Efficient expression of retroviral vector-transduced human low density lipoprotein (LDL) receptor in LDL receptor-deficient rabbit fibroblasts *in vitro*

(gene therapy/hypercholesterolemia/Watanabe heritable hyperlipidemic rabbit)

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ABSTRACT Familial hypercholesterolemia is caused by a genetic deficiency of the low density lipoprotein (LDL) receptor. The Watanabe heritable hyperlipidemic (WHHL) rabbit, which is also defective in LDL receptor activity, provides an excellent animal model of homozygous familial hypercholesterolemia. As a step toward development of effective gene therapy for familial hypercholesterolemia, we have constructed a transmissible retroviral vector containing a full-length human cDNA for the LDL receptor. WHHL fibroblasts infected *in vitro* expressed the human receptor efficiently, as indicated by RNA and ligand blotting studies. Infected fibroblasts bound and degraded a monoclonal antibody specific for the human LDL receptor (IgGC7) in a manner comparable to that seen with normal human fibroblasts. Human LDL was also degraded by infected WHHL cells and promoted cholesterol esterification to the same degree as seen in normal human fibroblasts. Although technical problems remain to be solved, these studies show that, in principle, gene therapy may be possible for familial hypercholesterolemia patients.

The recent development of methods for efficient and functional transfer of foreign genetic material (transgenes) into mammalian cells *in vitro* has led to a great increase in the understanding of mechanisms of eukaryotic gene regulation. Particularly powerful has been the transgenic mouse model, through which many important mechanisms involved in the regulation and tissue-specific expression of mammalian genes have been identified and characterized. Long-term *in vivo* studies and many *in vitro* gene-transfer systems are limited by the relative inefficiency of gene transfer by chemically mediated methods of transfection. These difficulties have been resolved by the development of viral vectors capable of delivering and expressing foreign genetic information in human and other mammalian cells with much greater efficiency. Vectors based on murine and avian retroviruses have become useful for many of these kinds of studies *in vitro*; their efficiency suggests their potential value in human beings to correct, partially or wholly, deficiencies in human genetic diseases (1).

One of the simplest models for human gene therapy involves the correction of a disease phenotype *in vitro* through retroviral gene transfer, followed by the introduction of the genetically modified cells into appropriate organs of a recipient animal. Such cells could then repopulate a large portion of an abnormal organ to provide a previously missing function, as in the potential correction of hemoglobinopathies and other defects of bone marrow cells (2). Alternatively, the introduced cells could provide physiologically useful amounts of circulating gene products such as hormones or

coagulation factors, even when implanted in ectopic sites such as the skin (3, 4).

An alternative model is that in which the implanted genotypically and phenotypically altered cells might act as "sinks" by providing a metabolic trap for an accumulating toxic product. In a sense the low density lipoprotein (LDL) that accumulates in the plasma in familial hypercholesterolemia is such a "toxic product"—at least for arteries. Familial hypercholesterolemia is caused by a genetic deficiency of cellular receptors for LDL (5), and Brown, Goldstein, and their colleagues have thoroughly defined the pathophysiology and molecular biology of this disorder (6). A closely related and useful animal model, the LDL receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbit (7, 8), is available to study the feasibility of *in vivo* delivery of the LDL receptor gene through implantation methods. The mammalian liver is the most important single site for LDL metabolism, both as the major site for net removal of LDL particles from plasma and as the only organ able to excrete cholesterol from the body. Thus the ideal gene therapy for this disease would be through introduction of receptors into the liver. However, it is conceivable that an ectopic organ could serve as a source of receptor-mediated clearance as well, providing that an efficient "reverse cholesterol transport" system exists *in vivo*. The latter must occur to some extent even in the WHHL rabbit, since cholesterol accumulation does not occur in the majority of extrahepatic tissues despite marked hypercholesterolemia. As a preliminary step in that direction, we report the use of an efficient retroviral vector system to correct the genetic and metabolic defects in receptor-defective WHHL rabbit fibroblasts *in vitro*.

