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An adenoviral vector deleted for all viral coding sequences results in enhanced safety and extended expression of a leptin transgene

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ABSTRACT **Adenoviral (Ad)-mediated *in vivo* gene transfer and expression are limited in part by cellular immune responses to viral-encoded proteins and/or transgene immunogenicity. In an attempt to diminish the former responses, we have previously developed and described helper-dependent (HD) Ad vectors in which the viral protein coding sequences are completely eliminated. These HD vectors have up to 37 kb insert capacity, are easily propagated in a Cre recombinase-based system, and can be produced to high concentration and purity (>99.9% helper-free vector). In this study, we compared safety and efficacy of leptin gene delivery mediated by an HD vector (HD-leptin) and a first-generation E1-deleted Ad vector (Ad-leptin) in normal lean and *ob/ob* (leptin-deficient) mice. In contrast to evidence of liver toxicity, inflammation, and cellular infiltration observed with Ad-leptin delivery in mice, HD-leptin delivery was associated with a significant improvement in associated safety/toxicity and resulted in efficient gene delivery, prolonged elevation of serum leptin levels, and associated weight loss. The greater safety, efficient gene delivery, and increased insert capacity of HD vectors are significant improvements over current Ad vectors and represent favorable features especially for clinical gene therapy applications.**

Adenoviral (Ad) vectors are currently among the most efficient gene transfer vehicles for both *in vitro* and *in vivo* delivery, but the utilization of current Ad vectors for many gene therapy applications is limited by the transient nature of transgene expression obtained by these vectors (1–7). Several factors have been shown to contribute to and modulate the duration of Ad-mediated gene expression and the immunogenicity of these vectors, including "leaky" viral protein expression and the transgene that is delivered (8–15). The development of Ad vectors that are deleted in all viral protein-coding sequences offers the prospect of a potentially safer, less immunogenic vector with an insert capacity of up to 37 kb (16–26). This vector is supplied in trans with the structural proteins required for packaging and rescue and is thus helper-dependent (HD) (24).

Leptin has been recently identified as a potent modulator of weight and food intake. Daily delivery of recombinant leptin protein was shown to induce weight reduction, suppress appetite, and decrease blood insulin and glucose levels in *ob/ob* (leptin-deficient) mice (27–29). It has been shown that delivery of the leptin cDNA by first-generation Ad vectors (Ad-leptin) may substitute for daily recombinant leptin protein treatment, although the effects were transient in both lean and *ob/ob* treated mice (7, 30). In the present study, we delivered the leptin cDNA using the HD virus (HD-leptin), testing the hypothesis that elimination of the viral protein coding sequences would diminish the vector's cellular immunogenicity and toxicity, and hence support its longevity *in vivo*. Because both the viral proteins and the transgene were factors implicated in the cellular immunogenicity of recombinant Ad viruses, we designed experiments to compare the HD and Ad vectors in ob/ob mice that are naive to leptin (in which the protein is potentially immunogenic), as well as in lean mice that normally express leptin.

In this study, we show that HD-leptin provided greater safety as reflected by absence of liver toxicity, cellular infiltrates, extended longevity of gene expression, and stability of vector DNA in livers of treated mice over that observed with Ad-leptin treatment.

MATERIALS AND METHODS

Construction of Vectors. Construction of Ad-leptin and Ad-β-galalactosidase (β-gal) recombinant vectors has been described (30). The expression cassettes contain the human cytomegalovirus (HCMV) promoter (Invitrogen), the transgene, and the bovine growth hormone poly(A) sequence. First generation vectors were propagated and titered as described (31, 32). The HD vector constructed for this study (HD-leptin) was prepared by releasing the linear backbone structure of HD-leptin from its plasmid pΔSTK120-HCMV-mOb-BGHpA (by *Pme*I digest) and transfecting the linear DNA into 293-cre4 cells followed by helper infection as described in HD-leptin propagation section below. Two different structures were used for rescuing HD viruses expressing leptin, HD-leptin (used in all the *in vivo* studies reported in this article), and HD-leptin-monomer. The structure of the HD-leptin plasmid is a pBluescript IIKS based plasmid that contains (in the following order) the Ad5 inverted terminal repeat (ITR) sequences and the packaging signal ψ, 440 bp, (nucleotides 1–440); a 5,072-bp fragment of hypoxanthine guanine phosphoribosyltransferase (nucleotides 12,373–17,781 in gb:humhprtb); the leptin expression cassette, 1,835 bp; a *Hin*dIII 9,063-bp fragment of C346 cosmid (nucleotides 12,421–21,484 in gb:L31948); and the right-end terminus of Ad5, composed of the ITR sequence, 117 bp (nucleotides 35,818–35,935); with the intervening multiple cloning sites between junctions of the different fragments the total size is 19.6 kb including 2.9 kb of the pBluescript IIKS. The 2.9 kb of pBluescript IIKS is eliminated before HD vector rescue by linearizing the plasmid with two *Pme*I flanking sites. HD-leptin-monomer plasmid (pSTK120-HCMV-mOb-BGHpA) differs in that the hypoxanthine guanine phosphoribosyltransferase "stuffer" is a larger fragment of 16,054 bp (nucleotides 1,799–17,853 in gb:humhprtb), total size ≈30 kb

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Abbreviations: Ad, adenoviral; HD, helper-dependent; Ad-leptin, leptin Ad vector; HD-leptin, leptin HD; ITR, inverted terminal repeat; β-gal, β-galactosidase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCMV, human cytomegalovirus.

