lead to reduced expression of these genes. A targeted insertion of a hygro' cassette into the human β-globin locus control region (LCR) in a mouse/human hybrid erythroid cell line inactivated the downstream located β-globin gene (21). The inactivation was suggested to be the result of interaction of LCR elements with the proximal enhancer/promoter introduced by the hygro' cassette, preventing the normal interaction between the LCR and the β-globin gene promoter. Similarly, competition between the genes in the Apoa1-c3-a4 gene cluster for a shared regulatory sequence has been proposed by Maeda et al. (22) as an explanation for the observed reduction of expression of the Apoa1 and Apoa4 genes in intestine of homozygous apoc3-deficient mice.

Which of the above mentioned options for the altered gene expression of the Apoe-cl-c2 cluster in the various knockout animals is true cannot be deduced from our data. A metabolic interaction between apoE and apoC1 is quite feasible, because work in transgenic mice already suggested that these two proteins may interact coordinately to modulate plasma lipid levels (16). Although there are multiple shared regulatory elements for the Apoe and Apoc1 genes (15), it is not clear whether these elements also regulate the expression of Apoc2.

The integration of the targeting vector did not delete any of the known regulatory sequences. If the hepatic control region does not regulate the expression of Apoc2 but only controls the expression of Apoe and Apoc1, then the reduced levels of these latter genes in the homozygous knockout mice could be explained by the competition model. As the expression of Apoc2 is decreased in homozygous apoc1- and double-deficient animals, normal in homozygous Apoe knockout mice and increased in heterozygous apoe-deficient mice, it is hard to explain these results with the competition model. If the expression of Apoc2 is regulated by the same cis-acting control region as the Apoe and Apoc1 genes, then the position of the enhancer relative to the gene (5' of Apoc2 and 3' of Apoe and Apoc1) and the distance between the enhancer and the gene might play a part.

The overt phenotype of the apoe-C1-deficient mice is similar to that of Apoe knockout mice (6–8), that is hypercholesterolemia on a chow diet, which becomes more severe on a high-fat/cholesterol diet. Interestingly, heterozygous double-deficient mice fed a chow diet display a lower serum cholesterol level than their wild-type littermates. This difference is significant for males. This unexpected finding was also observed in heterozygous apoe-deficient mice fed a chow diet (6), although in that case the difference was only significant for females. At this moment, we do not know the reason for these observations.

On a chow diet, serum cholesterol levels in the double-deficient mice seem to be somewhat lower than in apoE-deficient mice [12.5 ± 4.3 mM and 19.3 ± 5.5 mM (6), respectively], whereas serum cholesterol is comparable for both types of mice on the HFC diet. The difference on chow might be caused by difference in age between the apoE-deficient and the double-mutant 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. Plump, Rockefeller University, New York) was used as the source of ES cells. The Apoc1 gene was targeted with a replacement-type vector designed to disrupt solely the Apoc1 gene, as described previously (12).
ES cells were cultured and subsequently selected on hygrob<sup>+</sup> mouse embryonic fibroblasts derived from mice deficient in muscle creatine kinase (23). ES cells were electroporated in the presence of 25 µg/ml linearized vector DNA at 4.0 kV/cm in a TA750 transfection apparatus (Krüss GmbH Hamburg, Germany). Selection took place with 300 µg/ml hygromycin B (ICN Biochemicals, Cleveland, OH) and 0.2 µM FIAU (1-[2-deoxy, 2-fluoro-β-D-arabinofuranosyl]-5-iodouracil), Bristol Myers, New York, NY) 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 #236691 and Sigma GPO-Trinder kit #337-B, 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 HFD diet containing 15% coconut 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**

Apol, apolipoprotein; VLDL, very low density lipoprotein(s); HDL, high-density lipoprotein(s); kb, kilobase pairs; ES, embryonic stem; neor, neomycin resistance; hygroB, hygromycin B resistance; HSV-1-K herpes simplex virus thymidine kinase; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; LDL, low-density lipoprotein(s); FPLC, fast protein liquid chromatography; HFD, mild high fat/cholesterol diet; LCR, locus control region; FIAU, 1-[2-deoxy, 2-fluoro-β-D-arabinofuranosyl]-5-iodouracil; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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