MATERIALS AND METHODS

Vector Construction. Plasmid pLDLR3, derived from pLDLR2 (9), contains the complete coding region of the LDL receptor and was kindly provided by D. W. Russell, J. L. Goldstein, and M. S. Brown (University of Texas Health Science Center at Dallas). This was used to prepare a retroviral vector (Fig. 1) containing a full-length human LDL receptor cDNA expressed from the long terminal repeat (LTR) of Moloney murine leukemia virus (10) and the Tn5 neomycin-resistance gene (11) expressed from an internal Rous sarcoma virus (RSV) LTR. A second Moloney murine leukemia virus LTR was introduced to provide transcriptional termination and other sequences needed for preparation of transmissible viruses. This vector DNA was desig-

Abbreviations: LDL, low density lipoprotein; β -VLDL, β very low density lipoprotein; WHHL, Watanabe heritable hyperlipidemic (rabbit); NZW, New Zealand White (rabbit); LTR, long terminal repeat; LPDS, lipoprotein-deficient serum.

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FIG. 1. Structure of retroviral vector pLDRNL. Open boxes represent the Moloney murine leukemia virus long terminal repeat (LTR). The solid box represents the human LDL receptor cDNA. The stippled box represents the Rous sarcoma virus LTR. The hatched box represents the Tn5 neomycin-resistance gene. The sizes of restriction fragments with *Sst* I or *Eco* RI are shown; kb, kilobases.

nated pLDRNL (L = LTR, D = LDL receptor, R = RSV promoter, N = Tn5 neomycin resistance, and L = LTR). Some control experiments were performed with a vector in which the LDL receptor sequence was inserted in the reverse orientation, pLDR^RNL (D^R = reverse orientation).

Cell Lines and Culture. The ecotropic ψ -2 (12) and amphotropic PA317 (13) packaging mouse fibroblast cell lines, rat cell line 208F (14), and rabbit fibroblast lines established in our laboratory from New Zealand White (NZW) and WHHL rabbits were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Neomycin-resistant cells were selected in medium containing the neomycin analog G418 (GIBCO; 400 μ g/ml for ψ -2, PA317, and 208F and 600 μ g/ml for WHHL).

Transfection and Infection. To produce transmissible virus, vector DNA was transfected into ψ -2 cells with the calcium phosphate coprecipitation method (15). One day after transfection, culture medium was collected, filtered through a 0.22- μ m membrane filter, and applied to PA317 cells in the presence of Polybrene (Sigma; 4 μ g/ml). Single colonies were isolated by selection in G418-containing medium and expanded into mass cultures. To titer the virus produced by selected PA317 cells, dilutions of cell-free culture medium from each PA317 clone were applied to 208F cells in the presence of Polybrene, and G418 selection was begun 24 hr later as described above. After 2 weeks, G418-resistant cells were detected by Giemsa staining and viral titers were determined. PA317 clones producing high virus titers were then assayed for human LDL receptor expression as described below. Cell-free culture media from selected PA317 cells expressing high human LDL receptor activity were then applied to WHHL cells, and resulting G418-resistant WHHL cells were tested for LDL receptor activity as described below.

RNA Analysis. Poly(A)⁺ RNA samples were prepared as described and subjected to electrophoresis in a 1.0% agarose/2.2 M formaldehyde gel, transferred to a nylon membrane, and hybridized to a ³²P-labeled human LDL receptor cDNA probe (16).

Monoclonal Antibody Purification. Murine monoclonal antibody IgGC7 (17) was purified from mouse ascites fluid after intraperitoneal injection of hybridoma cells (American Type Culture Collection) into pristane-primed BALB/c mice (18). Antibody purified by FPLC chromatography on a staphylococcal protein A-Sepharose 4B column (Pharmacia) gave a single 150-kDa band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (NaDodSO₄/PAGE) in 10–15% minigels (Pharmacia Phast gels). Iodination was achieved with the lactoperoxidase technique. Specific activities were 2000–5000 cpm/ng.

