Treatment of Diabetes and Long-Term Survival After Insulin and Glucokinase Gene Therapy

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Diabetes is associated with severe secondary complications, largely caused by poor glycemic control. Treatment with exogenous insulin fails to prevent these complications completely, leading to significant morbidity and mortality. We previously demonstrated that it is possible to generate a "glucose sensor" in skeletal muscle through coexpression of glucokinase and insulin, increasing glucose uptake and correcting hyperglycemia in diabetic mice. Here, we demonstrate long-term efficacy of this approach in a large animal model of diabetes. A one-time intramuscular administration of adeno-associated viral vectors of serotype 1 encoding for glucokinase and insulin in diabetic dogs resulted in normalization of fasting glycemia, accelerated disposal of glucose after oral challenge, and no episodes of hypoglycemia during exercise for >4 years after gene transfer. This was associated with recovery of body weight, reduced glycosylated plasma proteins levels, and long-term survival without secondary complications. Conversely, exogenous insulin or gene transfer for insulin or glucokinase alone failed to achieve complete correction of diabetes, indicating that the synergistic action of insulin and glucokinase is needed for full therapeutic effect. This study provides the first proof-of-concept in a large animal model for a gene transfer approach to treat diabetes. ***Diabetes* 62:1718–1729, 2013**

Diabetes is a chronic disease for which there is currently no cure. Patients with type 1 diabetes need insulin replacement therapy to survive, but glycemia is not always properly regulated. Chronic hyperglycemia leads to development of diabetes-associated microvascular, macrovascular, and neurologic complications, which can be delayed by intensive insulin therapy (1). However, this treatment is not suitable for all diabetic patients because of its high risk of hypoglycemia secondary to excessive insulin dosage (1). Thus, precise regulation of glucose homeostasis is a major challenge in diabetes management. Therapeutic benefit has been obtained with islet transplantation (2), but access to human islets and the necessary immunosuppressive therapy are important limitations. Alternative cell- and gene-based therapies, centered around the engineering of nonpancreatic tissues to produce insulin, or the generation of stem cell-derived β cells, are undergoing investigation (3,4); however, long-term safety and efficacy data in large animal models are lacking.

Genetic engineering of skeletal muscle to counteract hyperglycemia is an attractive strategy to correct diabetes. Skeletal muscle is responsible for the disposal of ~70% of circulating glucose after a meal. In muscle, glucose utilization is controlled by insulin-stimulated glucose transport through the glucose transporter type 4 (GLUT4) (5) and phosphorylation by hexokinase II, which has a low K_m for glucose and is inhibited by glucose-6-phosphate, limiting glucose uptake (6). In diabetic muscle, because of the lack of insulin, GLUT4 translocation to the plasma membrane and hexokinase II activity both decrease. In contrast to hexokinase II, the liver enzyme glucokinase has a high K_m for glucose, is not inhibited by glucose-6-phosphate, and shows kinetic cooperation with glucose (7). When expressed in skeletal muscle of transgenic mice, glucokinase facilitates glucose uptake only when blood glucose is high (8). However, during diabetes, constant basal levels of insulin are required to ensure the presence of GLUT4 on the cell membrane (5). These observations led to the hypothesis that regulation of glycemia could be achieved by coexpression in skeletal muscle of glucokinase and low levels of insulin; in such a system, glucose influx is regulated by circulating glucose levels allowing glucose uptake only when hyperglycemia is present. Accordingly, intramuscular delivery of adeno-associated viral (AAV) vectors expressing insulin and glucokinase to diabetic mice resulted in disease correction (9).

AAV vectors are the vector of choice for in vivo gene therapy because of their excellent safety and efficacy profiles. Preclinical studies have shown that AAV-mediated gene transfer results in long-term gene expression in small and large animal models (10). Recently, these preclinical data have been successfully translated into humans (11,12). In the most clear-cut examples of success, clinical studies of hemophilia B (12) and Leber congenital amaurosis (11) were preceded by convincing studies of efficacy in large animal models (13,14). However, for most proof-of-concept studies in mice, no successful scale-up or long-term efficacy has been reported. This goal has yet to be demonstrated in large animal models of diabetes with gene and cell therapy approaches. Here, we used dogs treated with β-cell cytotoxic drugs as a model of experimental

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Received 17 August 2012 and accepted 28 January 2013.
DOI: 10.2337/db12-1113
This article contains Supplementary Data and videos online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db12-1113/-/DC1.
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See accompanying commentary, p. 1396.

diabetes (15), because large animal models of autoimmune diabetes are not available. We demonstrate that after a single intramuscular injection of AAV1 vectors, insulin transgene (Ins) and glucokinase (Gck) transgene act synergistically to achieve tight control of glycemia. This represents the first proof-of-concept study of long-term correction of diabetes in a large animal model using gene transfer.

RESEARCH DESIGN AND METHODS

Animals. Male Beagle dogs were purchased from Isoquimen (Barcelona, Spain) and housed at Servei de Granges i Camps Experimentals of the Universitat Autònoma de Barcelona. Animals were fed individually once daily at 9:00 A.M. with 30 g/kg body weight of standard dry food (Nestle, Vevey, Switzerland) or twice daily at 9:00 A.M. and 9:00 P.M. with 15 g/kg body weight of diabetic food (Prescription Diets w/d; Hills, Topeka, KS) when indicated. Dogs were monitored regularly at the Universitat Autònoma de Barcelona Veterinary Clinical Hospital. C56Bl6 male mice (Harlan Teklad, Barcelona, Spain) were fed ad libitum with a standard diet (Harlan Teklad) and maintained in the specific pathogen free (SPF) mouse facility at the Center of Animal Biotechnology and Gene Therapy under a 12-h light–dark cycle (lights on at 8:00 A.M.). The Ethics Committee on Animal and Human Experimentation approved all procedures.

Diabetes induction. Experimental diabetes was induced in 6- to 12-month-old dogs by a single intravenous injection of a mixture of streptozotocin (35 mg/kg) and alloxan (40 mg/kg) (Sigma, St. Louis, MO) as described previously (15). When hyperglycemia developed, dogs were maintained without exogenous insulin treatment, unless indicated. Dogs receiving exogenous insulin were injected subcutaneously with Lantus (Sanofi-Aventis, Paris, France). When indicated, exogenous insulin treatment was optimized individually, increasing gradually the insulin dose up to the maximum tolerated dose that did not cause hypoglycemia. To induce diabetes in mice, animals aged 8 weeks were administered, for 5 consecutive days, an intraperitoneal injection of streptozotocin (45 mg/kg body weight) dissolved in 0.1 mol/L citrate buffer (pH 4.5) immediately before administration.