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including 2.9 kb of the pBluescript IIKS, which as in the case of HD-leptin plasmid, is also eliminated by linearizing the plasmid with two *Pme*I flanking sites and releasing the HD-leptin-monomer fragment.

Propagation of the HD Viruses. For propagation of the HD viruses, we used a helper virus system consisting of a modified first-generation E1-deleted vector with *lox* sites flanking the packaging signals (AdLC8clucl), and a 293 cell line derivative expressing Cre recombinase (293-cre4) (24, 33). HD-leptin vector DNA was excised from the plasmid backbone (by *Pme*I digestion) and 4 μg were used to transfect semiconfluent 293-cre4 cells in 6-cm plates. After an overnight incubation, cells were infected at a multiplicity of infection of 1 with the helper virus AdLC8clucl. Cells were monitored for complete cytopathic effect, at which point cells were collected and lysate was used for serial propagation and expansion of viral stock by slight modification over what was described (24, 34). Two ml of lysate collected from P1 (the transfection/infection step) was used to infect 6-cm plates of semi-confluent 293-cre4 for 24 hours, supplemented with 1 ml of fresh medium. After the 24-hr incubation, the helper virus AdLC8clucl was added at a multiplicity of infection of 1, to the cells. P2 lysate was collected upon detection of cytopathic effect. The same procedure was repeated for another three propagation's (P3, P4, and P5) infecting 10-cm plates followed by 15-cm plates of semiconfluent 293-cre4 cells, respectively. Lysate collected from P4 was used to infect twenty 15-cm plates (1 ml of lysate added to 24 ml of fresh medium), and again upon detection of cytopathic effect the lysate was collected and cesium chloride banded as described (32). The banded viruses were analyzed by restriction mapping and the HD-leptin virus was sequenced for verification of structure.

The final stock of HD-leptin was harvested from $\approx 1.2 \times 10^9$ 293-cre4 cells and the cesium chloride banded viral stock yield was $\approx 8 \times 10^{12}$ particles (2×10^{12}/ml). The helper virus (AdLC8clucl) content in the HD-leptin stock was 1.5×10^7 plaque-forming units per ml. Fifty microliters ($1–2 \times 10^{11}$ OD particles per dose, containing $\approx 7.5 \times 10^5$ plaque-forming units helper, i.e., $<0.1\%$ contamination with helper per estimated infectious HD dose) of the stock were diluted with dialysis buffer to 100 μl for the mouse tail vein injections.

Repeat of HD-Leptin Viral Rescue. Three independent rescues of the HD-leptin recombinant virus, initiated at the first step (P1), which is the transfection of pΔSTK120-HCMV-mOb-BGHpA resulted in an identical, and stable structure of HD-leptin. Seven different enzymes were used for verifying the structures of the recombinant HD viruses; Asp-718, *Eag*I, *Fse*I, *Hin*dIII, *Pac*I, *Sma*I, and *Xho*I. Digested viral DNA (50–100 ng) was analyzed by Southern blot analysis, fragments were radiolabeled using T4 DNA polymerase, DNA fragments were viewed on a 1.0 or 0.5% (for sizing purposes in case of undigested DNA extracted from HD-leptin and Ad-leptin) agarose gels in Tris/acetate/EDTA (TAE) buffer, and identified by radioautography or ethidium bromide staining.

PCR Amplification of the Junction Fragment and Sequencing. A primer flanking the junction fragment was used for PCR, primer J4-F: 5′-CTCTTCTTCTGTCACACCCCTCCCUC-3′ was used individually to amplify the junction-fragment of HD-leptin, the fragment generated was ≈300 bp, and was cloned into PCR 2.1 vector (Invitrogen) and sequenced.

Mouse Colony. *ob/ob* (C57BL/J6-*ob/ob*) mice and homozygous normal lean (C57BL/J6) litter mates (age-matched females), were purchased from The Jackson Laboratory for use in this study. Animals were free of all common murine pathogens. Eight- to twelve-week-old mice (*ob/ob* ≈70 g and lean ≈28 g) were redistributed based on equal representation of weight and caged in groups of five on day 0, immediately preceding treatment. After a series of baseline blood samples were obtained by tail incision from conscious mice, animals were divided into four groups and received by tail vein injection a single 100-μl aliquot containing $1–2 \times 10^{11}$ particles of HD-leptin, Ad-leptin, Ad-β-gal (control), or dialysis buffer (control). Body weight and food intake were measured daily, and blood was collected 2–3 times weekly, pre- and posttreatment. Animals were killed by carbon dioxide inhalation and organs removed for immunohistochemistry and RNA analysis. All animals used in this study were maintained in accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute for Laboratory Animal Resources, National Research Council, 1996). The protocol was approved by the Institutional Animal Care and Use Committee, Merck.

