Post-transcriptional regulation of retroviral vector-transduced low density lipoprotein receptor activity

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Abstract We have reported the use of a retroviral vector to introduce the low density lipoprotein (LDL) receptor gene into receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbit fibroblasts (Miyanohara, A., M. F. Sharkey, D. Steinberg, J. L. Witztum, and T. Friedmann. 1988. *Proc. Natl. Acad. Sci. USA.* **85:** 6538-6542). Because the cDNA for the LDL receptor did not contain the 5' sterol regulatory element that confers sterol-mediated inhibition of LDL receptor transcription, we did not anticipate that LDL receptor activity transduced by this vector would be sterol-responsive. However, we now demonstrate sterol-mediated down-regulation of receptor protein in the infected cells by a mechanism that appears to be mediated at a post-transcriptional level. Down-regulation of LDL receptor activity occurred when infected WHHL cells were preincubated with either LDL or cholesterol plus 25-hydroxycholesterol. Identically organized vectors bearing cDNAs encoding irrelevant genes such as firefly luciferase or bacterial β-galactosidase exhibited no sterol regulation of reporter gene activity. Insulin receptor activity in WHHL fibroblasts and in WHHL fibroblasts infected with the LDL receptor retroviral vector was also unchanged by sterol. Transfection of LDL receptor-deficient Chinese hamster ovary (CHO) cells with a nonretroviral vector containing the same LDL receptor cDNA also resulted in sterol responsiveness of the transduced LDL receptor. These experiments suggest that the effect of sterol was specific for the LDL receptor transcript. Transgene LDL receptor mRNA levels from the infected cells were unaffected by sterol, indicating that the sterol-mediated reduction in LDL receptor activity did not result from alterations in steady state mRNA levels. These data suggest the existence of post-transcriptional level mechanisms that are responsible for sterol regulation of expression of the transduced LDL receptor gene.—**Sharkey, M. F., A. Miyanohara, R. L. Elam, T. Friedmann, and J. L. Witztum.** Post-transcriptional regulation of retroviral vector-transduced low density lipoprotein receptor activity. *J. Lipid Res.* 1990. **31:** 2167-2178.

Supplementary key words Watanabe rabbits • sterol regulation of LDL receptor gene

One of the most common inborn errors of metabolism is familial hypercholesterolemia (FH) (1). In homozygous FH, a complete deficiency in LDL receptors results in marked hypercholesterolemia and accelerated atherosclerosis, often producing fatal myocardial infarction even in the first decade of life. Currently, there are no effective therapeutic modalities short of heroic measures such as long term plasmapheresis (2) or liver transplantation (3). Theoretically, a preferable mode of treatment for homozygous FH patients would be "replacement gene therapy," leading to the introduction of a normal complement of fully functional cellular LDL receptors (4).

An inbred strain of laboratory rabbits, the Watanabe Heritable Hyperlipidemia Rabbit (WHHL), has a genetic defect in LDL receptors similar to that found in one class of homozygous FH patients (5) and consequently provides an excellent animal model for studies of gene replacement therapy in FH (6). Previous studies in our laboratory have shown that a retroviral vector can be used to transfer the LDL receptor gene to WHHL fibroblasts in vitro, and lead to functional LDL receptor activity (4). Wilson et al. have shown similar results using WHHL hepatocytes in culture (7).

To obtain efficent expression of LDL receptor activity, we prepared a retroviral vector containing a full-length human LDL receptor cDNA expressed from the long terminal repeat (LTR) of the Moloney murine leukemia virus (vector LDRNL, **Fig. 1**). This construct does not contain the recognized sterol regulatory element (8) that confers sterol-dependent inhibition of transcription of the endogenous LDL receptor gene. Therefore we anticipated that the transduced LDL receptor activity would be equivalent or even greater than the endogenous LDL receptor activity found in wild-type rabbit fibroblasts and

Abbreviations: LDL, low density lipoprotein; WHHL, Watanabe heritable hyperlipidemic rabbit; FH, familial hypercholesterolemia; CHO, Chinese hamster ovary; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LTR, long terminal repeat; LDS, lipoprotein-deficient serum; FCS, fetal calf serum; RSV, Rous sarcoma virus; SV40, simian virus 40; h-LDL-R, human LDL receptor; luc, luciferase; β-gal, β-galactosidase; Neo, Tn5 neomycin-resistance; DHFR, dihydrofolate reductase.

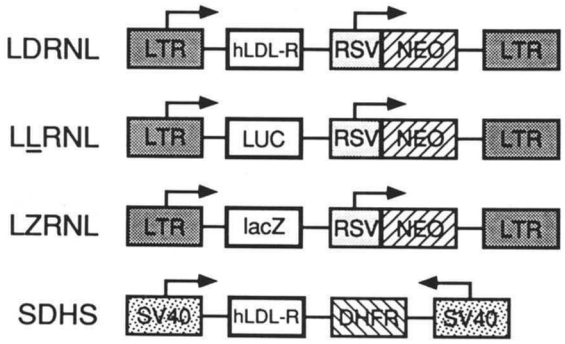

Fig. 1. Structure of LDRNL, LLRNL, LZRNL, and SDHS vectors. Letters and short abbreviations are used as follows: L or LTR, Moloney murine leukemia long terminal repeat; D or hLDL-R, human LDL receptor; R or RSV, Rous sarcoma virus; N or NEO, Tn5 neomycin resistance gene; L or LUC, Luciferase; Z or lacZ, *E. coli* β-galactosidase; S or SV40, Simian virus-40 early promoter/enhancer; H or DHFR, mutant dihydrofolate reductase.

that the transduced receptor activity would not be regulated by the presence of sterol in the medium. Indeed, two WHHL cell lines were established that stably expressed LDL receptor activity similar to that found in wild type cells (4). The transduced LDL receptor activity was down-regulated by sterols, but transduced LDL receptor mRNA levels were unaffected by sterol preincubation. These observations suggest that sterols can inhibit LDL receptor expression not only at the transcriptional level, but also at the level of translation or protein stability.

