Gene Correction of the Apolipoprotein (Apo) E2 Phenotype to Wild-type ApoE3 by in Situ Chimeraplasty*

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Apolipoprotein (apo) E is a polymorphic plasma protein, synthesized mainly by liver. Here, we evaluate whether synthetic DNA-RNA oligonucleotides (chimeraplasts) can convert a dysfunctional isoform, apoE2 (C→T, R158C), which causes Type III hyperlipidemia and premature atherosclerosis, into apoE3. First, we treated recombinant Chinese hamster ovary cells stably secreting apoE2 with a 68-mer apoE2 to apoE3 chimeraplast. About one-third of apoE2 was converted to apoE3, and the repair was stable through 12 passages. Subcloning treated cells produced both apoE2 and apoE3 clones. Direct sequencing and reverse transcription polymerase chain reaction confirmed the genotype, whereas phenotypic change was verified by isoelectric focusing and immunoblotting of secreted proteins. Second, we established that the APOE2 gene can be targeted both in vivo, using transgenic mice overexpressing human apoE2, and in chromosomal context, using cultured lymphocytes from a patient homozygous for the e2 allele. We conclude that chimeraplasty has the potential to convert the apoE2 mutation in patients with Type III hyperlipidemia to apoE3.

Apolipoprotein (apo) E is a 34-kDa polymorphic protein that has anti-atherogenic actions by clearing remnant lipoproteins and promoting cholesterol efflux from cells (1–4). Low apoE is a risk factor for coronary artery disease (5), and apoE deficiency results in severe hyperlipidemia and atherosclerosis (6, 7). By contrast, infusing apoE into hyperlipidemic rabbits resists atherosclerotic lesions (8), and apoE transgenic mice resist diabetic or dietary hyperlipidemia (9, 10). The three common isoforms of apoE differ in two amino acid positions; apoE2 (Cys-112 and Cys-158), apoE3 (Cys-112 and Arg-158), the most prevalent or wild-type protein, and apoE4 (Arg-112 and Arg-158). The rarest variant, apoE2, is the primary molecular cause of Type III hyperlipidemia (11), which is characterized by accumulation of remnant lipoproteins and premature heart disease.

Approaches to treat or prevent atherosclerosis include systemic gene therapy, using viral or non-viral delivery strategies, to overexpress proteins that inhibit atherogenesis or stabilize lesions. Indeed, adenoviral-mediated apoE gene transfer ameliorates experimental hyperlipidemia and atherosclerosis (12, 13). The recent development of targeted gene repair, using synthetic DNA-RNA oligonucleotides (chimeraplasts), offers another possibility (14–16). Chimeraplasts contain short regions of correcting DNA bounded by long stretches of 2′-O-methyl RNA; hairpin loops and GC clamps are also incorporated to make the structure self-associating. These features promote strong, specific binding to the target genomic sequence and allow the DNA repair machinery of the cell to identify and correct the point mutation in situ (15).

Here, we apply chimeraplasty to recombinant Chinese hamster ovary (CHO) cells secreting human apoE2 and show that the gene conversion of apoE2 to apoE3 is stable and permanent at the DNA, mRNA, and protein level. In addition, we demonstrate gene repair in vivo, using transgenic mice overexpressing human apoE2, and also verify that the human genome can be targeted using lymphocytes from a patient homozygous for the e2 allele. We conclude that it is feasible to correct the apoE2 mutation in patients with Type III hyperlipidemia by chimeraplasty.

EXPERIMENTAL PROCEDURES

Recombinant CHO Cells—CHO×apoE2 cells were cultured in Iscove’s modified Dulbecco’s medium with 10% dialyzed fetal bovine serum (FBS; Sigma), supplemented with 2 mM glutamax, 100 μM hypoxanthine, 16 μM thymidine, and 1% non-essential amino acids (Life Technologies, Inc.). Human apoE2 cDNA was cloned into p7055 (17), an expression vector with the selectable DHFR gene, and then 6 μg of purified p7055.E2 were complexed with cationic liposomes to transfect CHO×apoE2 cells (2×106). After 2–3 weeks of growth in Iscove’s selection medium, which lacks hypoxantine and thymidine, clones of CHO-E2 cells were isolated by limiting dilution; as controls, CHO-E3 cells were produced similarly.

Transgenic Mice Expressing Human ApoE2—Male transgenic mice expressing human apoE2 (E−/E2) (18) and female E−/E2 mice were mated. DNA was extracted from tail tips of offspring and screened by PCR at three loci (19); animals positive for human APOE and the hygromycin-resistance marker, but negative for murine APOE, were selected and maintained on standard chow.

Cultured Human Lymphocytes—Lymphocytes were isolated from the heparinized blood of a patient homozygous for the e2 allele and immortalized with Epstein-Barr virus (20). Transformed cells were cultured in RPMI 1640 medium supplemented with 10% FBS (Sigma) and 2 mM L-glutamine.