Histopathology Studies. Mice ($n = 3$ per treatment per time point) were humanely killed, and liver samples were collected and fixed in 10% buffered formalin. Tissues were routinely processed through paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin. Replicate unstained slides also were prepared using standard procedures for immunohistochemistry and stained for the presence of CD3 (T cell) and CD45R (B cell) determinants on infiltrating or intrinsic cells (not shown).

Blood Measurements. Blood samples were obtained by tail incision and collected into heparinized microhematocrit tubes (VWR Scientific) every 2–3 days during the course of the study. Tubes were centrifuged at $13{,}700 \times g$ for 2 min, and hematocrit values were monitored. Plasma was collected for measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), leptin, glucose, and insulin levels. ALT and AST were measured using ALT/serum glutamic oxaloacetic transaminase and AST/serum glutamic pyruvic transaminase, DT slides, respectively (Vitros Chemistry Products, Johnson & Johnson). Leptin and insulin levels were measured by radioimmunoassay performed by Linco Research Immunoassay (St. Charles, MO). Glucose levels were measured using Kodak Ektachem DT slides (Eastman Kodak).

Northern and Southern Blot Analysis. For Northern blot analysis, total RNA was extracted (Trizol, GIBCO) from livers of Ad-leptin-treated and HD-leptin-treated mice at 1-, 2-, 4-, and 8-week intervals, and untreated mice. Leptin RNA message was detected by Northern blot analysis (35) using leptin cDNA as a probe that recognizes a single ≈500 bp band (30). A probe for β-actin was used as the internal control (≈1 kb) (Biochain, San Leandro, CA). Southern blot analysis (35) was used to investigate the stability of vector DNA. Genomic DNA was extracted from livers (pooled DNA, $n = 3$ per treatment per time point) of *ob/ob* and lean mice were treated with Ad-β-gal, Ad-leptin, or HD-leptin. Control animals were injected with similar volumes of dialysis buffer. Pooled ($n = 3$) genomic DNA was digested with *Hin*dIII restriction enzyme, and 20 μg of digested DNA were loaded on 0.8% TAE agarose gels. For copy number estimation, 20 μg control DNA were spiked with HD vector DNA equivalent to 2.0, 1.0, 0.2, and 0.1 copies per cell, and the mixture digested with *Hin*dIII restriction enzyme followed by Southern blot analysis. The filters were hybridized with a mouse leptin cDNA (≈500 bp) probe, which hybridized to a single *Hin*dIII fragment containing the leptin insert in both the HD-leptin (≈6 kb), and Ad-leptin (≈1.2 kb) vectors. Developed autoradiographs were scanned (Personal Densitometer SI, Molecular Dynamics) and the relative band densities quantitated (IMAGE QUANT software, Molecular Dynamics). To normalize DNA concentration and to estimate relative vector DNA stability between treatment time points, a detected internal leptin genomic DNA signal was used as control. Copy number equivalence were assigned based on comparisons to the relative density ratio between the internal genomic signal and the leptin signal of spiked vector DNA in a copy number control experiment.

RESULTS AND DISCUSSION

The HD viruses rescued and propagated were analyzed both for structure verification and for helper-load contamination. HD-leptin (used in this study) was generated from a 16.7-kb vector