AAV production and administration. AAV vectors were produced by triple transfection of HEK293 cells and purified by a CsCl-based gradient method (16). Expression of an engineered human insulin gene containing an endoprotease furin cleavage signal and expression of rat glucokinase were driven by the cytomegalovirus (CMV) promoter in both vectors (9). For certain experiments as indicated, codon-optimized versions of human insulin (oIns; with furin cleavage sites) and human glucokinase cDNAs (oGck) were used. Vectors were delivered to a total of 12–25 sites on the lateral aspect of the thigh (with a five-prong needle syringe) and the craneolateral face of the leg (single point injections) of both hind limbs, with maximal vector dose per site of injection being $<6 \times 10^{11}$ vg (Supplementary Fig. 1). Dogs received a vector dose of 1×10^{12} vg/kg or 2×10^{12} vg/kg 2–4 weeks after diabetes induction (Supplementary Table 1). Mice were intramuscularly treated with 4×10^{12} vg/kg of AAV1-oGck 2 weeks after diabetes induction, distributed into tibialis cranealis, gastrocnemius, and quadriceps muscles of both hindlimbs.

Biodistribution. Total DNA was isolated from Dog3 with MasterPureDNA Purification Kit (Epicentre Biotechnologies, Madison, WI). Vector genome copy number was determined in 20 ng of genomic DNA by TaqMan quantitative PCR with primers and probe specific for the cytomegalovirus (CMV) promoter: forward primer, 5′-CACCAATGGGCGTGGATAGC-3′; reverse primer, 5′GCAGTTGTTACGACATTTTGGAAA-3′; and probe, 5′-ATTTCCAAGTCTC CACCC-3′.

RNA analysis. Liver and quadriceps total RNA was extracted (Qiagen, Valencia, CA) from dog and mouse samples and analyzed by Northern blot with radiolabeled (GE Healthcare UK, Buckinghamshire, UK) insulin or glucokinase cDNA probes or by quantitative RT-PCR for detection of glucokinase regulatory protein: forward primer, CAAGCACCAAGCGGTATCA; and reverse primer, GTCAGTGGGTTGGACTTCTCT.

Western blotting. Nuclear and cytoplasmatic protein fractions were obtained from skeletal muscle and liver extracted from starved mice, or 15 min after intraperitoneal glucose injection (3 g/kg body weight) as described (17). Fractioned or total protein lysates were subjected to SDS-PAGE, electrotransferred on polyvinylidene fluoride (Millipore) membranes, and probed with Akt (Cell Signaling, Danvers, MA), pAkt (Cell Signaling), Gck (Sigma), Histone 3 (Abcam, Cambridge, UK), and α-tubulin (Abcam) as previously described (18). All rabbit primary antibodies were immunodetected using horseradish peroxidase–conjugated polyclonal swine anti-rabbit immunoglobulins (Dako, Glostrup, Denmark). Loading was normalized by α-tubulin.

Morphological and immunohistochemical analysis. Dog samples were fixed in 10% formalin, embedded in paraffin, and sectioned. Double glucagon and insulin immunostaining was performed with mouse antiglucagon (Sigma) and guinea pig anti-insulin (Sigma) antibodies. Biotinylated horse anti-mouse (Vector Laboratories, Burlingame, CA) followed by streptavidin-conjugated Alexa488 (Molecular Probes, Leiden, the Netherlands) and Alexa568-conjugated goat anti-guinea pig (Molecular Probes) were used as secondary antibodies. The β-cell area was measured on four sections of pancreas biopsy samples, or multiple areas from the whole pancreas in necropsy samples, stained with anti-insulin and horseradish peroxidase–conjugated rabbit anti-guinea pig immunoglobulin (Dako, Glostrup, Denmark). Pancreatic β-cell area was calculated by dividing the area of insulin-positive cells by total pancreatic area of each section. To analyze muscle integrity, cross-sections were stained with hematoxylin and eosin or rabbit antilaminin (Dako, Glostrup, Denmark). Periodic acid-Schiff staining was used to evaluate muscle glycogen content (Sigma).

Hormone and metabolite determinations. Serum insulin was measured with human insulin radioimmunoassay (Millipore, Billerica, MA). Serum human C-peptide was determined with C-peptide radioimmunoassay (Millipore) that does not cross-react with insulin or with canine C-peptide. Serum glucagon was measured by radioimmunoassay (Millipore). Blood glucose levels were determined using a Glucometer Elite analyzer (Bayer, Leverkusen, Germany). Serum fructosamine concentration was measured by nitroblue tetrazolium reduction test. The concentration of glycogen in skeletal muscle was measured as previously described (9). Urine was analyzed by Multistix 10 SG Urinalysis Strips (Siemens, Munich, Germany).

Oral glucose tolerance test. Oral glucose tolerance tests (OGTTs) were performed in either 12- or 24-h fasted dogs. Briefly, animals were administered an oral gavage of glucose 1.75 g/kg body weight. Glycemia was determined at times 0, 15, and 30 min, and then every half hour up to 3 h after glucose administration.

Exercise test. Fasted dogs (24 h) were subjected to 37 min of exercise under increasing speed and slope on a variable speed belt treadmill (Starker Hund S.A.S., Padua, Italy). The protocol was as follows: 1) 5 min, 0 degrees, 4 km/h; 2) 5 min, 0 degrees, 8 km/h; 3) 5 min, 2.5 degrees, 8 km/h; 4) 5 min, 5 degrees, 8 km/h; 5) 5 min, 7.5 degrees, 8 km/h; 6) 5 min, 10 degrees, 8 km/h; 7) 5 min, 0 degrees, 10 km/h; and 8) 2 min, 0 degrees, 4 km/h. Dogs were allowed a recovery time equal to the exercise period (Video 1).

Glucokinase activity determination. To measure glucokinase activity in skeletal muscle, tissue biopsy samples were obtained from mice or from necropsy samples from Dog3. Frozen mouse gastrocnemius or dog quadriceps samples were homogenized in an ice-cold buffer (pH 7.4) containing 100 mmol/L Gly-Gly, 200 mmol/L ClK, 5 mmol/L DTT, and 65 mmol/L Tris. Samples were then centrifuged to pellet insoluble material. The glucose phosphorylation capacity was assayed in the supernatants at 30°C in a buffer containing 50 mmol/L Gly-Gly, 100 mmol/L KCl, 2.5 mmol/L DTT, glucose-6-phosphate dehydrogenase (1 units/mL), 0.5 mmol/L NADP, and 4.5 mmol/L ATP-Mg. Glucokinase activity was calculated as the difference between the glucose phosphorylation capacity at 100 and 0.5 mmol/L glucose. Protein content was measured by Bradford assay (Pointe Scientific) and glucokinase activity was expressed as mU/mg of protein.

Insulin sensitivity test in mice. Awake, fed AAV1-oGck– or AAV1-null–treated diabetic mice were intraperitoneally injected with 0.75 IU/kg body weight of insulin (Humulin regular; Eli Lilly). Glucose concentration was determined in blood samples obtained from tail vein before and at 0, 15, 30, 45, and 60 min after the insulin injection.

Statistical analysis. All values are expressed as means ± SEM. Differences between groups were compared by unpaired Student t test. $P < 0.05$ was considered statistically significant.