Antibody Binding Experiments. Specific binding of IgGC7 was determined with modifications of the techniques of Beisiegel *et al.* (17). Cells (12,000–14,000 per well) were plated in 96-well flat-bottom microtiter plates in DMEM/10% fetal calf serum for 48 hr. LDL receptor activity was up-regulated by growth for 24–48 hr in DMEM containing 5

mg/ml lipoprotein-deficient serum (LPDS). Ice-cold medium containing labeled IgGC7 in the presence or absence of a 100-fold excess of unlabeled IgGC7 was then incubated with the fibroblast monolayers for 1 hr. Medium was removed, the cells were washed (19) and solubilized with Bio-Rad Lowry buffer *in situ*, and cell protein content was determined by using a Titertek Multiskan plate reader. An aliquot of solubilized cells from each well was then assayed for ¹²⁵I radioactivity and specific binding was calculated as the difference in amounts bound in the presence and absence of excess unlabeled antibody (17).

Degradation Experiments. Specific degradation of labeled IgGC7 (or LDL) was measured as previously described (17, 20) except that experiments were performed on cells in 96-well microtiter plates as noted above. DMEM containing LPDS (or fetal calf serum), with iodinated IgGC7 (or LDL) in the presence or absence of excess unlabeled IgGC7 (or LDL), was incubated with cells at 37°C for 5 hr. The amount of trichloroacetic acid- and silver nitrate-soluble radioactive material in the medium was determined, and specific degradation was calculated as difference between values obtained in the presence and absence of excess unlabeled antibody (or LDL).

Cholesterol Esterification. Cellular cholesterol esterification was determined as described (21). Fibroblasts grown in 35-mm plates were exposed to LPDS for 48 hr. Cholesterol esterification was measured by determining the net incorporation of oleate into cellular cholesterol esters in the presence and absence of added LDL.

Ligand Blots. Ligand blots were performed with rabbit β very low density lipoprotein (β -VLDL) (22) labeled with Iodo-Gen (Pierce) to give a specific activity of 300 cpm/ng. Fibroblasts, grown in 100- to 150-mm plates, were exposed to LPDS for 48 hr. Cells were chilled to 4°C, scraped from the plates, and solubilized in a Triton X-100- and urea-containing buffer (23), and 100 μ g of protein was separated on NaDodSO₄/4–10% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes, "blocked" with 5% bovine serum albumin in phosphate-buffered saline and then incubated at 27°C with ¹²⁵I- β -VLDL (5 μ g/ml) for 4 hr in fresh blocking buffer in the presence or absence of 10 mM EDTA. The nitrocellulose membranes were washed and air-dried, and autoradiograms were prepared (24).

RESULTS

The retroviral vector pLDRNL, expressing both a full-length copy of the human LDL receptor cDNA and the Tn5 neomycin-resistance gene, was constructed as shown in Fig. 1. In addition, a control construct, pLDR^RNL, which was the same as pLDRNL except that the LDL receptor cDNA was placed in reverse orientation, was prepared. These vectors were used to transfect ecotropic mouse ψ -2 "packaging" cells. One day after transfection, culture media were applied to a second amphotropic "packaging" mouse fibroblast cell line, PA317, to produce high titer amphotropic virus (13). Of 18 single colonies that survived G418 selection for 2 weeks, 5 were found to produce viral titers greater than 10⁵ colony-forming units/ml.

To detect expression of human LDL receptor activity in infected, virus-producing PA317 cells, specific binding and degradation of IgGC7, a murine monoclonal antibody specific for the human LDL receptor (17), were measured. Beisiegel *et al.* (17) have previously shown that the LDL receptor binds, internalizes, and degrades IgGC7 much like its natural ligand, LDL. Human fibroblasts, grown in the absence or presence of lipoproteins (to up- or down-regulate receptors, respectively), as well as uninfected PA317 and a clone of PA317 infected with the reverse LDL construct, pLDR^RNL, were also studied as controls. As shown in Fig. 2 (column 3),