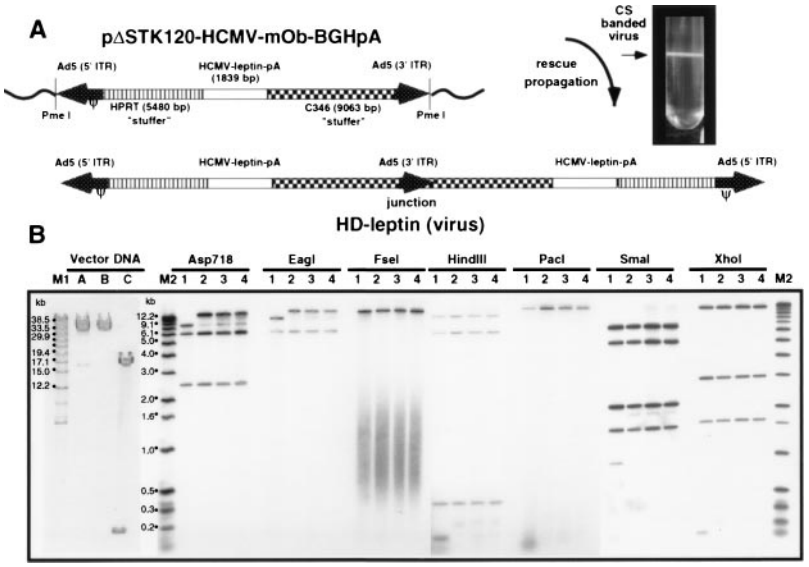

FIG. 1. HD-leptin construct. (*A*) The DNA composite fragments of pΔSTK120-HCMV-mOb-BGHpA (≈19.6 kb total size) are from left to right: the left end terminus of Ad5, composed of the ITR sequences and the packaging signal ψ (nucleotides 1–440, solid arrow); the 5,072-bp fragment of hypoxanthine guanine phosphoribosyltransferase (HPRT) (nucleotides 12,373–17,853 in gb:humhprtb, ▨); the leptin expression cassette (1,835 bp), composed of the HCMV promoter, the murine leptin cDNA (500 bp) and the bovine growth hormone poly(A) tail (□) (inserted in the complementary orientation); the *Hin*dIII 9063-bp fragment of C346 cosmid (nucleotides 12,421–21,484 in gb:L31948, ▨); and the right end terminus of Ad5, composed of the ITR sequence (nucleotides 35,818–35,935). The ITRs are flanked by unique *Pme*I restriction sites used to liberate the vector fragment from the plasmid backbone before the initial transfection into 293-cre4 cells for viral rescue and propagation (released fragment is 16.7 kb). To the right of the vector structures is a representative cesium chloride banded HD-leptin vector stock (see *Materials and Methods*), at the final stage of band collection. The band is single, compact, and thick. (*B*) The structure and similarity of HD-leptin vectors (33 kb), which are all tail-to-tail concatamerizations (junction is at the 3′ ITR ends of ΔSTK120-HCMV-mOb-BGHpA), is verified by the restriction enzyme pattern of the three independently rescued viruses. The gel, labeled Vector DNA, shows 0.5 μg of DNA extracted from the HD-leptin viral stock (lane A), Ad-leptin stock (lane B) and the *Pme*I cut pΔSTK120-HCMV-mOb-BGHpA (lane C) compared on a 0.5% agarose gel for sizing. Both HD-leptin (33 kb) and Ad-leptin (34 kb) extracted DNA migrate, as expected, between 38.5–29.9 kb, and the cut ΔSTK120-HCMV-mOb-BGHpA (16.7 kb) migrates between 17.1 and 15.0 kb, the smaller band corresponds to the plasmid backbone (2.9 kb), and the faint band in lane A represents the trace amount of the propagated 16.7-kb linearized vector. Structures of ΔSTK120-HCMV-mOb-BGHpA (lane 1) (gel extracted after separation of plasmid backbone by *Pme*I digestion) and the three HD-leptin vectors (lanes 2–4) are compared by restriction analysis, as described in *Materials and Methods*. The expected fragment sizes for HD-leptin are: for Asp-718: 15,391-single band (s), 6,296-double band (d), and 2,501-d; *Eag*I: 20,445-s and 6,270/6,266-d; *Fse*I: 16,523/16,458-d; *Hin*dIII: 10,207/10,174-d, 5,845-d, and 454/450-d; *Pac*I: 16,516/16,465-d; *Sma*I: 6,701-d, 5,163-d, 2,180-d, 1,715-s, and 1,589-d, and *Xho*I: 11,833-d, 2,964/2,953-d; and 1,701/1,697-d bp. The expected fragment sizes for ΔSTK120-HCMV-mOb-BGHpA are: for Asp-718: 7,837-s, 6,296-s, and 2,501-s; *Eag*I: 10,364-s and 6,266-s; *Fse*I: 16,458-s and 172-s; *Hin*dIII: 10,174-s, 5848-s, 450-s, and 158-s; *Pac*I: 16,465-s and 165-s; *Sma*I: 6,701-s, 5,163-s, 2,180-s, 1,589-s and 997-s, and *Xho*I: 11833-s, 2964-s, 1697-s and 136-s bp. M1 and M2 are DNA markers (8–48 kb, Bio-Rad, and 1-kb DNA ladder, GIBCO/Life Technologies, Gaithersburg, MD, respectively).

fragment (Fig. 1). This fragment when transfected and propagated in the presence of a helper virus resulted in an HD-virus with a full-length of ≈33 kb (Fig. 1). The full-length structure is a tail-to-tail concatamerization recombinant virus (Fig. 1*A*). This virus has several interesting characteristics. (*i*) The HD-leptin structure contains a duplicated 5′ITR and packaging signal sequence, one copy at each end of the recombinant virus (Fig. 1*A*). (*ii*) This structure is very stable throughout multiple propagations (originating from viral stock). In addition, repeated rescue (originating from the 16.7-kb DNA fragment) results in a single concatamerization species (Fig. 1*B*). The three HD-leptin recombinant viruses shown, are all independent results of a tail to tail (3′ ITR - 3′ITR) concatamerization of two ΔSTK120-HCMV-mOb-BGHpA molecules. The HD-leptin structure contains two copies of the 5′ITR and packaging signals that flank the recombinant virus at both ends and the 3′ITR (one copy only) at the junction of the two molecules. No other concatamerization species were identified in any of the three independent rescues (by restriction mapping and analysis of radiolabeled digestion fragments). (*iii*) The helper virus contamination load is consistently very low; <0.1%/infectious HD unit; <1 plaque-forming unit of helper virus per 100,000 OD particles per ml of HD stock (minimum estimated HD infectious unit:OD particle is 1:100). HD-leptin expressed leptin at levels comparable to its counterpart first generation Ad-leptin (data not shown).

In contrast, the HD-leptin monomer, containing a single copy of the packaging signal sequence at the left arm only, consistently results in at least 3–10 fold higher load of helper virus contamination (1 plaque-forming unit of helper virus per 10^3–10^4 OD particles per ml) in HD-leptin monomer stock. Given that all the various viral stocks were prepared following the same standard laboratory procedures, the consistently lower levels of helper contamination in the concatamerized HD-leptin viral stocks may be attributed to either the duplicated copy of packaging signal sequence or the differences in backbone composition, possibilities now under investigation.