RESULTS

Glycemic control by insulin alone. After experimental diabetes induction, three dogs (DogDb1–3) were subjected to glycemic control with subcutaneous exogenous insulin according to hormone administration regimens and feeding protocols indicated for diabetic companion dogs. Despite therapy, fasting normoglycemia was not achieved in any of the animals (Fig. 1*A*–*C*); one of the animals, DogDb3, experienced severe fasting hyperglycemia. Exogenous insulin therapy prevented weight loss in all dogs (Fig. 1*A*–*C*). All these animals showed elevated fructosamine levels (Fig. 2*A*), a marker of glycosylated proteins in blood and an indicator of recent (3 weeks) glycemic control used in veterinary medicine (19–21). Finally, diabetic

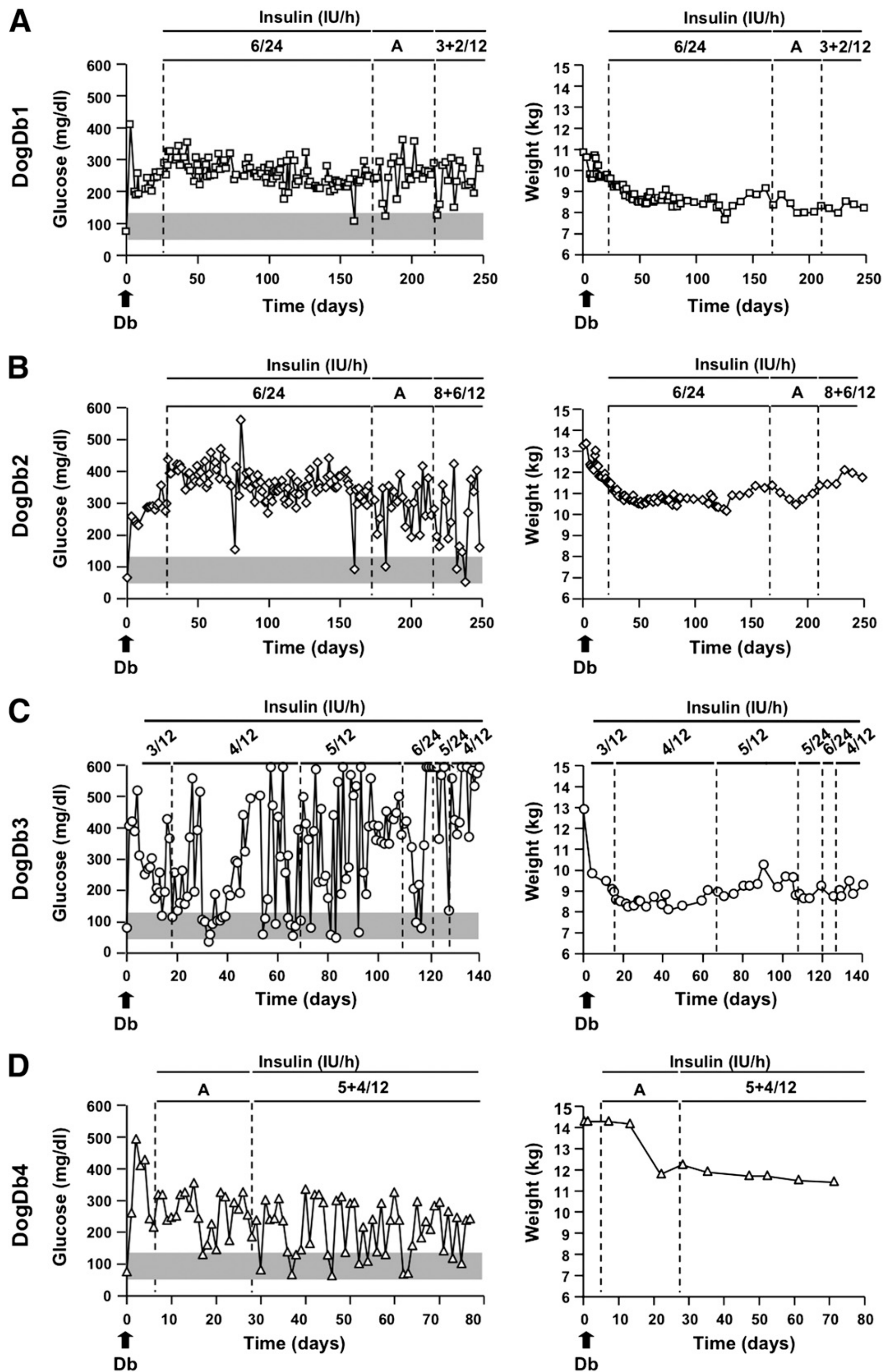

FIG. 1. Glycemic control by exogenous insulin. *A–D*: Follow-up of fasting glycemia and body weight of diabetic control dogs (DogDb1–4) treated daily with exogenous insulin (dosage [IU] and timing [24 h vs. 12 h] are shown). DogDb1, square; DogDb2, diamond; DogDb3, circle; DogDb4, triangle. Db, dog treatment with streptozotocin (STZ) plus alloxan. A indicates the period of adjustment of the insulin dosing regimen. Gray bar indicates fasting normoglycemia range in dogs (20).