EXPERIMENTAL PROCEDURES

Vector construction and preparation of retroviruses

The retrovirus vector pLDRNL (4) contains a full-length human LDL receptor cDNA (9) expressed from the long terminal repeat (LTR) of Moloney murine leukemia virus (MoMLV) and the Tn5 neomycin-resistant gene expressed from an internal Rous sarcoma virus (RSV) LTR. The h-LDL-R cDNA in this vector is a 2.6 kb HindIII fragment from pTZ1 obtained from Drs. Russell, Brown, and Goldstein. It includes 14 bp of 5'-noncoding sequence and terminates after the A residue of the TGA translational termination codon. Similar vectors were prepared in which the *E. coli* β-galactosidase or firefly luciferase genes were substituted in place of h-LDL-R. The lacZ vector (LZRNL) contains a 3.1 kb SalI-SmaI fragment of the *E. coli* β-galactosidase gene (a gift from C. Cepko) derived from plasmid pSP6-β-GAL (10, 11) including 16 bp of 5'-noncoding sequence. The luciferase vector (LLRNL) contains a 1.7 kb HindIII-SspI fragment derived from plasmid pJD204 (12) including 22 bp of 5'-noncoding region. Because the cDNA fragments encoding for lacZ or luciferase were inserted into the same restriction enzyme site of the vector as h-LDLR cDNA, the structure of these plasmids is exactly the same as pLDRNL except for the inserted reporter genes.

The vector SDHS (Fig. 1) used in the CHO cell experiments was kindly provided by Drs. William Chen and Michael G. Rosenfeld. The human LDL receptor transcription unit utilized the SV40 early promoter/enhancer and the SV40 late splice/polyadenylation site. A mutant dihydrofolate reductase gene provided a dominant selectable marker (13). It also utilized an SV40 early promoter/enhancer.

Cell culture

Cell lines utlized in these studies were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cell lines include: ψ-2 (14) and PA317 (15) mouse fibroblast packaging cell lines; rabbit fibroblast cell lines established in our laboratory from New Zealand White (NZW) rabbits and WHHL rabbits, respectively; a human fibroblast line established from normal infant foreskin; and an LDL receptor-deficient Chinese hamster ovary (CHO) cell line ldlA-7 (16), kindly provided by Dr. M. Krieger. Neomycin-resistant cells were maintained in DMEM with 10% FCS containing the neomycin analog G418 (400 μg/ml for PA317 and 600 μg/ml for WHHL). Methotrexate-resistant CHO ldlA-7 cells were maintained in DMEM with 10% dialyzed FCS containing 10 μM methotrexate.

Transfection, infection, and selection

Retroviral vector (Fig. 1, LDRNL, LLRNL, and LZRNL) transfection into ψ-2 cells, collection of transmissible virus, and subsequent infections of PA317 and WHHL cells were performed exactly as described previously (4). The calcium phosphate co-precipitation technique (17) was used for transfection of the nonretroviral vector SDHS (Fig. 1) into CHO ldlA-7 cells. Methotrexate-resistant CHO ldlA-7 cells were selected in 400 nM methotrexate in DMEM with 10% dialyzed FCS. Gene amplification was accomplished using stepwise increases in methotrexate to a final concentration of 10 μM.

Lipoproteins

LDL (d 1.025–1.063 g/ml) and lipoprotein-deficient sera (LDS) were isolated from normal human plasma under sterile conditions by density gradient ultracentrifugation (18), and then dialyzed against phosphate-buffered saline (PBS, 0.154 M NaCl, 21 mM Na_2HPO_4, 15 mM NaH_2PO_4, 0.3 mM EDTA, pH 7.35). Rabbit β-very low density lipoprotein (β-VLDL) (d < 1.006 g/ml) was isolated from New Zealand White rabbit plasma after the rabbits had been fed a high cholesterol diet for several months. The protein concentrations were determined by a modification of the Lowry technique using a bovine serum albumin standard (19).

Antibodies

Purification of human LDL receptor specific monoclonal antibody IgGC7 (20) and control monoclonal antibody AmL-43-F11 directed against acetyl LDL was performed as described (4) from murine ascites using staphylococcal protein A chromatography on a fast protein liquid chromatography unit (Pharmacia, model LCC-500).

Binding assays

Specific binding of labeled IgGC7 was performed using a 96-well microtiter plate format as previously described (4). Cells were plated at 12,000 cells per well in 96-well flat-bottom microtiter plates in DMEM/10% FCS. Forty eight hours after plating, the cell media were replaced with the indicated preincubation media containing DMEM supplemented with either 5 mg/ml LDS or 10% FCS for 24 h. The cells were placed on ice for 20 min, then washed twice with 200 μl ice-cold PBS containing 5 mg/ml BSA, then incubated for 1 h on ice trays with iodinated ligand in the presence or absence of 100-fold excess unlabeled ligand in PBS with 5 mg/ml BSA. IgGC7 was iodinated using lactoperoxidase technique (21); a specific activity of 1000–3700 cpm/ng was obtained. Unbound antibody was removed by washing the cells once with 200 μl PBS with 5 mg/ml BSA and then twice with 200 μl PBS. Cell protein content of individual wells was determined using Bio-Rad Lowry buffer and a Titertek Multiskan plate reader according to the manufacturer's directions. An aliquot of the solubilized cells was then assayed for ^{125}I radioactivity.

Degradation assays

Specific degradation of labeled LDL or IgGC7 was performed using a 96-well microtiter plate format as described previously (4). LDL was labeled with Iodogen (22) or iodine monochloride (23) and had a specific activity of 250–650 cpm/ng. IgGC7 was labeled as described above. The cells were plated as described above. After a 24-h preincubation, cells were washed twice with 200 μl DMEM, then incubated for 5 h at 37°C with iodinated ligand in the presence or absence of 50- to 100-fold excess unlabeled ligand in 200 μl DMEM supplemented with 5 mg/ml LDS. The amount of trichloroacetic- and silver nitrate-soluble radioactivity in the medium was then determined. Cell protein content of individual wells was determined as described above.

Luciferase and β-galactosidase assays

Quantitative assays for firefly luciferase and *E. coli* β-galactosidase were performed as previously described (10, 12). Subconfluent cells were incubated in DMEM supplemented with either 5 mg/ml LDS or 10% FCS for 24 h prior to preparation of cells extracts. The protein concentrations of each extract were measured using the Coomassie Protein Assay Reagent (Pierce Chemical Co., Rockford, IL).