As a first measure of the difference between first generation Ad and HD vectors, safety studies were conducted in control and treated lean and *ob/ob* mice. Mice were treated with a single tail intravenous infusion of 1–2 × 10^{11} particles of either HD-leptin, Ad-leptin, control Ad-β-gal vector or an equal volume of dialysis buffer. Figure 2 shows the levels of AST and ALT in the sera of lean mice at one, two and four weeks posttreatment (similar results were observed in treated *ob/ob* mice; data not shown). Liver toxicity, as reflected by the significant elevation in AST and ALT serum levels over basal control levels, was observed only in mice treated with Ad-β-gal and Ad-leptin, but not HD-leptin. Ad-vector-associated toxicity observed in both the lean and *ob/ob* treated mice was most significant at one week, was present but to a less significant extent at two weeks, and was resolved by 4 weeks posttreatment. In contrast, HD-treatment was not associated with liver toxicity as reflected by the AST and ALT serum levels that were essentially indistinguishable from controls.

Liver sections of HD-leptin-treated lean mice (Fig. 3) were histologically indistinguishable from control liver sections (Fig. 3*A*) at all timepoints tested posttreatment [1 (Fig. 3*C*), 2 (Fig. 3*E*), and 4 (Fig. 3*G*) weeks). Occasional perivascular clusters of 50:50 T and B cells and small foci of cellular infiltrates in HD-leptin-

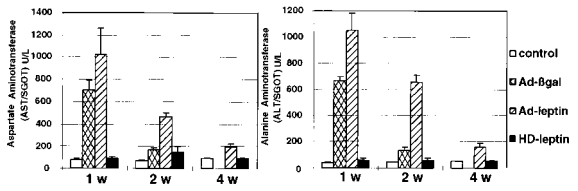

FIG. 2. Mice were treated with Ad-β-gal, Ad-leptin, and HD-leptin ordialysis buffer (controls). AST and ALT levels in the sera of lean control and treated mice are plotted at 1, 2, and 4 weeks posttreatment.