FIG. 2. Binding (A) and degradation (B) of ^{125}I -labeled IgGC7 in human fibroblasts and in PA317 cells grown in LPDS (unless otherwise stated) before and after retrovirus vector infection. The amount of specific binding or degradation is expressed as a percentage of IgGC7 bound or degraded by human fibroblasts grown for 48 hr in LPDS. Column 1, human fibroblasts; column 2, human fibroblasts grown in the presence of lipoproteins (fetal calf serum); column 3, uninfected PA317 fibroblasts; column 4, PA317 fibroblasts after infection with the virus pLD^RRNL; columns 5–9, PA317 fibroblast clones after infection with pLD^RRNL. The clone represented by column 5 is later referred to as PA317 clone 12 and that represented by column 7 is referred to as PA317 clone 22. The value for 100% binding in A was 45.3 ng/mg; 100% degradation in B was 85.6 ng/ml. Each point is the mean $\pm$ SD of quadruplicate wells.

uninfected PA317 failed to bind or degrade IgGC7, demonstrating that IgGC7 does not bind to the native mouse LDL receptor. PA317 cells infected with the reverse LDL construct also failed to bind IgGC7 (column 4). However, all five clones infected with the correctly oriented LDL construct showed significant amounts of IgGC7 binding, degradation, or both compared to uninfected cells (columns 5–9). Two of these five clones, clones 12 and 22 (columns 5 and 7), showed the highest degree of binding and degradation of IgGC7 and were selected for further study.

To document the integration of the human LDL receptor cDNA into the genome of infected PA317 cells, a Southern blot analysis of clones 12 and 22 was performed, using the human LDL receptor cDNA probe (16). Both blots showed the identical 5.8- or 2.1-kb band after digestion with *Sst* I or *Eco*RI, respectively (data not shown; see Fig. 1).

Culture media of PA317 clones 12 and 22 were collected and filtered, and viral titers were confirmed to be 2×10^5 colony-forming units/ml. Viruses from these two clones were used to infect cultures of WHHL cells and, after 2 weeks of selection with G418, surviving infected cells were tested for expression of LDL receptor activity. As shown in Fig. 3, wild-type WHHL fibroblasts (column 3) neither bound nor degraded IgGC7. However, fibroblasts infected with virus from either PA317 clone 12 or PA317 clone 22 (columns 4 and 5) bound and degraded IgGC7 at levels similar to the level

seen with human fibroblasts (column 1). Infected WHHL fibroblast clones were also shown to contain a full-length proviral transcript of 5.8 kb on RNA blots when hybridized with an LDL receptor cDNA probe, while uninfected WHHL cells did not contain such a transcript (Fig. 4A).

The size of the LDL receptor protein present in the infected WHHL cells was estimated from ligand blots of solubilized membrane protein using labeled rabbit β -VLDL, a ligand that binds with high affinity to the LDL receptor (22). As shown in Fig. 4B, uninfected WHHL cells showed only a trace amount of a 110-kDa protein (lane 3), consistent with previous reports that wild-type WHHL fibroblasts contain only a trace of functional LDL receptors (8, 22). Normal rabbit fibroblasts (lane 2) showed a binding protein of molecular mass similar to that of the WHHL cells. However, WHHL fibroblasts infected with virus from either clone 12 or clone 22 (lanes 4 and 5) showed considerable binding of β -VLDL to a 130-kDa protein, consistent with the molecular mass of the LDL receptor present in human fibroblasts (lane 1). In addition there was a small amount of binding to a smaller (approximately 110-kDa) protein.