Insulin receptor assays

Insulin receptor activity was studied with specific binding of labeled insulin as described by McClain and colleagues (24). Cells were plated at 50,000 cells per 35-mm plate in DMEM supplemented with 10% FCS. Three days after plating the cell media were replaced with DMEM supplemented with either 5 mg/ml LDS or with 10% FCS. After a 48-h preincubation, cells were cooled on ice trays, washed twice with binding buffer (24), and then incubated for 3 h at 4°C with 0.2 ng/ml ^{125}I-labeled porcine insulin in the presence or absence of 500,000-fold excess unlabeled insulin. The insulin had a specific activity of 408,000 cpm/ng and was kindly provided by Dr. J. Olefsky. The cells were washed twice with binding buffer, once with BSA-free binding buffer, and then removed from the plates with 0.2 N NaOH. Cell protein content of each plate was determined and aliquots were assayed for ^{125}I radioactivity. Specific binding was calculated as described (24).

Ligand blots

Ligand blots were performed with rabbit β-VLDL as described previously (4). Fibroblasts were plated in 150-mm plates in DMEM with 10% FCS, and then incubated for another 48 h with either 5 mg/ml LDS or fresh FCS. The cells were solubilized in a Triton X-100- and urea-containing buffer and 100 μg of solubilized protein was separated on $NaDodSo_4$ (SDS)–7% polyacrylamide gels. The separated proteins were transferred to nitrocellulose membranes, blocked with PBS with 5% nonfat dry milk, and then incubated with ^{125}I-labeled β-VLDL as described in the legend. Autoradiograms were prepared as described (4).

RNA analysis

Cytoplasmic RNA samples from subconfluent cells incubated for 24 h in DMEM supplemented with either 5 mg/ml LDS or 10% FCS were prepared as described (25, 26), subjected to electrophoresis in a 1.0% agarose–0.66 M formaldehyde gel, and transferred to a nylon filter (4, 24). RNA transfer blots were hybridized with an h-LDL-R cDNA fragment that was labeled to a specific activity of 10^9 cpm/μg with [^{32}P]dATP using the random primer method (26). The filter was stripped by boiling in SDS–sodium citrate solution and rehybridized with a cDNA probe for chicken β-actin (27). The autoradiogram was quantitated by a scanning laser densitometer interfaced with an integrator.

[^{32}S]Methionine pulse-chase

The time course of LDL receptor processing was studied with a pulse-chase protocol using incorporation of

[^{35}S]methionine into LDL receptor protein followed by immunoprecipitation with IgGC7 as described by Tolleshaug et al. (28). A control precipitation was performed using monoclonal antibody AmL-43-F11, which is directed against an irrelevant antigen (acetyl-LDL) but which has an IgG subclass identical to that of IgGC7. Sucrose gradient precipitates of immune complexes were separated with SDS-6% polyacrylamide gels, and processed as described (29).

RESULTS

When WHHL fibroblasts expressing the transduced LDL receptor were grown in the grown in the presence of fetal calf serum (FCS), which contains LDL and other lipoproteins, significant sterol-mediated down-regulation of LDL receptor activity was found compared to cells grown in lipoprotein-deficient serum (LDS) (**Fig. 2**). This was not anticipated since the retroviral LTR promoter driving LDL receptor expression in the vector LDRNL (Fig. 1) was not known to be sterol-responsive, and because the LDL receptor cDNA used in making this vector was not known to contain any endogenous sterol-responsive regions.

With normal wild-type cells it is known that sterol in the media is capable of down-regulation of LDL receptor activity (1). These sterols could be either in the form of LDL, which enters the cell via the LDL receptor, or in the form of solubilized cholesterol plus 25-hydroxycholesterol, which does not require LDL receptor for its entry into the cell. To determine whether similar events occurred in the infected WHHL fibroblasts, normal human fibroblasts, WHHL fibroblasts, and two infected WHHL fibroblast lines (WHHL-12 and WHHL-22 (4)) were preincubated for 24 h with either LDS, FCS, LDL, or cholesterol plus 25-hydroxycholesterol, and then assayed for their ability to bind, internalize, and degrade LDL. As shown in Fig. 2, preincubation in sterol-containing media, whether FCS, LDL, or cholesterol plus 25-hydroxycholesterol, substantially suppressed LDL degradation in normal human fibroblasts. This was also found in both infected WHHL cell lines, although the degree of receptor suppression was less than that found in normal cells. Thus, as in normal cells, cholesterol delivery to the infected WHHL cells via either an LDL receptor-dependent, or via an LDL receptor-independent mechanism, led to sterol suppression of receptor activity.

Sterol regulation of LDL receptor activity has been documented in many different cell lines (30). To determine whether sterol regulation of the receptor transgene would occur if the LDL receptor transgene was inserted into another cell line, murine fibroblast PA317-22, a helper cell line that had been infected with the vector LDRNL, was assayed for changes in transduced human LDL receptor expression after preincubations with sterol-