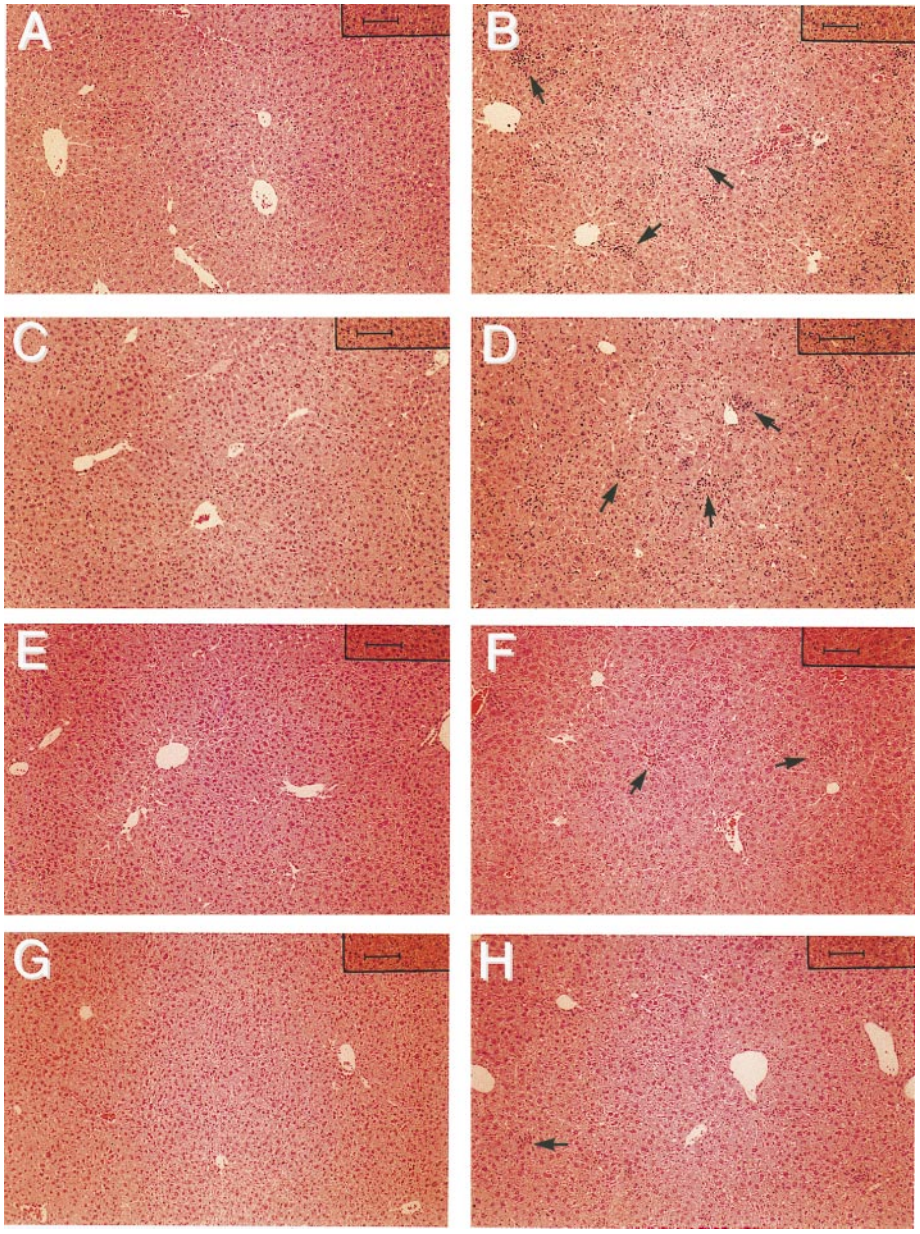

FIG. 3. Photomicrographs illustrating liver histopathology in lean mice. (*A*) Untreated control lean. (*B*) Positive Ad-β gal-treated control lean, 1 week posttreatment. (*C*) HD-leptin-treated lean, 1 week posttreatment. (*D*) Ad-leptin-treated lean, 1 week posttreatment. (*E*) HD-leptin-treated lean, 2 weeks posttreatment. (*F*) Ad-leptin-treated lean, 2 weeks posttreatment. (*G*) HD-leptin-treated lean, 4 weeks posttreatment. (*H*) Ad-leptin-treated lean, 4 weeks posttreatment. (Bar = 100 μM.)

treated as well as in untreated control mice were observed. In contrast, Ad-leptin and Ad-β-gal treated mice displayed hepatic pathology throughout the posttreatment intervals. At 1 week posttreatment, both Ad-β-gal (Fig. 3*B*) and Ad-leptin-treated (Fig. 3*D*) mice display degenerative hepatic pathology characterized by foci of round cell infiltration (solid arrows) composed almost entirely (>98%) of T-cells (data not shown), individual liver cell necrosis, increased liver cell mitotic activity, and dissociation of hepatic cords. At 2 weeks posttreatment, Ad-leptin-treated (Fig. 3*F*) mice display a similar, but less pronounced hepatic pathology. The cellular infiltration observed resolved by the fourth week posttreatment; there is almost an absence of lesions in the Ad-leptin treated (Fig. 3*H*) mice, with only a trace of individual cell death present, which is within normal ranges. Examination of liver sections obtained from *ob/ob* mice reflected similar Ad-vector associated histopathology. Similar to the observations in lean mice, evidence of toxicity associated with Ad vectors was not observed with HD-leptin treatment in *ob/ob* mice, however, a slight cellular infiltrate was detected, which may be attributed to the immunogenicity of leptin in these leptin-deficient mice. Nonetheless, the extent of inflammation and cellular infiltrates remained significantly less than that observed with Ad-leptin (data not shown).

In the lean mice, treatment with Ad-leptin resulted in a transient increase in serum leptin levels and weight loss that lasted for only 7–10 days (Fig. 4 *A* and *B*). In contrast, treatment with HD-leptin resulted in high serum leptin levels (6- to 10-fold over background) and ≈20% weight loss that persisted at least 2 months (Fig. 4 *A* and *B*). Weight loss in HD-leptin-treated mice was associated with satiety that persisted over a longer period (2–3 weeks, data not shown) than in those treated with Ad-leptin (5–7 days) (30). Vector DNA in the livers of Ad-leptin treated mice was rapidly lost and fewer than 0.2 copy per cell was detected, compared with 1 or 2 copies per cell after HD-leptin treatment at 8 weeks postinjection (Fig. 4*C*). These effects can be correlated with the duration of gene expression obtained with these two vector types. Gene expression mediated by Ad-leptin was transient and almost undetectable as early as 1 week posttreatment as seen by northern blot analysis of total liver RNA, whereas that mediated by HD-leptin persisted for at least eight weeks (Fig. 4*D*). No changes in serum glucose or insulin levels in the treated lean mice were detected throughout the study (Fig. 4 *E* and *F*). Vector DNA levels were stable at 1 to 2 copies per cell at 1, 2, 4, and 8 weeks posttreatment.