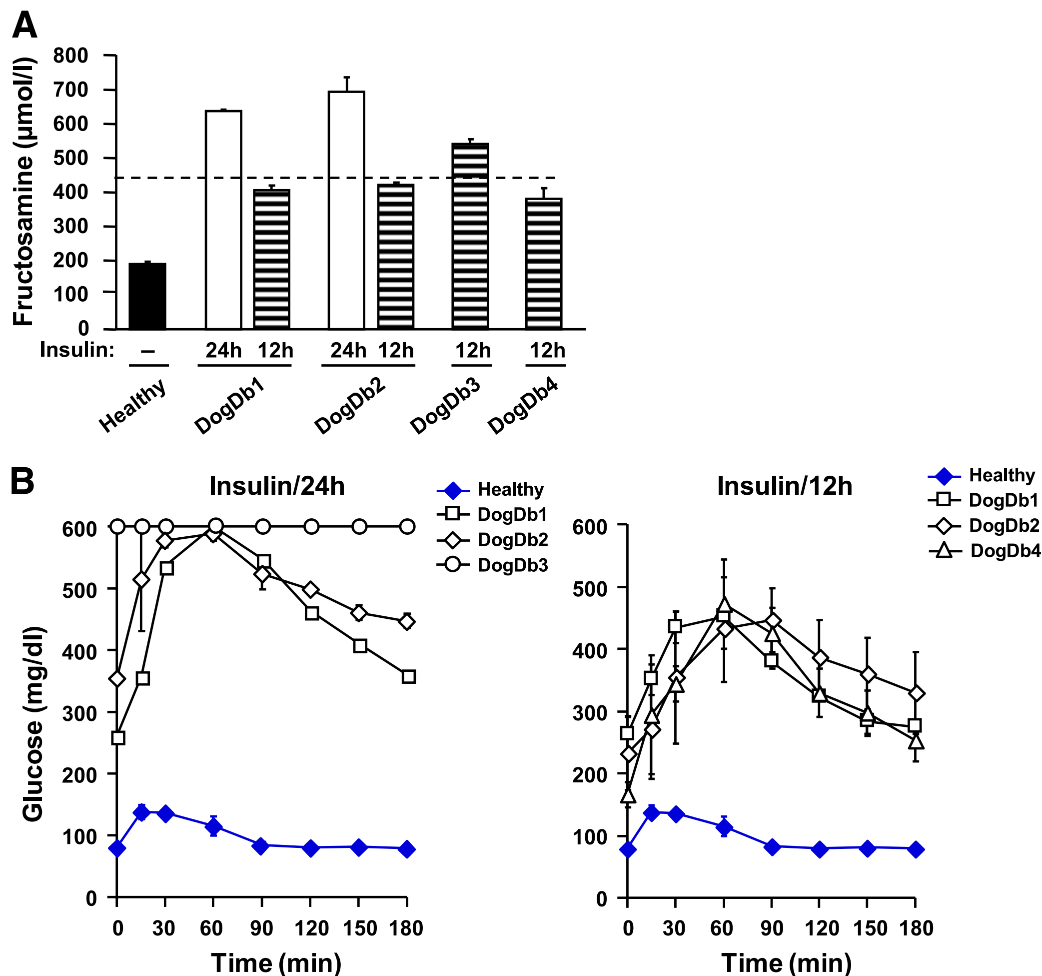

FIG. 2. Fructosamine levels and glucose disposal after a load in dogs treated with exogenous insulin. *A*: Monitoring of serum fructosamine in DogDb1–4 receiving exogenous insulin every 24 or 12 h. The fructosamine upper limit for good diabetes control in dogs is indicated with the dashed line. Fructosamine concentrations between 350 and 400 µmol/L indicate excellent glycemic control, concentrations between 400 and 450 µmol/L indicate good glycemic control, concentrations between 450 and 500 µmol/L indicate fair glycemic control, and concentrations >500 µmol/L indicate poor glycemic control (20). Results are shown as mean ± SEM of 2–4 determinations. *B*: OGTTs were performed at 1.75 g/kg glucose before (healthy) and after diabetes induction in DogDb1–3 treated with exogenous insulin every 24 h (*left*) or in DogDb1, DogDb2, and DogDb4 receiving insulin every 12 h (*right*). Mean ± SEM, *n* = 3. (A high-quality color representation of this figure is available in the online issue.)

dogs showed a marked and sustained increase in blood glucose compared with healthy animals when subjected to an OGTT at the American Diabetes Association–recommended standard dose of glucose (1.75 g/kg) (22) (Fig. 2*B*).

In an effort to further improve glycemic control, a new treatment regimen with exogenous insulin was established. In DogDb1 and DogDb2, and in an additional diabetic dog (DogDb4), insulin dosing was increased as much as possible without causing severe hypoglycemia; this was performed on an individual basis with twice-daily administrations. In addition, dogs were fed with diabetic food distributed in two servings. Despite treatment optimization, no major changes were observed in fasted glycemia (Fig. 1*A*, *B*, *D*), whereas the improvement in glycemic control resulted in lower fructosamine levels, which decreased to below the upper limit of good glycemic control (Fig. 2*A*) in veterinary medicine (450 µmol/L) (19–21) and improved OGTT (Fig. 2*B*).

When insulin alone was expressed in skeletal muscle of a diabetic dog by administration of 1×10^{12} vg/kg of AAV1-Ins vector (DogIns), partial correction of fasting glycemia was achieved (Fig. 3*A*). This animal recovered the body weight initially lost after diabetes induction and had fructosamine levels that ranged between 250 and 300 µmol/L after gene transfer (Fig. 3*B*, *C*). Despite achieving normal levels of fasting insulinemia (Fig. 3*D*), the ability of DogIns to dispose of glucose after an OGTT was only moderately improved compared with diabetic dogs (Fig. 3*E*).

Ins and Gck gene transfer to skeletal muscle corrects diabetes in dogs. Five diabetic dogs (Dog1–4 and DogDb3+Ins/Gck) were intramuscularly administered with AAV1-Ins and AAV1-Gck vectors. Administration of 1×10^{12} vg/kg of each vector to Dog1 and Dog2 resulted in rapid return to fasting normoglycemia and normoinsulinemia, recovery of body weight (Fig. 4*A*, *B*, and Video 2), and long-term survival (>4 years, observation ongoing). The administration of a twice-high vector dose (2×10^{12} vg/kg) of each vector to Dog3 was also safe and resulted in correction of diabetes with no episodes of hypoglycemia (Fig. 4*C*, *D*). Thus, Ins and Gck gene transfer results in better control of diabetes than exogenous insulin therapy or gene transfer for Ins only. This result was further confirmed in DogDb3 (Fig. 1*C*), which was treated with AAV1-Ins and