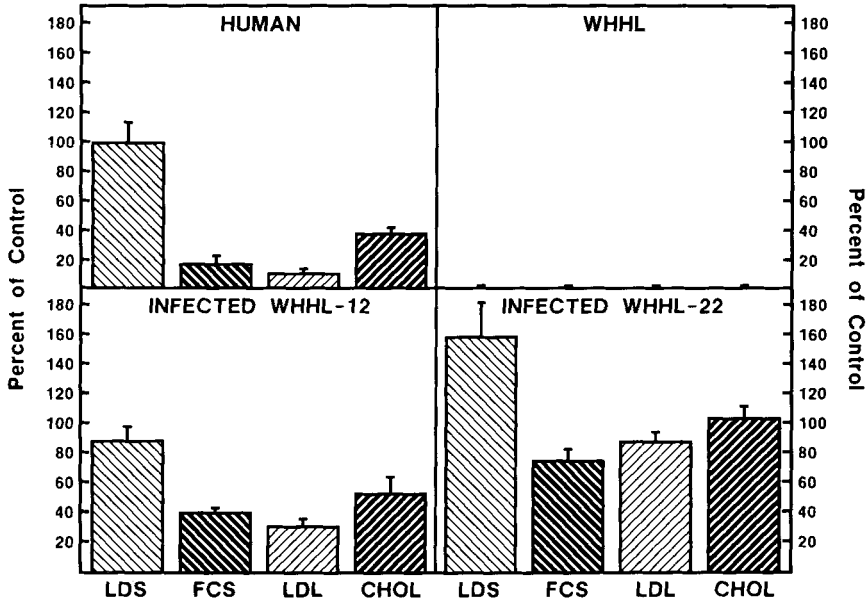

Fig. 2. LDL receptor activity in various cells grown in the presence of sterol. The figure displays the degradation of ^{125}I-labeled LDL in human, WHHL, infected WHHL-12, and infected WHHL-22 fibroblasts. Cells were preincubated for 24 h in either LDS, FCS, LDL (100 μg/ml), or cholesterol (10 μg/ml) plus 25-hydroxycholesterol (0.5 μg/ml), and then incubated with ^{125}I-labeled LDL (5 μg/ml) for 5 h in the presence and absence of 100-fold excess unlabeled LDL in DME supplemented with LDS. For each cell type the amount of specific LDL degradation is expressed as a percentage of the specific LDL degradation noted in the human fibroblasts grown in LDS. The value for 100% degradation was 4.1 μg/mg per 5 h. Each point is the mean and SD of quadruplicate wells.

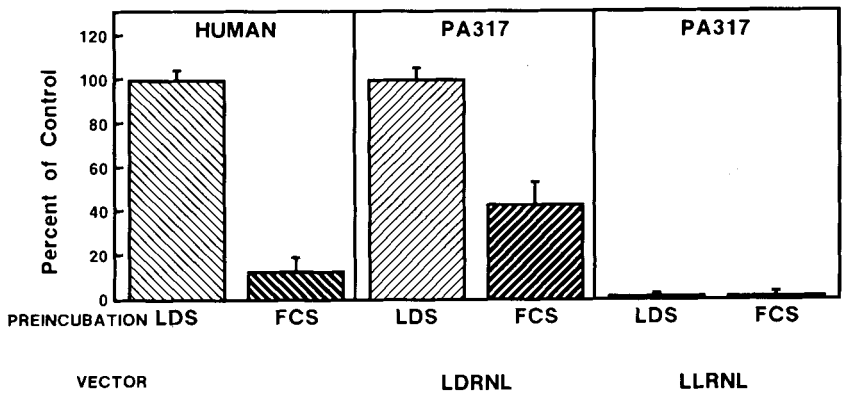

Fig. 3. Specific degradation of ^{125}I-labeled IgGC7 by human fibroblasts, PA317 fibroblasts infected with vector LDRNL, and PA317 fibroblasts infected with vector LLRNL. Indicated cells were preincubated with LDS or FCS for 24 h and then incubated with ^{125}I-labeled IgGC7 (0.6 μg/ml) in the presence or absence of a 100-fold excess of unlabeled ligand in media containing DME supplemented with LDS. The amount of specific degradation is expressed as a percentage of IgGC7 degraded by human fibroblasts grown in LDS. The value for 100% degradation in human fibroblasts preincubated in LDS was 108 ng/mg per 5 h. Each point is the mean and SD of quadruplicate wells.

containing media. IgGC7 (20), a monoclonal antibody specific for the human LDL receptor that is taken up and degraded by cellular LDL receptors as is LDL, was used in this experiment because it does not cross-react with the endogenous murine PA317 LDL receptor (4). **Fig. 3** shows that normal human fibroblast degradation of IgGC7 fell from 100% to 13% after a 24-h preincubation with FCS, while PA317-22 degradation of IgGC7 dropped from 100% to 43%. In a control experiment, PA317 cells infected with the vector LLRNL (Fig. 1), had, as expected, no detectable human LDL receptor activity. Thus, sterol regulation of transduced receptor is found to occur in the PA317-22 producer cell line as well as in infected WHHL-12 and WHHL-22 cell lines.

The effect of sterol on cell surface LDL receptor activity was similar to the suppression by sterol of LDL receptor-mediated degradation of both LDL and IgGC7. **Table 1** shows the effect of sterol preincubation on the amount of specific binding of IgGC7 to cell surface LDL receptor on normal human fibroblasts and infected WHHL fibroblasts. The presence of sterol in the preincubation media reduced IgGC7 binding from 100% to 30% in normal human fibroblasts, and from 72% to 33% in infected WHHL fibroblasts. The degree of cell surface LDL receptor down-regulation by sterol found using the IgGC7 binding assay is very similar to that found when either LDL or IgGC7 degradation assays are used.

To determine whether total LDL receptor content of the cells was regulated by sterols, we performed ligand blots using Triton-solubilized extracts of cell pellets obtained from normal human fibroblasts and from infected WHHL fibroblasts grown in the presence or absence of sterol (**Fig. 4**). Rabbit β-VLDL was used as a ligand in these experiments, since it binds to the LDL receptor with high affinity (4, 31). Presumably, these blots measure the total cell content of functional LDL receptors, including membrane-bound cell surface receptor and receptor in the cell interior. The intensity of the ligand blot of the 130 kD LDL receptor band in the infected cells preincubated in the absence of sterol was greater than the intensity of the receptor band prepared from cells grown in the presence of sterol, confirming that total cellular LDL receptor expression was influenced by cholesterol in the medium. Similar results were seen with the normal human fibroblasts. Thus, measurements of the LDL receptor transgene expression, either with LDL degradation, IgGC7 binding or degradation, or by β-VLDL ligand blots of total cellular protein, all indicate that sterols down-regulated LDL receptor protein expressed from the transgene in the infected WHHL cell lines.

To explore the possibility that sterol-responsive sequences in the viral LTR used to provide the promoter for the LDL receptor were involved in regulating the expression of the transduced LDL receptor gene, two different vectors were studied, LLRNL and LZRNL (Fig. 1), in

TABLE 1.

Preincubation Media	% IgGC7 Bound	
	Normal Human Cells	Infected Human Cells
LDS	100 ± 18	72 ± 7
FCS	30 ± 13	33 ± 13

Indicated cells were preincubated with LDS or FCS for 24 h and then incubated with ^{125}I-labeled IgGC7 (2.4 μg/ml) in the presence or absence of a 100-fold excess unlabeled ligand. The amount of specific binding is expressed as a percentage of IgGC7 bound by human fibroblasts grown in LDS. Each point is the mean ± SD from quadruplicate wells. The value for 100% binding was 21 ng/mg.