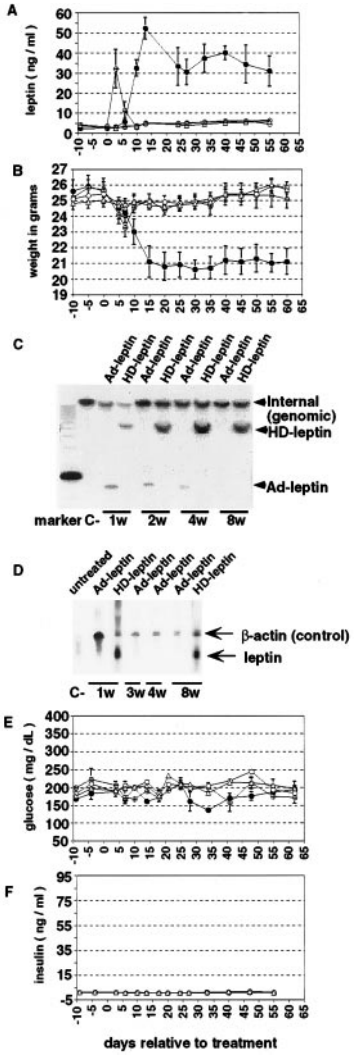

FIG. 4. HD-leptin and Ad-leptin effects in lean mice. Animals were injected via the tail vein with a single dose of 1–2 × 10^{11} particles of HD-leptin (n = 5), Ad-leptin (n = 10), Ad-β-gal (n = 10), or the equivalent volume of dialysis buffer (n = 10). The time course shows (*A*) serum leptin levels, collected 2–3 times weekly (ng/ml, mean ± SEM); (*B*) weight (g, mean ± SEM). (*C*) Southern blot analysis, the arrows refer to the single HD-leptin and Ad-leptin bands. Three internal genomic bands were also detected in treated and control DNA: one strong (uppermost, greater than 12 kb), and two faint (lower) bands. (*D*) Total RNA from livers of Ad-leptin-treated and HD-leptin-treated mice at 1-, 2-, 4-, and 8-week intervals, and at 1 and 8 weeks in HD-leptin-treated mice. Arrows refer to the leptin message (≈500 bp) band, β-actin was used as the internal control (≈1 kb). (*E* and *F*) Serum glucose (mg/dl) and insulin (ng/ml) were measured in all animal groups (mean ± SEM).

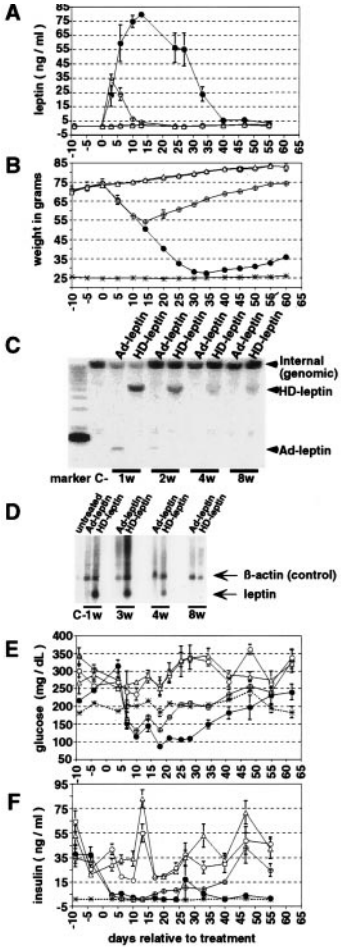

FIG. 5. HD-leptin and Ad-leptin effects in ob/ob mice. Essentially as described in Fig. 4, animals were injected in the tail vein with a single dose of 1–2 × 10^{11} particles of HD-leptin (n = 5), Ad-leptin (n = 10), and Ad-β-gal (n = 10), or the equivalent volume of dialysis buffer (n = 10). Lean control values are plotted for comparison. The time course shows (*A*) serum leptin levels (ng/ml, mean ± SEM), collected 2–3 times; (*B*) weight (g, mean ± SEM). (*C*) Southern blot analysis, the arrows refer to the single HD-leptin and Ad-leptin bands. Three internal genomic bands were also detected in treated and control DNA: one strong (uppermost, >12 kb), and two faint (lower) bands. (*D*) Total RNA from livers of Ad-leptin-treated and HD-leptin-treated mice at 1-, 2-, 4-, and 8-week intervals, and in untreated mice. Arrows refer to the leptin message (≈500 bp) band, β-actin was used as the internal control (≈1 kb). (*E* and *F*) Serum glucose (mg/dl) and insulin (ng/ml) were measured in all animal groups (mean ± SEM).

The *ob/ob* mice are naive to leptin and thus transgene immunogenicity is not an unexpected finding. In these animals, similar to what was observed in the lean mice, HD-leptin was found to be more effective than the first-generation Ad-leptin vector. In the *ob/ob* mice treated with Ad-leptin, serum levels of leptin increased only for a short period during the first 4 days of treatment, returning to baseline levels within 10 days postinjection (Fig. 4*A*). Increased leptin levels were associated with transient body weight loss of ≈25%, followed by weight gain, 2 weeks after treatment (Fig. 5 *A* and *B*). Similar to the results obtained in lean mice, the Ad-leptin vector DNA (Fig. 5*C*) was also rapidly lost (<0.2 copy per cell were detected by 2 weeks posttreatment, and undetectable by 8). In contrast, the *ob/ob* HD-leptin-treated mice had increased serum leptin levels up to ≈15 days posttreatment, after which the levels gradually dropped to baseline over the subsequent 25 days (Fig. 5*A*). The initial rise in leptin levels correlated with rapid weight reduction resulting in >60% weight loss (reaching normal lean weight) by 1 month (Fig. 5*B*). Weight loss was maintained for a period of 6–7 weeks posttreatment. As leptin levels dropped to baseline, a gradual increase in body weight was observed. Satiety was observed in association with increased leptin levels, and appetite suppression was sustained for a longer period (≈1 month) compared with the short transient effect induced by Ad-leptin (≈10 days) (data not shown). Leptin-specific antibodies were detected in the sera of *ob/ob* Ad-leptin- and HD-leptin-treated mice (data not shown); therefore, it was essential to determine whether the drop observed in serum leptin levels was due to interference of the antibodies with the ELISA assay used to measure leptin or a loss of vector DNA and/or gene expression. Although by Southern blot analysis greater stability of HD-vector DNA was observed over Ad-vector DNA in livers of *ob/ob* treated mice compared at similar time points, the analysis revealed eventual loss of the HD-vector DNA over the 8 week time interval (Fig. 5*C*). Approximately 75% less vector DNA was detected in the livers of HD-leptin-treated *ob/ob* mice at 4 and 8 weeks posttreatment compared with the persistent levels found in the livers of HD-leptin-treated lean littermates at similar time points (Fig. 5*C* and 4*C*, respectively). Gene expression in *ob/ob* Ad-leptin-treated mice correlated with the DNA findings, RNA levels were below the sensitivity level of detection at 1 week posttreatment, whereas in HD-leptin-treated mice, gene expression was detected up to 4 weeks postinjection and was undetectable at 8 weeks (Fig. 5*D*). Serum glucose and insulin levels dropped during the first one or