Chimeraplast—The 68-mer oligonucleotides were synthesized commercially by MWG-Biotech (Ebersberg, Germany) with ten (fifteen for 88-mers) complementary 2′-O-methyl RNA residues flanking each side of the 5-base-DNA stretch; four Thr residues in each loop and a 5-bp GC
clamped. Cells were transfected for 24 h with the selectable expression vector p7055.E2, the PCR-RFLP pattern was unchanged when CHO-E3 cells were treated with the chimera-plast (Fig. 2B, lane 3), whereas an E3 to E2 chimera-plast as a negative control did not affect the CHO-E2–16 cells.

When CHO-E2–16 cells, previously treated with chimera-plast (400 nm for 4 h), were targeted a second time the initial conversion of 37.3% was increased dramatically to nearly 60% (Fig. 2C). To confirm that the apoE2 to apoE3 correction was not clone-specific, three other CHO-E2 clones (numbers 7, 17, and 18; see Fig. 1) were studied. Each clone was found to be efficiently converted to a mixture of apoE2/E3 cells with 400 nm of the chimera-plast (Fig. 2D), and whereas treatment with 200 nm had little effect on line 7, the conversion of lines 17 and 18 was readily apparent (Fig. 2D).

**Modifying the Chimera-Plast Design**—Chimeraplasts of two additional designs were evaluated. The first, an 88-mer had 10 extra RNA residues to strengthen hybridization and potentially enhance conversion by the enzymatic mismatch repair machinery. This 88-mer was indeed found to give higher conversion, 23.3% at 400 nm compared with 14.8% by our standard 68-mer (Fig. 3A). A second chimera-plast with only a single mismatch (in the all-DNA strand) against the targeted double-stranded apoE DNA (Fig. 2A) was also examined, but, though effective at both 400 and 200 nm (47.7 and 16.7%; see Fig. 3B), it proved inferior to the standard double-mismatched 68-mer (54.8 and 45.8%; see Fig. 3B).

**Clonal Analysis of Chimera-plast-treated CHO-E2 Cells at the Level of Genomic Sequence, mRNA, and Protein**—Chimeraplast-treated CHO-E2–16 cells were passaged 12 times using 1:10 splits, and the apoE DNA was reanalyzed by PCR-RFLP; the gene correction of apoE2 to apoE3 was unchanged (Fig. 4A, center lane). Next, the passaged cells were cloned, and after expansion and analysis of nine clones, three different genotypes were found; four clones were apoE2/3, three were apoE2 (uncorrected), and two were apoE3 (corrected). ApoE2 (line 8) and apoE3 (line 3) clones were then compared with the starting apoE2/apoE3 mixed cell population (Fig. 4A). Direct DNA sequencing confirmed the genotypes, in particular, that the apoE3 genotype had been created by chimera-plast (Fig. 4B). Moreover, the expected pattern for true apoE2 and apoE3 clones was found when polyadenylated RNA was analyzed by RT-PCR-RFLP (Fig. 4C). Importantly, PCR-independent confirmation of gene conversion was obtained by separating the secreted apoE...
isoforms using i.e.f. As expected, non-cloned apoE2/3 cells gave two bands, identical to a plasma control sample. By contrast, the apoE2 and apoE3 clones gave the predicted single bands separated by one charge unit (Fig. 4), confirming that chimera-plasty had produced a phenotypic change.

Targeting the APOE2 Gene in Transgenic Mice and Human Lymphocytes—The APOE2 gene was targeted in vivo by injecting 1000 nM of the standard 68-mer PEI complex into a transgenic mice overexpressing human apoE2; a second animal received PEI alone. DNA extracted from liver was analyzed 7 days later, and about 25% of hepatic APOE2 was found to be converted to APOE3 (Fig. 5A). We also targeted APOE2 within the human genome, treating cultured lymphocytes from a homozygous e2/e2 patient for 16 h with increasing concentrations (0–1000 nM) of 68-mer PEI complexes (amine:phosphate molar ratios between 5:1 and 8:1). Although low chimeraplast concentrations were ineffective, a clear conversion of APOE2 to APOE3 was seen at 800 and 1000 nM with a 7:1 ratio of PEI to oligonucleotide giving the highest conversion (for example, using 800 nM, 34% conversion was seen versus 22 and 3% at 8:1 and 6:1, respectively; see Fig. 5B).

**DISCUSSION**

Diverse techniques are being developed to introduce transgenes into various tissues to express therapeutic proteins. There are many problems to overcome, achieving efficient gene transfer and specific gene delivery to target tissues, sustaining levels of gene expression, preventing adverse immunological responses, and ensuring safety, particularly with viral vectors (22). By contrast, when defective proteins result from point mutations in the wild-type allele, the genes that encode them can potentially be repaired by synthetic RNA-DNA oligonucleotides, termed chimeraplasts. Chimeraplasty, or targeted gene correction, is an exciting alternative to viral gene therapy. It has the great advantage of retaining existing gene promoters and enhancers and cell-specific context. Although a new technology, there is already evidence that chimeraplast-induced corrections are permanent and produce phenotypic conversions. Examples in vitro include the restoration of tyrosinase activity in melanocytes of albino mice (23) and correction of the b^+ hemoglobin allele that causes sickle cell anemia (24). Phenotypic changes are also reported in vivo using natural and transgenic preclinical models of disease, including rat and dog.
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hemophilia B, which have defective clotting factor IX (25), the Gunn rat model of Crigler-Najjar syndrome (26), and mouse and dog models of muscular dystrophy (27, 28). As the technique has also been used in bacteria (29) and plants (30), there appears no obvious restriction to DNA sequences that can be targeted.