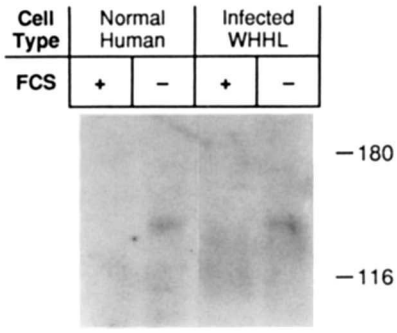

Fig. 4. ^{125}I-labeled β-VLDL ligand blot of detergent-solubilized fibroblast protein. The nitrocellulose membrane containing 100 μg per lane of solubilized cell protein was incubated at room temperature with ^{125}I-labeled β-VLDL 5 μg/ml (iodinated with ICl (22), sp act 160 cpm/ng) for 4 h in fresh blocking buffer. The membranes were washed, air-dried, and autoradiograms were prepared.

which cDNAs encoding firefly luciferase and bacterial β-galactosidase were inserted in place of the LDL receptor cDNA. The orientation and content of all other vector sequences were similar to those of LDRNL (Fig. 1). Two different cell lines infected with these reporter gene vectors, PA317 and WHHL, were then tested to determine whether sterol in the media would lead to suppression of transduced reporter gene expression. As shown in **Fig. 5**, neither the PA317 cells expressing a luciferase transgene nor the WHHL cells expressing a lacZ transgene demonstrated reporter gene regulation by sterol.

To examine the hypothesis that sterol regulation of the transduced LDL receptor activity was specific for the LDL receptor itself and not secondary to interaction with retroviral vector sequences, we used a nonretroviral vector, SDHS (Fig. 1), prepared by Drs. William Chen and Michael G. Rosenfield, to transfect the human LDL receptor into receptor-deficient ldlA-7 Chinese hamster ovary (CHO) (16) cells. In this case, LDL receptor transcription was directed by the SV40 early promoter/enhancer. LDL receptor activity in the transfected cells was then assayed to determine whether sterol preincubation down-regulated the transfected LDL receptors (**Fig. 6**). CHO cells prior to transfection showed minimal LDL degradation. The transduced cells had 50% of the activity found in normal human fibroblasts. The presence of sterol in the media suppressed the transduced LDL receptor activity, although the extent of suppression in the CHO cells was somewhat less than that noted in normal human cells. Thus, use of a nonretroviral vector for transfer of the LDL receptor gene also led to significant down-regulation of the expression of the LDL receptor transgene by sterol despite absence of any recognized 5' sterol responsive element sequences.

To rule out any nonspecific effects on membrane protein expression due to retroviral infection, neomycin analogue selection, or the presence of cholesterol in the media, cell surface insulin receptor numbers were measured with an insulin binding assay. As shown in **Fig. 7**, specific insulin binding was unaffected by sterol or by the acquisition of the human LDL receptor transgene.

To investigate the cellular mechanism responsible for sterol-mediated regulation of the transduced LDL receptor activity, we examined Northern blots to determine whether sterol affected the steady-state level of LDL receptor proviral transcripts in the infected WHHL cells. Total RNA was isolated from normal human fibroblasts and from infected WHHL fibroblasts grown in the presence or absence of FCS for 24 h and was then hybridized with ^{32}P-labeled human LDL receptor cDNA (**Fig. 8**). The normal human fibroblasts showed a marked reduction in endogenous LDL receptor mRNA after growth in FCS, consistent with previous studies, (Fig. 7A, lanes 3

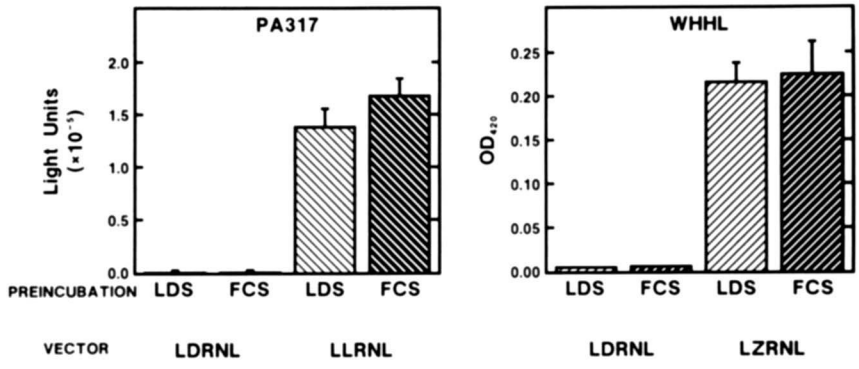

Fig. 5. Absence of sterol regulation of other reporter genes β-galactosidase and luciferase. Retroviral vectors LZRNL and LLRNL (Fig. 1), similar in design to vector LDRNL used to transduce LDL receptor activity, were used to transduce reporter genes β-galactosidase (Z) and luciferase (L) into PA317 and WHHL fibroblasts, respectively. Cells were preincubated with either LDS or FCS for 24 h and then β-galactosidase or luciferase activity was measured. As controls, similar activity studies were done in cells infected with the LDL receptor gene vector LDRNL.

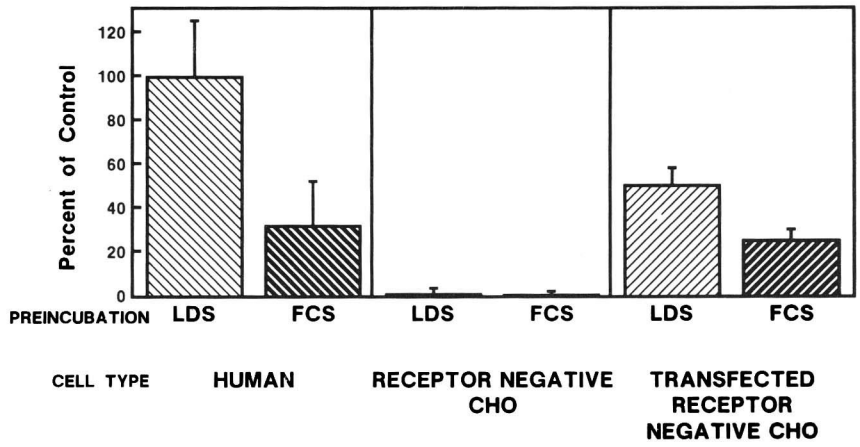

Fig. 6. Degradation of ^{125}I-labeled LDL in human fibroblasts, receptor-deficient CHO cells, and SDHS-transfected CHO cells. Cells were preincubated for 24 h with LDS or FCS and then incubated with ^{125}I-labeled LDL (10 μg/ml) in the presence of absence of 50-fold excess unlabeled LDL for 5 h. The amount of specific degradation is expressed as a percentage of LDL degraded by human fibroblasts grown in LDS. The value for 100% degradation in LDS-up-regulated human fibroblasts was 3.51 μg/mg. Each value is the mean and SD of quadruplicate wells.