To demonstrate that the introduced LDL receptors were functional with respect to their natural ligand, we examined the ability of the infected cells to process human LDL. Both of the infected WHHL fibroblast lines approached the capacity of normal human fibroblasts to degrade human LDL

FIG. 3. Binding (A) and degradation (B) of ^{125}I -labeled IgGC7 in human fibroblasts and in WHHL fibroblasts grown in LPDS (unless otherwise stated) before and after retrovirus vector infection. Specific binding or degradation of IgGC7 is expressed as a percentage of IgGC7 bound or degraded by human fibroblasts grown for 48 hr in LPDS. Column 1, human fibroblasts; column 2, human fibroblasts down-regulated with fetal calf serum; column 3, wild-type WHHL fibroblasts; column 4, WHHL fibroblasts after infection with virus derived from PA317 clone 12; column 5, WHHL fibroblasts after infection with virus derived from PA317 clone 22. The value for 100% binding in A was 44.9 ng/mg; 100% degradation in B was 226.9 ng/ml. Each point is the mean $\pm$ SD of quadruplicate wells.

FIG. 4. (A) Blot hybridization analysis of poly(A)⁺ RNA prepared from infected or uninfected WHHL cells. The filter was probed with ³²P-labeled human LDL receptor cDNA. Lane 1, uninfected cells; lanes 2 and 3, cells infected with virus from PA317 clone 12 or 22, respectively. (B) ¹²⁵I-labeled β-VLDL ligand blot of detergent-solubilized fibroblast membrane protein. Blot 1 was performed in the absence of EDTA and blot 2 was performed in the presence of EDTA. Lane 1, human fibroblasts; lane 2, NZW fibroblasts; lane 3, uninfected WHHL fibroblasts; lanes 4 and 5, WHHL fibroblasts after infection with virus from PA317 clone 12 or 22, respectively. Note the absence of binding in the presence of EDTA.

(Fig. 5). When the LDL receptor pathway is intact, uptake of LDL leads to enhancement of cellular cholesterol esterification. Incubation of wild-type WHHL cells with LDL did not promote cellular cholesterol esterification (Fig. 5 *Inset*), but when the two infected WHHL cells were incubated with LDL, stimulation of cholesterol esterification occurred to a degree comparable to that seen in normal human fibroblasts (Fig. 5 *Inset*).

DISCUSSION

We report here the development of a retroviral vector system that transduces and expresses the human LDL receptor cDNA with surprising efficiency in receptor-defective WHHL rabbit fibroblasts *in vitro*. Ligand blots performed with β-VLDL demonstrated a 130-kDa membrane protein in the WHHL fibroblast, similar to the known size of the LDL receptor in human fibroblasts, suggesting the faithful synthesis and membrane insertion of an intact human LDL receptor protein. The molecular mass of the endogenous rabbit receptor appears to be ≈110 kDa, and it is interesting to note that there appeared to be a small amount of binding to a 110-kDa protein as well in the infected WHHL cells. It is unclear whether this represents a precursor or alternatively processed form of the human receptor or stabilization of endogenous rabbit LDL receptor.

The introduced human receptor appears to be fully competent in the WHHL cell. Binding and degradation of IgGC7

were similar to those in human fibroblasts. The ratio of IgGC7 degraded over 5 hr to that bound (at 1 hr) in the two infected WHHL fibroblast lines was 10.1 and 10.6, comparable to (actually even greater than) the ratio of 5.7 seen in human fibroblasts, suggesting (but not proving) that the newly introduced LDL receptors in the WHHL fibroblasts were recycling normally (or possibly even faster than normal). The infected WHHL cells were also able to internalize and degrade human LDL at rates comparable to the rate observed in normal NZW rabbit fibroblasts and normal human fibroblasts. LDL-stimulated enhancement of cellular cholesterol esterification is a sensitive indicator of an intact LDL receptor pathway. Thus, the observation that LDL stimulated cholesterol esterification in infected WHHL cells but not in wild-type cells further substantiates the functional integrity of the LDL receptors expressed from the transduced cDNA. Since the introduced cDNA contains only coding sequences of the LDL receptor, these studies confirm that the coding portions of the gene are sufficient for effective synthesis, insertion into the membrane in correct orientation, and normal cellular processing (22).