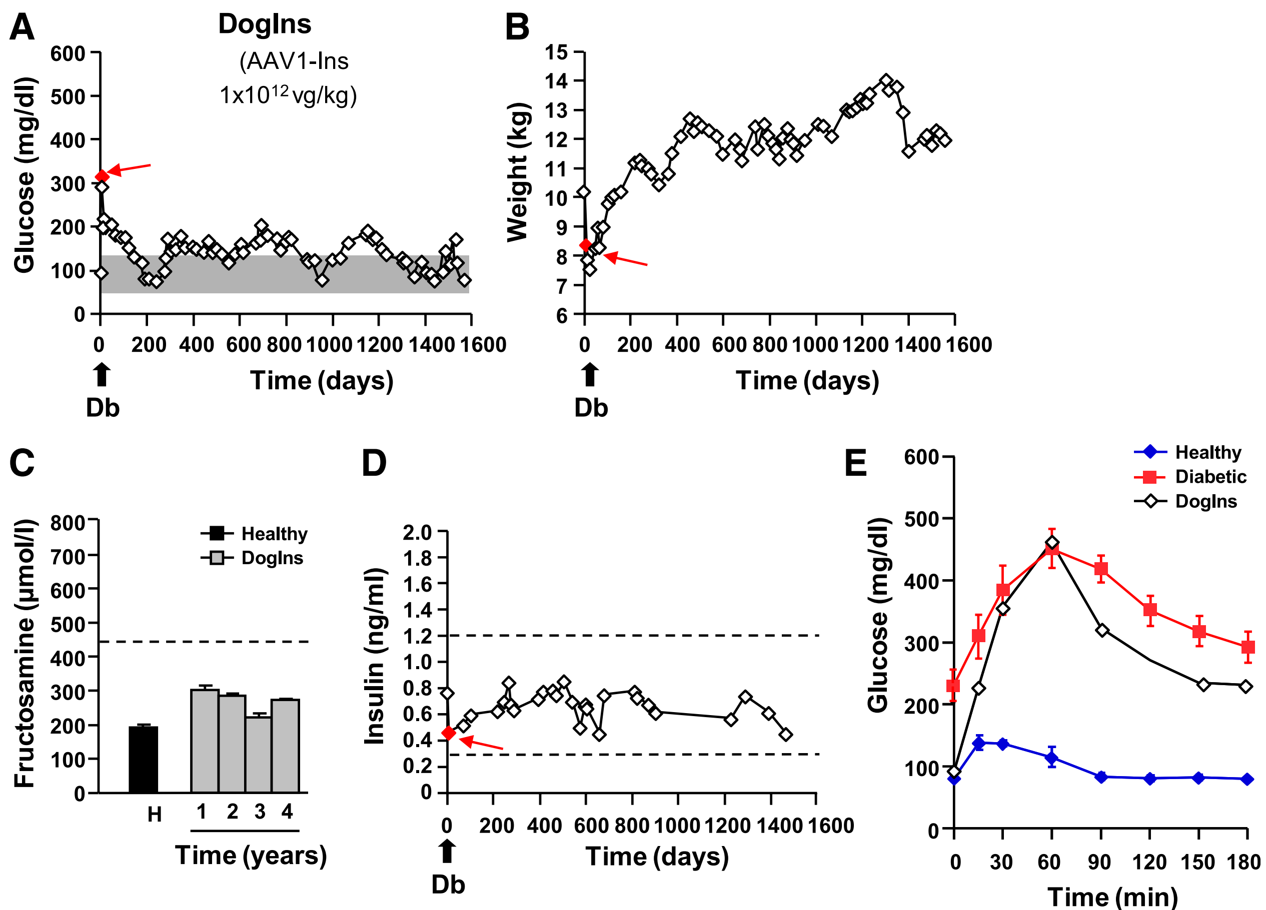

FIG. 3. Glycemic control by AAV1-Ins alone. Diabetic dog treated with AAV1-Ins (1×10^{12} vg/kg; DogIns) showed partial normalization of fasting blood glucose levels (*A*) and recovery of body weight loss (*B*). *C*: Fructosamine monitoring in DogIns. Results are shown as mean ± SEM of more than four measurements per year. *D*: Fasting insulinemia of DogIns. Dashed lines indicate average maximum and minimum fasting insulinemia values obtained by random measurements in six healthy dogs. *E*: No major improvement was observed in the ability of DogIns to dispose of glucose during an OGTT at 1.75 g/kg performed 4 years after gene transfer when compared with diabetic dogs under optimized exogenous insulin treatment. Db indicates dog treatment with streptozotocin (STZ) plus alloxan. Gray bar indicates fasting normoglycemia range in dogs (20). DogIns, diamond symbols in panels *A*, *B*, and *D*.

AAV1-Gck after 5 months of poorly controlled glycemia (now named DogDb3+Ins/Gck), resulting in normalization of glycemia without exogenous insulin administration. Codon-optimized versions of human transgenes also were tested to achieve better glycemic control at doses of 1×10^{12} vg/kg, because use of codon-optimized genes increases the production of proteins (23). Delivery of AAV1-oIns and AAV1-oGck to Dog4 led to rapid recovery of normoglycemia, normoinsulinemia, and body weight (Fig. 4*E*), with no signs of fasting hypoglycemia detected during the 5-month follow up.

In all Ins- and Gck-treated dogs, fructosamine levels remained within the range of 250–350 µmol/L (Fig. 5*A*). When an OGTT was performed at a dose of 1.75 g/kg, treated dogs showed only a small increase in glycemia after the load, followed by return to normoglycemia within 2 hours, a profile considered nondiabetic by American Diabetes Association guidelines (2-h plasma glucose <200 mg/dL) (22), with the exception of DogDb3+Ins/Gck, which was ~200 mg/dL at 120 min (Fig. 5*B*). Furthermore, Ins- and Gck-treated dogs showed improved glucose disposal even after high-dose (3 g/kg) glucose load; peak glycemia was lower than that of diabetic dogs and 2-h glycemia declined to <200 mg/dL (Supplementary Fig. 2). Importantly, Dogs1–4 and DogDb3+Ins/Gck showed good glycemic control with exercise, with no development of hypoglycemia (Fig. 6). In agreement with normalization of glycemia, ketone bodies were never detected in the urine of these dogs (data not shown). Finally, Ins- and Gck-treated dogs did not show signs of secondary complications, whereas DogDb1–3 developed cataracts a few months after hyperglycemia development (Supplementary Table 2).

Both transgenes were expressed in skeletal muscle (Fig. 7*A*), and glucokinase was active in this tissue (Fig. 7*B*). Circulating insulin in AAV1-treated dogs derived from expression of Ins transgene in muscle, as documented by the lack of surviving β cells in pancreas biopsy samples (Fig. 7*A*, *C*, *D*) and with the detection of human C-peptide in serum (Fig. 7*E*). In contrast to healthy dogs, and in agreement with the lack of pancreatic insulin-producing cells, the first phase peak of insulin release after a meal was not observed in AAV1-treated dogs (Fig. 7*F*). Together, these findings suggest that circulating insulin detected in AAV1-treated dogs derives from the expression of the Ins transgene in skeletal muscle and not from residual expression from surviving β cells. All AAV1-Ins +Gck–treated diabetic dogs showed normal circulating glucagon levels (Fig. 7*G*), indicating preservation of α-cell function.