and 4), while the infected fibroblasts showed no detectable suppression of LDL receptor mRNA (Fig. 8A, lanes 1 and 2). Note that under the conditions used, 30 μg of RNA from the normal human fibroblasts was required per lane on the Northern blot to produce a faint 5.3 kb band representing the endogenous receptor transcript. In contrast, under the same conditions, only 10 μg of RNA from the infected WHHL fibroblasts was needed to produce a heavy 5.8 kb band representing the transduced LDL receptor transcript. Hybridization (Fig. 8B) of the same filter used for the LDL receptor with the β-actin probe confirmed the differences in the amounts of RNA used. Thus, sterol preincubation apparently had no effect upon steady-state LDL receptor mRNA in infected WHHL cells, while it markedly reduced endogenous mRNA levels in normal human cells. Furthermore, despite the fact that LDL receptor activity was similar in the infected WHHL cells and in the normal human cells (Fig. 2), there was several-fold more h-LDL receptor mRNA in the infected WHHL cells than was found in the human cells. Quantification of the Northern blot with scanning laser densitometry confirmed that the infected WHHL cells grown in LDS had approximately a 12-fold increase in LDL receptor mRNA compared with normal human fibroblasts grown in LDS. Furthermore, the amount of LDL receptor mRNA in normal cells grown in LDS was 7-fold higher than that found in normal cells grown in FCS. In contrast, infected WHHL cells had the same amount of LDL receptor mRNA whether grown in LDS or FCS.

Possibly, some of the discrepancy between the transduced LDL receptor mRNA levels and LDL receptor activity in the infected WHHL cells might be the result of species-related inefficiency of processing of the human receptor gene product in rabbit cells. To test this idea, normal human fibroblasts were infected with the LDRNL virus (Fig. 1) and grown in G418-containing media for 2 weeks to select for stably infected cells. They were then assayed for LDL receptor mRNA levels and for LDL receptor activity. Levels of the proviral transcript encoding the LDL receptor (the 5.8 kb band) were indeed found to be several-fold higher in the infected cells than LDL receptor mRNA in the uninfected human cells (Fig. 8A, lanes 5 and 6 versus lanes 3 and 4). Quantification of the LDL receptor transcripts indicated that the level of

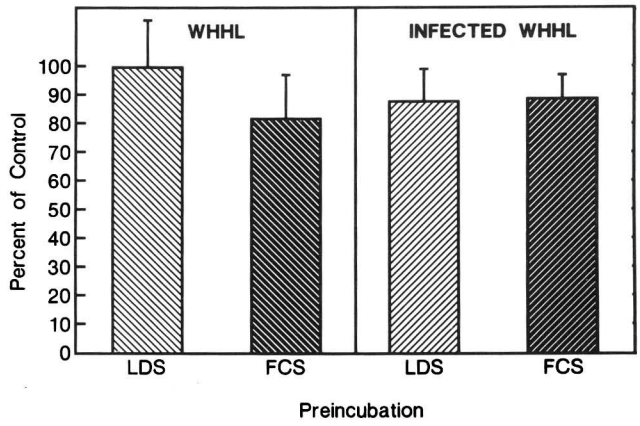

Fig. 7. Absence of sterol regulation of endogenous cell surface insulin receptor. ^{125}I-labeled porcine insulin binding was determined in uninfected and LDRNL-infected WHHL fibroblasts that were preincubated with either LDS or FCS for 48 h. Specific binding was determined as previously described (23) and represents the difference between binding in the presence and absence of excess unlabeled insulin. Each value is the mean and SD from triplicate dishes. Values are expressed as a percentage of specific binding to wild type WHHL cells grown in LDS (absolute value 1.03 nmol insulin bound/μg/cell protein).