Here, we have used chimeraplast to correct a dysfunctional variant of apolipoprotein E, termed apoE2, that causes the genetic disorder of Type III hyperlipidemia. As established human cell lines secreting apoE2 are unavailable (we found that hepatoblastoma HepG2 cells (31) and THP-1 monocyte macrophages (32) were derived from apoE3/E3 donors), we studied recombinant CHO cells. Initial experiments with 400 nm of a standard 68-mer chimeraplast consistently gave conversion efficiencies of about 30%, whereas retargeting substantially increased this number. Moreover, the correction was specific; CHO-E3 cells were unaffected, while an apoE3 to apoE2 chimeraplast did not convert CHO-E2 cells. Although short stretches of DNA can enter cells through endocytosis, we used PEI as carrier. This polycation is a very efficient delivery system for DNA. Our analyses confirmed the genotyping data; chimeraplast-induced conversion of apoE2 to wild-type protein. This was important; it established that all-DNA strand of the chimeraplast. An 88-mer chimeraplast also gave a higher conversion, presumably because the additional RNA bases strengthened hybridization and formed a more stable complex with the target sequence (16). However, this does not necessarily indicate greater practical value; the increased size of an 88-mer means a more expensive reagent that is also probably of lower purity than a 68-mer.

Having established that chimeraplasts can target the apoE gene in our model system, we critically evaluated whether the technique might repair the apoE2 mutation in patients with Type III hyperlipidemia. First, we determined whether the gene correction was permanent and passed to future generations of cells and whether wild-type protein was secreted. Chimeraplast-treated cells were maintained in continuous culture for 2 months (12 passages at 1:10 splits) and then clones were isolated. PCR-RFLP analysis showed an unchanged conversion efficiency and the presence of corrected apoE3. Direct DNA sequencing and RT-PCR analysis confirmed that chimeraplast had produced permanent changes at genomic and mRNA levels, respectively. Conveniently, the single nucleotide polymorphism distinguishing apoE2 from apoE3 results in an amino acid change, Cys-158 to Arg-158, and a charge difference between the two isoforms. This is exploited in routine phenotyping using i.e.f. to separate plasma apoE isoforms with detection by immunoblotting (37). We used an identical protocol to detect apoE secreted into culture medium and to monitor chimeraplast-induced conversion of apoE2 to wild-type protein. Our analyses confirmed the genotyping data; chimeraplast-treated CHO-E2 cells secreted both apoE2 and apoE3 isoforms, whereas the corrected apoE3 clone secreted only wild-type protein.

Next, we assessed whether our chimeraplast could efficiently target the liver, which synthesizes >90% of plasma apoE (38). We used an animal model of Type III hyperlipidemia, the doubly transgenic E−/−/hE2 mouse, produced by crossing transgenic mice overexpressing human apoE2 with apoE-deficient mice (18), and observed clear conversion of hepatic apoE2 to apoE3. This was important; it established that another cell type can be effectively targeted and that the apoE2 gene, rather than apoE2 cDNA, can be corrected. We now have studies underway to confirm that this finding is consistent, to improve targeting efficiency, and to study any reversal of the hyperlipidemia and associated atherosclerosis. Finally, we also wanted to show that our chimeraplast could target the APOE gene in context within human chromosome 19. We achieved this by successfully converting EBV-transformed lymphocytes from a homozygous e2/e2 patient to the apoE3 genotype.
Many inherited point mutations in liver-derived proteins are associated with hyperlipidemia and premature cardiovascular diseases, whether secreted like apolipoproteins, enzymes, and transfer proteins or retained like the LDL receptor. Nevertheless, apoE is a good candidate for generic gene therapy as it has multiple antiatherogenic actions, both within the circulation and at lesion sites (39). Indeed, adenoviral-mediated expression of apoE3 from liver significantly regresses early fatty streaks in the arterial wall, as well as advanced lesions (40, 41). The apoE2 isoform has defective binding to the LDL receptor and LDL receptor-related protein, leading to impaired clearance of atherogenic remnant lipoproteins from the circulation (42). ApoE2 is also much less efficient at sequestering cellular cholesterol than apoE3 (3). We predict, therefore, that using chimeraplasty to convert apoE2 into apoE3 will reduce progression of atherosclerotic lesions or even promote regression in patients with Type III hyperlipidemia; heterozygous apoE2/E3 subjects do not have premature atherosclerosis. Thus, our present finding that the APOE2 gene can be targeted and efficiently converted to APOE3, resulting in secretion of apoE3 protein, is significant. Furthermore, it implies that apoE4 can be converted to apoE3, an intriguing possibility as the $e^4$ allele is strongly associated with Alzheimer’s disease and a variety of other neurodegenerative disorders (43). Clearly, further studies in vitro and in preclinical models will be needed to realize such long-term goals.

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