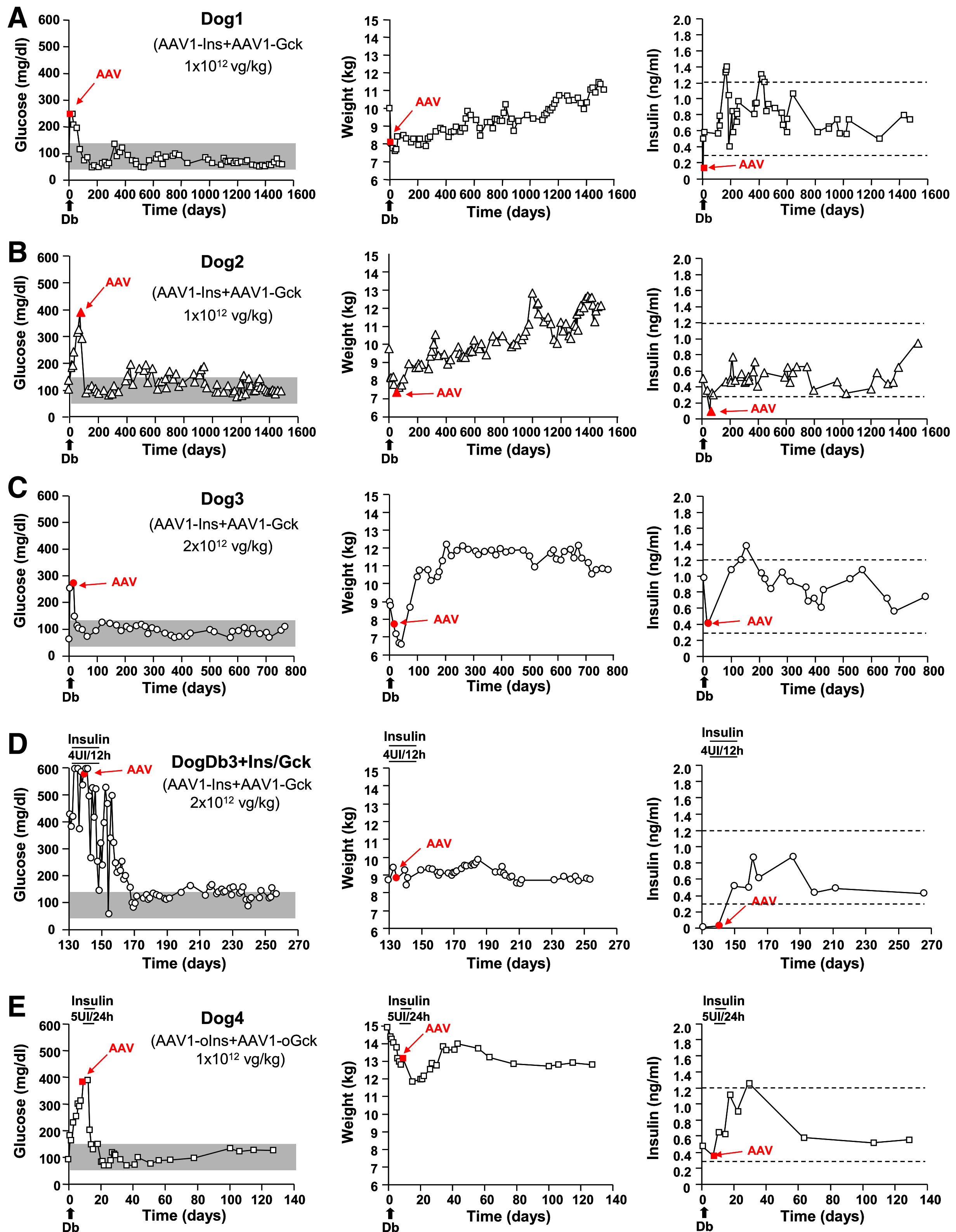

FIG. 4. Treatment with AAV1-Ins and AAV1-Gck corrects diabetes in dogs. *A–D*: Follow-up of glycemia, body weight, and insulinemia. Five diabetic dogs (Dog1–4 and DogDb3+Ins/Gck) were treated with AAV1-Ins and AAV1-Gck vectors at 1×10^{12} vg/kg each for Dog1 and Dog2 (*A* and *B*), at 2×10^{12} vg/kg each for Dog3 and DogDb3+Ins/Gck (*C* and *D*), or with AAV1-oIns and AAV1-oGck vectors at 1×10^{12} vg/kg each for Dog4 (*E*). Dogs had serum insulin levels that remained within the range of fasted healthy animals (dashed lines). Db indicates dog treatment with streptozotocin (STZ) plus alloxan. Gray bars indicate fasting normoglycemia range in dogs (20).

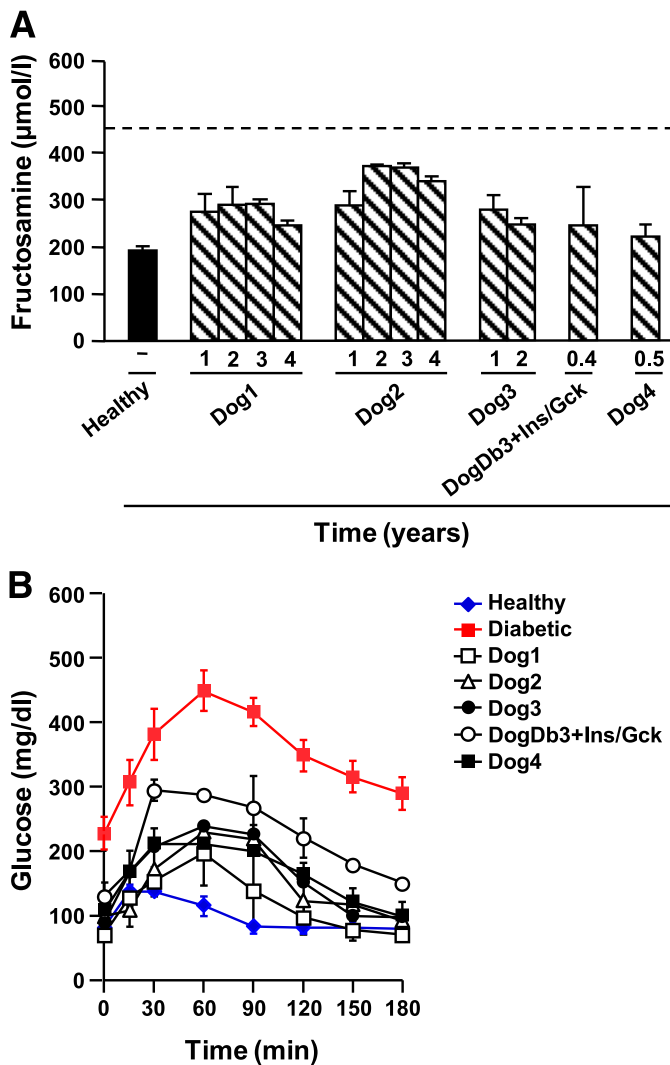

FIG. 5. AAV1-Ins and AAV1-Gck treatment normalizes serum fructosamine and recovers glucose disposal after a load. *A*: Dog1–4 and DogDb3+Ins/Gck had levels of serum fructosamine that ranged from 200 to 350 µmol/L. *B*: OGTT at a dose of glucose of 1.75 g/kg. Ins- and Gck-treated dogs showed a glucose profile similar to healthy animals and below the range for diabetes diagnosis according to American Diabetes Association guidelines (2-h plasma glucose <200 mg/dL). Data are represented as mean ± SEM of 2–3 OGTTs performed every year or during the study period in dogs with shorter follow-up. OGTTs for diabetic control dogs (red line) were performed during the period of intensive exogenous insulin treatment.