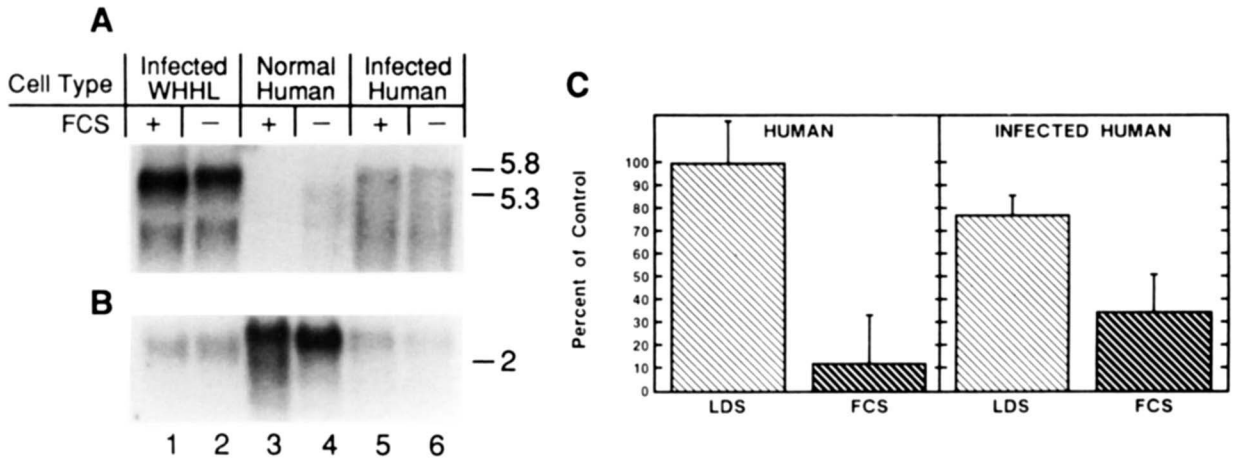

Fig. 8. Comparison by Northern blot analysis of the level of endogenous LDL receptor mRNA in normal human fibroblasts with that of LDL receptor transgene transcript in infected WHHL cells and infected normal human cells. A Northern blot of total cytoplasmic RNA prepared from normal human, infected WHHL, or infected human fibroblasts was hybridized with (A) ^{32}P-labeled h-LDL-R cDNA, then stripped of label and then rehybridized with (B) ^{32}P-labeled β-actin cDNA. Lane 1, infected WHHL cells grown in FCS; lane 2, infected WHHL cells grown in LDS; lane 3, normal human cells grown in FCS; lane 4, normal human cells grown in LDS; lane 5, infected human cells grown in FCS; lane 6, infected human cells grown in LDS. Ten μg of RNA was applied to lanes 1, 2, 5, and 6 and 30 μg was applied in lanes 3 and 4. (C) ^{125}I-labeled IgGC7 degradation in normal human fibroblasts and infected human fibroblasts grown in LDS and FCS. The experimental procedure was similar to that described in Fig. 3 except that 1.1 μg/ml ^{125}I-labeled IgGC7 was used. Specific IgGC7 degradation is expressed as a percentage of antibody degraded by normal human fibroblast grown in LDS. The value for 100% degradation was 720 ng/mg per 5 h. Each point is the mean ± SD of quadruplicate wells.

LDL receptor transcript was 8-fold higher in the infected human fibroblasts than in the normal human fibroblasts. Despite this, LDL receptor activity in the infected human cells was no greater than that found in normal cells (Fig. 8C). Furthermore, sterol suppression of receptor activity was again demonstrable, although at a reduced magnitude compared with that seen in normal fibroblasts (Fig. 8C). LDL receptor mRNA in the infected human fibroblasts did not increase with removal of sterol from the growth media (Fig. 8A, lanes 5 and 6). These data further support the hypothesis that sterol regulates the transduced LDL receptor at a post-transcriptional level. These experiments also suggest that species differences between provirus-expressed genes and infected cells are not responsible for the discrepancy between receptor mRNA levels and receptor activity noted in the infected WHHL cells.

In order for elevated receptor mRNA levels to result in physiological amounts of receptor activity, either receptor transcript translation, processing, or degradation must be altered in the infected cells. Schneider, Brown, and Goldstein (32) have demonstrated that WHHL fibroblasts possess an abnormality in the LDL receptor protein that results in its delayed processing and increase in size from 120 kD to 160 kD in the Golgi. Yamamoto et al. (5) demonstrated that LDL receptor cDNA from WHHL rabbits, as well as from a class II FH patient with abnormal LDL receptor processing, both contained small deletions in the ligand binding region. They postulated that abnormal folding of the mutant LDL receptor was responsible for the observed impairment of receptor protein processing. To rule out the possibility that the infected WHHL cells had an inherent defect in processing even normal LDL receptors, we studied the kinetics of synthesis of the normal transduced human LDL receptor in the WHHL cells. [^{35}S]Methionine pulse-chase experiments were performed with the infected WHHL cells (**Fig. 9**) to examine the likelihood of abnormal processing of the

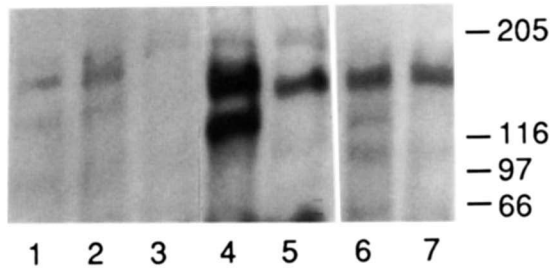

Fig. 9. LDL receptor processing in uninfected WHHL (lanes 1–3), infected WHHL (lanes 4, 5), and normal human (lanes 6, 7) fibroblasts. One 150-mm plate per lane of fibroblasts was plated at 1.5 × 10^6 cells per plate. Three to four days later media were replaced with DMEM supplemented with LDS 5 mg/ml for 48 h prior to labeling. The media were changed to methionine-free DMEM supplemented with LDS 5 mg/ml for 20 min, and then 208 μCi/ml of [^{35}S]methionine was added to the individual plates for a 2-h pulse incubation period followed by 0 (lanes 1, 2, 4, and 6) and 2 h (lanes 3, 5, and 7) chase incubation periods with DMEM supplemented with LDS 5 mg/ml. Each lane contains equivalent amounts of [^{35}S]methionine-labeled solubilized protein. IgGC7 was used for immunoprecipitation of lanes 2 through 7 while the control antibody was used for lane 1. The autoradiogram was produced by exposing the dried gel to Kodak X-Omat RP film for 72 h.

wild-type receptor. In WHHL cells there is persistence of predominantly immature 120 kDa [^{35}S]methionine-labeled receptor after a 2-h chase period. In contrast, in the infected WHHL cells, all of the [^{35}S]methionine found in the precursor 120 kDa receptor at the beginning of the chase period (lane 4) had become converted to the mature 160 kDa receptor (lane 5) after a 2-h chase period. The normal human control cells (lanes 6 and 7) also display both precursor and mature receptor forms before the chase period, but only mature receptor after a 2-h chase period. Note that the uninfected WHHL cells showed only faint bands in this region (lanes 2 and 3) similar to those found after immunoprecipitation with a nonspecific antibody (lane 1). These data confirm the original hypothesis of Yamamoto et al. (5) and demonstrate that WHHL cells can process the normal, wild-type LDL receptor in a normal fashion.

DISCUSSION

LDL receptor regulation is thought to occur primarily at the transcriptional level through interaction of sterol with the LDL receptor 5' upstream promoter (8). This promoter region includes an octanucleotide "sterol regulatory element" that has also been found in the promoters for 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (33) and HMG-CoA-synthase (34). While sterol affects both HMG-CoA reductase transcription and protein degradation (35), studies in normal cells indicate that sterol does not increase LDL receptor degradation (36, 37). As yet unidentified short-lived proteins that are important in LDL receptor gene transcription (38) and LDL receptor protein degradation (37) have been described. LDL receptor gene promoter elements are sensitive to growth activating agents (platelet-derived growth factor, insulin) as well as to sterol (38). Second messengers of the diacylglycerol-protein kinase C, inositol 1,4,5-triphosphate-calcium, and cyclic AMP pathways have been demonstrated to increase LDL receptor gene transcription transiently in the human monocytic leukemia cell line THP-1 and in human hepatocarcinoma cell line HepG2 (39, 40), suggesting that LDL receptor expression may by sensitive to external influences as well as to the effect of intracellular cholesterol.