To correct LDL deficiency in a receptor-deficient animal model or in a receptor-deficient patient, the hepatocyte would be the most appropriate cell into which functional LDL receptors should be introduced, as discussed above. The liver is the single most active organ in the body for serum LDL clearance (25). Indeed, hepatocytes are suitable target cells for retrovirus-mediated gene transfer (26). Phenotypically corrected hepatocytes infected *in vitro* could be im-

FIG. 5. LDL receptor functional assays. To assay LDL degradation, ¹²⁵I-labeled human LDL (¹²⁵I-LDL; specific activity of 600–800 cpm/ng) was prepared as previously described (19). Increasing amounts of ¹²⁵I-LDL were added to indicated fibroblasts in the presence or absence of a 25-fold excess of unlabeled LDL, and specific LDL degradation was determined (19). FCS, fetal calf serum. Each point is the mean ± SD of quadruplicate determinations. (*Inset*) Cholesterol esterification. Cholesterol esterification in the indicated fibroblasts was determined in the absence (solid bar) or presence (open bar) of 100 μg of LDL per well. The amount of cholesterol esterification is expressed as nmol of cholesterol ester formed per mg of cell protein. Each bar is the mean ± SD of triplicate determinations.

planted into the liver itself or other organs to complement a genetic defect (27). Unfortunately, the number of receptors that must be introduced may be very large compared, for example, to the number needed to correct an enzyme deficiency. Even though each receptor recycles every 5 or 10 min, it still can transport only a few hundred LDL molecules daily. Fortunately, the LDL receptor cDNA can and would be introduced with a 5' region *unresponsive* to the normal regulatory signals. Thus, transcription of the introduced DNA would not be down-regulated by high plasma LDL, a decided advantage if one were to attempt to restore receptor activity in a hyperlipidemic animal. It may even be possible to achieve over-expression through the use of suitable enhancers or tissue-specific regulatory signals. Even if one could introduce receptor activity equivalent to one-half the normal level, one would have simply converted a homozygote to a heterozygote. Although that might be considered only a limited therapeutic triumph, the difference in longevity of the homozygote and the heterozygote is 2–3 decades. Furthermore, traditional pharmacological treatments (that are receptor independent) may become more effective. For example, drugs that suppress production of cholesterol or VLDL synthesis might then further lower LDL levels or even normalize them.

Would anything be accomplished by introducing the receptor into extrahepatic tissues? Peripheral tissues are capable of removing LDL from the plasma compartment and thus lowering plasma LDL. However, the delivered cholesterol then has no exit except through "reverse cholesterol transport" (i.e., transport of the cholesterol back to the liver for disposal). The crucial question is whether the reverse cholesterol transport system in receptor-deficient subjects could return cholesterol to the liver at a sufficient rate to prevent damage through cholesterol deposition in tissues. The very fact that receptor-deficient animals and patients develop atherosclerosis suggests that reverse cholesterol transport does not work fast enough, at least in the case of the aorta. Yet the arterial lesion (and the xanthoma) may be peculiar in that macrophages accumulate there and take up excess LDL via the scavenger or acetyl-LDL receptor. Other tissues do not accumulate cholesterol to any significant extent even though the absolute rate of LDL degradation is considerably increased in receptor deficiency (25, 28). Thus, reverse cholesterol transport from most peripheral tissues is "keeping up" at ambient rates of LDL degradation and might keep up even in the face of a further increase in rate of degradation. Even if this were accomplished at the cost of an increase in steady-state cholesterol content in subcutaneous tissues, for example, it might have no ill effects. In short, it may not be *a priori* unreasonable to introduce LDL receptors into both liver and extrahepatic tissues.

The implantation of genotypically and phenotypically corrected fibroblasts may provide an effective approach to the therapy of some forms of genetic disease, not only by providing a source of circulating serum proteins, as proposed for hormone deficiencies and defects of coagulation proteins, but also by providing a source of functional ectopic receptors for other kinds of metabolic disease. The WHHL rabbit provides one particularly useful model system for the study and development of the methods for efficient and stable gene transfer and for cell implantation that will be necessary if such an approach is to become feasible. Clearly there are many conceptual and technical questions that still need to be answered. However, these studies suggest that gene therapy may become possible for the correction of familial hypercholesterolemia.

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