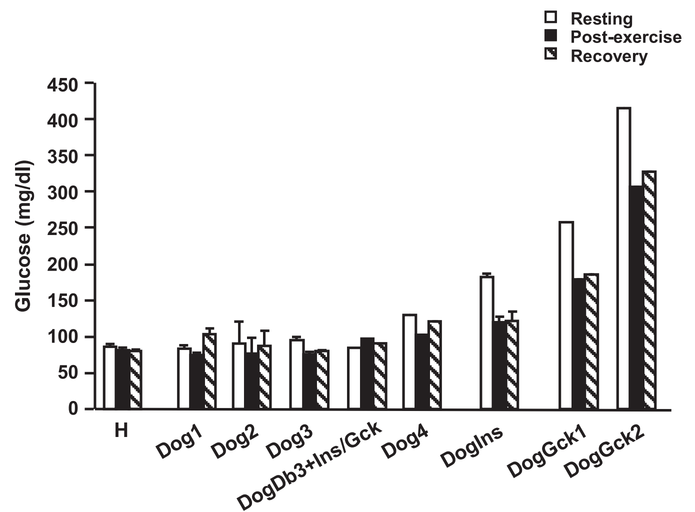

FIG. 6. Effect of exercise on glycemia. Blood glucose was measured in 24-h fasted dogs before, immediately after exercise, and during postexercise recovery. None of the dogs was hypoglycemic over the periods analyzed. Dog1–4 and DogDb3+Ins/Gck showed good glycemic control under exercise, comparable with that of healthy dogs. A decline in blood glucose was observed after exercise in dogs treated with AAV1-Ins or with AAV1-oGck vectors only; these dogs had baseline glucose levels much higher than those of all other animals treated with AAV1-Ins and AAV1-Gck vectors.

The production of insulin in skeletal muscle increased the phosphorylated AKT/total AKT ratio in muscle fibers, indicating that insulin activated its signaling in an autocrine/paracrine manner (Supplementary Fig. 3). Also, in agreement with the absence of glucokinase regulatory protein in skeletal muscle (data not shown) (24), glucokinase was detected only in the cytosol of AAV1-treated muscle fibers, even after a glucose challenge (Supplementary Fig. 4).

Dog3 was euthanized at 2.2 years after gene transfer, and vector genome biodistribution analysis confirmed that most of the detectable vector was present in injected muscles (Supplementary Table 3). Normal muscle morphology without glycogenosis was documented in AAV1-injected animals (Supplementary Fig. 5 and Supplementary Table 4).

Role of the glucokinase transgene in glycemic control. Two diabetic dogs were treated with AAV1-oGck vectors alone at 2×10^{12} vg/kg (DogGck1 and DogGck2). After vector delivery, both dogs remained hyperglycemic and required administration of exogenous insulin to reduce hyperglycemia and to stabilize weight loss (Fig. 8*A*, *B*), demonstrating that glucokinase expression alone is not sufficient to counteract hyperglycemia. Consistently, fructosamine was elevated in both dogs (Fig. 8*C*) and they developed cataracts a few months after hyperglycemia development (Supplementary Table 2). Dogs expressing glucokinase alone showed an impaired OGTT (1.75 g/kg), with glycemia curves similar to those of diabetic nontreated animals (Fig. 8*D*).

Similar to what it was performed with diabetic control dogs, exogenous insulin treatment was optimized on an individual basis in DogGck1 and DogGck2 with twice-daily administrations. Although no decline in fasted glycemia or fructosamine levels was observed (Fig. 8*A*–*C*), an improvement in OGTT was documented (Fig. 8*D*).

The higher sensitivity to exogenous insulin of DogsGck was, however, evidenced when an OGTT was performed with simultaneous subcutaneous injection of insulin. DogGck1 and DogGck2 had a faster glucose disposal than diabetic control dogs, with glycemia declining to <200 mg/dL in both animals (Fig. 8*E*). Increased sensitivity to exogenous insulin was also observed in diabetic mice treated with AAV1-oGck (Supplementary Fig. 6).

DISCUSSION

Since the 1922 breakthrough discovery of Banting and Best, who corrected hyperglycemia in dogs using pancreatic extracts, exogenous insulin administration has been the mainstay of diabetes therapy. Alternative therapies have been studied, but thus far only a few approaches, mainly involving allotransplantation or xenotransplantation of pancreatic islets, have reached clinical application (2).