While transcriptional level control undoubtedly has a profound impact on LDL receptor regulation, post-transcriptional level control of LDL receptor protein may also be important. For example, cholestyramine or pravastatin treatment does not result in enhanced rat hepatic LDL receptor expression, though estradiol treatment results in markedly increased hepatic LDL receptor levels. Schafer et al. (41) found elevated hepatic LDL receptor mRNA after treatment with estradiol, cholestyramine, and pravastatin, while LDL receptor protein was elevated only in the estradiol-treated animals, suggesting a novel post-transcriptional form of LDL receptor regulation in cholestyramine- and pravastatin-treated rats.

A priori we had expected to find no sterol regulation of the transduced LDL receptor, since, as detailed earlier, the LDL receptor cDNA used in creating these vectors does not contain the 5' promoter region and the retroviral LTR driving expression of the receptor is not known to be affected by sterol. Yet, our experiments showed substantial sterol regulation of transduced LDL receptor activity, as measured by degradation of LDL (Fig. 2), by degradation of the LDL receptor specific monoclonal antibody IgGC7 (Fig. 3), by binding of IgGC7 (Table 1), and by binding of β-VLDL using a ligand blot technique (Fig. 4). Sterol suppression of the LDL receptor transgene did not require receptor-dependent sterol uptake (Fig. 2), as has been found in normal cells (36). We also used a nonretroviral construct to introduce the LDL receptor cDNA into receptor-deficient CHO cells. In these cells we were also able to demonstrate sterol regulation of the transfected LDL receptor activity (Fig. 6). However, Davis et al. (42) did not find sterol-mediated regulation of the human LDL receptor after transfection of receptor negative CHO ldlA-7 cells with an SV40 driven human LDL receptor cDNA. The reason for the discrepancy between these earlier results and our own is not clear.

The remainder of our experiments attempt to define the mechanism for sterol suppression of the transduced LDL receptor. Northern blot analysis of LDL receptor steady-state proviral transcript levels showed that sterols did not affect mRNA levels in either infected WHHL fibroblasts or in infected normal human fibroblasts (Fig. 8). The possibility that other portions of the LDRNL vector were responsible for sterol regulation was ruled out by a combination of experiments, including infections with similarly oriented luciferase and β-galactosidase reporter gene vectors (Fig. 5) and by LDL receptor expression after receptor-negative CHO cells were transfected with a nonretroviral vector (Fig. 6). Insulin binding assays were used to rule out any nonspecific effects on cell surface membrane proteins caused by infection, G418 selection, or sterol deprivation (Fig. 7). These experiments indicated that sterol specifically regulates only LDL receptor transgene expression, rather than nonspecifically influencing the expression of several different transduced genes or endogenous proteins. Because sterol exposure did not affect the steady-state level of the transcript from the LDL receptor transgene, but did affect total receptor activity, we conclude that sterol regulation of the transduced receptor activity must occur at a post-transcriptional level.

As with HMG-CoA reductase, control of LDL receptor expression in normal cells may be dominated ordinarily by the effect of sterol on the control of receptor transcription. In our experiments, transcriptional control was

removed, as the vector with the LDL receptor cDNA contained only the LDL receptor coding region under constitutive transcriptional control of a viral promoter. Under these conditions we postulate that post-transcriptional mechanisms involved in LDL receptor regulation may be uncovered. These mechanisms may not be readily apparent in normal cells and yet their effect in infected cells may be substantial.

Our experiments have not established the precise mechanism of the post-transcriptional mechanism(s) responsible for how sterol results in down-regulation of the transduced receptor activity. It is not known whether sterol exposure could result in modulation of the rate of translation of the receptor transcript once the dominant effect of transcriptional control by sterol has been removed, as is the case in the infected cells. Likewise, although receptor degradation does not appear to be regulated by sterol in normal cells (36, 37), small alterations in LDL receptor half-life could become apparent in the infected cells. The cytoplasmic domain of the LDL receptor is known to undergo phosphorylation by a casein kinase (43). While no physiological role has been demonstrated, phosphorylation of the cytoplasmic region by intracellular kinases has been shown to be important in receptor turnover for other coated pit receptors (24, 44). If degradation of the provirus-expressed LDL receptor is altered by sterols, determination of the role of phosphorylation in regulation of the transduced receptor will be important. These infected cells should provide an useful model system for the study of LDL receptor expression when the dominant effect of transcriptional control has been removed.

In our infected cells we detected a significant difference in the levels of receptor mRNA present and in the amounts of LDL receptor activity actually expressed. While mRNA levels of the transgene were increased 6- to 10-fold, levels of receptor activity were only equal to or slightly above those found in wild type rabbit fibroblasts. The reasons for this relative inefficiency in expression of mRNA are likely to be complex. In part, it could relate to the chimerical nature of the construct used. However, it is unlikely that this latter point is the explanation for the sterol-mediated regulation of LDL receptor activity as two identical constructs containing two other reporter genes (lacz and β-galactosidase) were not regulated by sterol. Since the ultimate goal of these studies is to develop vectors that will lead to in vivo insertion of functional LDL receptor activity in receptor-deficient cells, it will be important to understand the mechanisms affecting translation of the transgene's mRNA and for processing of the expressed receptor protein.

The use of similar vectors for re-implantation of genetically corrected cells for the phenotypic correction of the reception deficiency or the development of similar vectors that can be targeted specifically to the LDL-receptor deficient hepatocytes in vivo might still be feasible, despite significant down-regulation of inserted receptors by sterol. Hepatic transplantation in homozygous FH succeeds in lowering plasma cholesterol levels despite markedly elevated recipient LDL levels, which presumably should suppress the activity of LDL receptors in the transplanted liver (2). While the absence of transduced receptor regulation by sterol might be of value immediately following receptor introduction when LDL levels are still massively elevated, in the long run, retaining some degree of sterol responsiveness in the newly introduced receptors might prevent excessive intracellular sterol accumulation or even hypocholesterolemia that might occur following unregulated LDL receptor introduction.

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