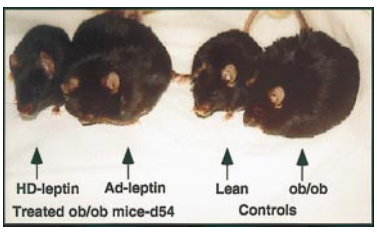

FIG. 6. Phenotypic correction of HD-leptin-treated *ob/ob* mice. On the left is a representative *ob/ob* mouse treated with HD-leptin at day 54 posttreatment, next to a littermate treated with Ad-leptin. The Ad-leptin-treated mouse initially lost weight during the first 2 weeks after the treatment, and subsequently gained weight. At 54 days post-Ad-leptin treatment, *ob/ob* mice are indistinguishable from untreated *ob/ob* control litter mates, whereas HD-leptin-treated mice remained indistinguishable from untreated lean control mice. Untreated *ob/ob* and lean control mice are shown for comparison as labeled.

two weeks posttreatment to normal lean values in both HD-leptin- and Ad-leptin-treated mice, although the effects of HD-leptin treatment were sustained for longer periods, which parallels what was seen with weight loss, satiety, DNA stability and leptin gene expression (Fig. 5 *E* and *F*). The subsequent increase in glucose and insulin levels in both vector treatments correlated with the drop observed in serum leptin levels and eventual loss of vector DNA. The overall HD-leptin-mediated prolonged effect was also reflected in the accompanying phenotypic correction, which lasted longer than that seen in litter mates treated with Ad-leptin (6–7 vs. 2–3 weeks) (Fig. 6).

It has been reported that Ad vectors and/or immunogenic transgenes can be associated with cytotoxic T lymphocyte cell responses that result in elimination of vector DNA infected cells and loss of gene expression (8–15). In some cases the response is influenced by the mouse strain used (15). In this study we used littermates to control against strain variation in our comparisons of the Ad-vector vs. the HD-vector immunogenicity in both lean animals that normally express leptin and *ob/ob* mice that are leptin deficient. Our studies clearly illustrate that HD-leptin achieved a substantial improvement in the safety profile and longevity of gene expression over that achieved with the first-generation Ad-leptin vector. The differences observed in the extent of cellular infiltrate in the liver, together with the pronounced liver toxicity as measured by ≈10- and 5-fold increases in AST and ALT serum values, respectively, associated with Ad-leptin but not HD-leptin treatment in lean mice, can be directly attributed to the elimination of the Ad protein-coding DNA sequences, because the leptin expression cassette was identical in both vectors. The appearance of a leptin-specific antibody response, gradual loss of gene expression and vector DNA observed in the *ob/ob* (leptin-deficient) but not in the lean mice (leptin-wild type) treated with HD-leptin may suggest an independent immune response event related to leptin tolerance.

The leptin model used in these studies provided a very instructive animal model to investigate the influence of both vector design and transgene product on the duration of expression after gene transfer. The differences between the longevity of expression mediated by the HD-deleted vector in the lean mice in this study and the very short lived effects reported by others may reflect variations in the vector construction features (23, 36).

The HD-vector system is a significant advance over existing Ad vectors with regards to safety and insert capacity (up to 37 kb). In addition to the gain of these two valuable properties, the HD vectors have not lost the features that contributed to the general attractiveness of Ad vectors that include: (*i*) efficient *in vivo* gene delivery, and (*ii*) high titer production. The concatamerization of the 16.7 vector fragment to generate a ≈33 kb recombinant virus is a phenomenon that has been previously observed by others (20, 34). The recombinant virus preferentially propagates at higher efficiencies when its genome length is at least 75% that of wild type (34). And although we detected traces of propagated 16.7 kb HD-leptin, the prevalence of this species was overwhelmingly surpassed by the 33-kb recombinant vector (Fig. 1*B*, Vector DNA A). Replacements of leptin by other transgenes in the pΔSTK120 are ongoing to determine the universality of this vector backbone. The generation of other backbones with the duplicated left arm is being tested to determine the extent to which the two copies of packaging signal sequences is contributing to the efficient propagation and possible advantage of the HD-recombinant virus over the helper virus leading to the exceedingly low levels of helper contamination in the HD stocks. The unique characteristics of HD-leptin together with the utilization of the 293-cre4 cells and the *lox* containing helper virus provides a biological method for generation of highly purified HD vectors. These advanced vectors improve the prospect of Ad vehicles for wide application in clinical gene therapy.

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