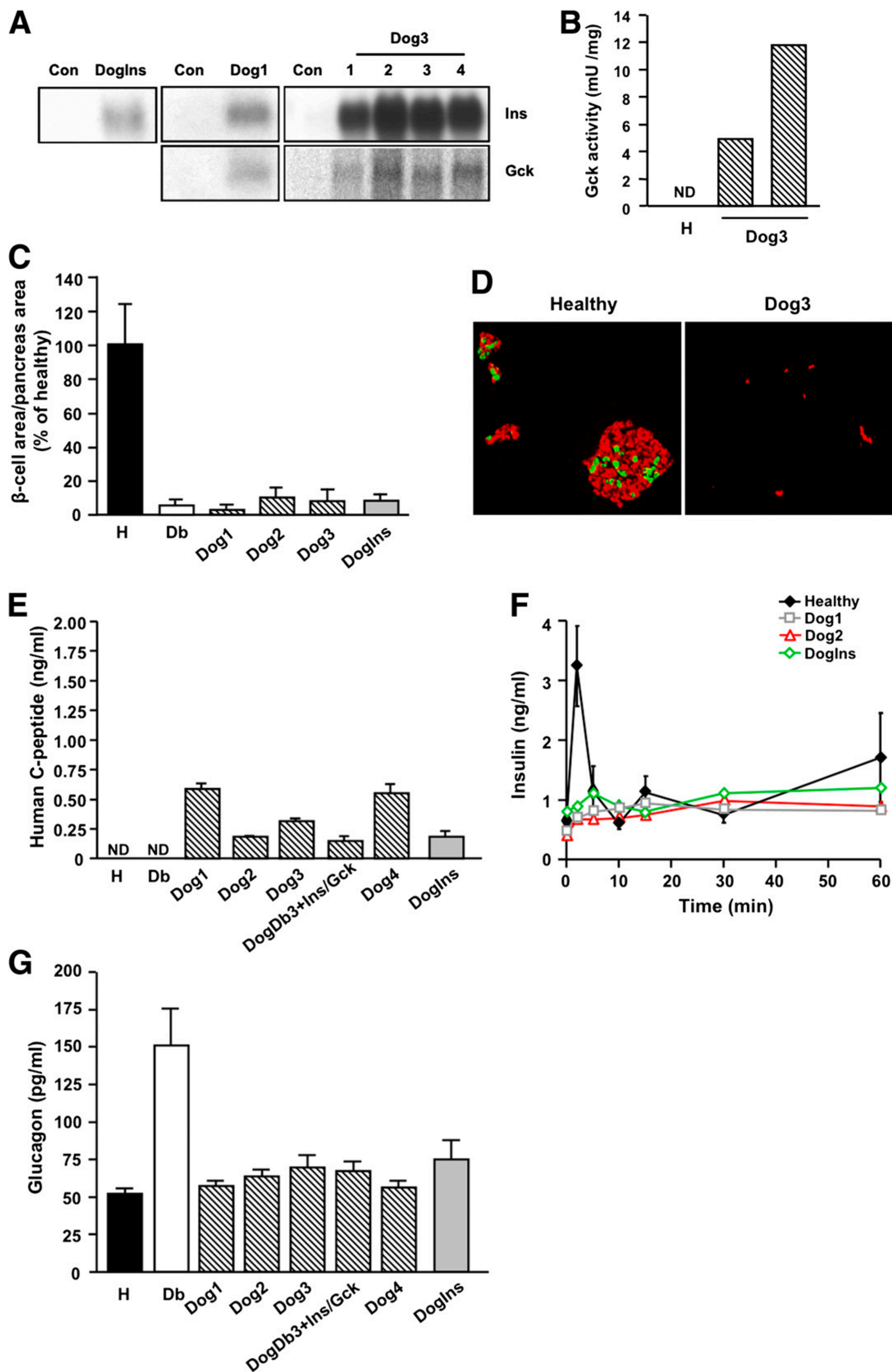

FIG. 7. Skeletal muscle is the source of insulin after AAV1-Ins and AAV1-Gck treatment. *A*: Transgene expression in skeletal muscle of AAV1-treated dogs. Northern blot analysis showing expression of insulin and glucokinase in skeletal muscle biopsy specimens from DogIns and Dog1, and necropsy samples from Dog3, obtained 9 months and 2.2 years after treatment, respectively. Uninjected muscle was used as a control. *B*: Measurement of glucokinase activity in skeletal muscle of healthy (H) dog and two different necropsy samples from Dog3. *C*: The β-cell area was quantified in necropsy samples (whole pancreas) from a healthy dog, a diabetic dog, and Dog3 (2.2 years after treatment), and in pancreas biopsy samples from Dog1, Dog2, and DogIns obtained 9 months after AAV1 administration. Diabetes induction led to >90% reduction in β-cell mass in all dogs. *D*: Representative images of pancreatic sections stained with insulin (red) and glucagon (green) illustrating the marked reduction of insulin-producing cells in Dog3 by 2.2 years after diabetes induction. Original magnification 200×. *E*: Human C-peptide was detectable in serum of all AAV1-Ins–treated dogs, but not in healthy (H) or untreated diabetic (Db) dogs, indicating that proinsulin was produced and processed in skeletal

In the clinical translation of bench results, the scale-up to a large animal model represents perhaps one of the most critical steps. This was demonstrated by the work performed in gene transfer for hemophilia B (12,25–27) and Leber congenital amaurosis (13,28), in which results for dogs were fully predictive of the outcome in humans.

Our novel approach to control hyperglycemia, through genetic engineering of a “glucose sensor” in skeletal muscle using AAV1 vectors, has permitted long-term, clinically meaningful regulation of glycemia in a large animal model of diabetes. Currently, the goal of normalization of glycemia is pursued through intensive insulin therapy, which can delay the onset and slow the progression of secondary complications of diabetes (1). However, this treatment is not suitable for all diabetic patients because of its high risk of hypoglycemia secondary to excessive insulin dosage (1). Additionally, cell therapies, including cadaveric and stem cell–derived islet transplantation, require life-long immunosuppression (2,29). Our approach circumvents a number of the challenges of diabetes therapy. In contrast to allotransplantation, in which supplies of human pancreatic islets are limiting, AAV vector manufacturing is robust and unlimited. Moreover, long-term (>10 years) transgene expression has been documented in humans after AAV vector administration to skeletal muscle (30).

The intramuscular delivery of AAV1 vectors to engineer the skeletal muscle results in expression of both transgenes in the target tissue, and not in the liver, the other tissue where vector genomes are detected, probably because of the silencing of the cytomegalovirus promoter (31). Our results support a model in which the production of insulin in skeletal muscle activates insulin signaling in an autocrine/paracrine manner, and the constant insulin production deriving from AAV1-Ins–transduced fibers is crucial for the system to work as a glucose sensor. Low basal levels of insulin are required to keep muscle capable of uptaking glucose by ensuring continuous GLUT4 translocation to the plasmatic membrane (9). Thus, glucose transport does not become a rate-limiting step to the system before a glucose challenge. However, because of the absence of glucokinase regulatory protein in muscle, the glucokinase produced in this tissue through gene transfer remains active in the cytosol (24). As a result, in a situation of hyperglycemia, glucokinase phosphorylates glucose efficiently, driving the uptake of large amounts of glucose through GLUT4 into the engineered muscle fibers. Then, AAV1-Ins alone is able to maintain good control of fasted glycemia, but AAV1-Gck is required to cope with a glucose overload, as shown by the OGTTs. The advantage of expressing both genes in the same muscle cell would be that by acting in an autocrine/paracrine manner, the levels of insulin required to achieve sufficient GLUT4 expression in the muscle fiber are low and therefore safe. Regarding the systemic action of insulin derived from the skeletal muscle, we have previously demonstrated that transgenic mice expressing insulin and glucokinase under the control of a muscle-specific promoter or diabetic mice treated with AAV1-Ins+Gck gene transfer have restored glucokinase and markedly reduced PEPCK expression in the liver (9). PEPCK is the most important enzyme in the control of gluconeogenesis and is regulated mainly at transcriptional level, with its expression upregulated in starvation or diabetes (32). This evidence suggests that hepatic gluconeogenesis is reduced in Ins- and Gck-treated diabetic animals when compared with untreated diabetic controls, which would contribute to better glycemic control.

Insulin alone, either provided as exogenous therapy or expressed in muscle by an AAV1 vector, did induce glucose uptake; however, it did not guarantee tight control of glycemia, especially after a glucose challenge. Expression of glucokinase alone did not correct hyperglycemia either. Diabetic dogs treated with high doses of AAV1-Gck were hyperglycemic in fasting conditions. However, expression of glucokinase in muscle results in greater sensitivity to insulin, either when insulin is exogenously administered or when it is expressed at low, safe levels from an AAV1 vector. Furthermore, even if an intensive exogenous insulin treatment is adjusted on an individual basis for each diabetic control or AAV1-Gck–treated dog, the glycemic control achieved by Ins and Gck gene therapy is superior, as evidenced by the lower fasted glycemia and fructosamine levels, and improved glucose disposal in AAV1-Ins+Gck–treated animals. Thus, only the synergistic action of insulin and glucokinase allows for tight control of diabetic hyperglycemia.

Skeletal muscle has a number of key advantages as a target tissue for AAV-mediated gene transfer. Muscle is easily accessible by noninvasive procedures, and vector delivery leads to minimal systemic biodistribution (Supplementary Table 2). Moreover, gene delivery to muscle is not limited by the presence of preexisting neutralizing antibodies against AAV (33), a key aspect given the relatively high prevalence of anti-AAV antibodies in the general population (34). In addition, muscle has a metabolism highly based on glucose consumption and can efficiently secrete proteins into the bloodstream. Insulin can be produced, processed, and secreted as a mature protein from skeletal muscle, provided the gene has a genetic modification allowing the cleavage by the endoprotease furin (9). We have been unable to detect insulin within muscle fibers of transgenic or AAV1-treated mice, suggesting that the insulin produced in muscle fibers is not accumulated in vesicles and that its secretion is unlikely to be regulated by vesicle-mediated exocytosis (as in pancreatic β cells). In contrast, secretion from the skeletal muscle seems to be constitutive.

Long-term follow-up of treated dogs suggests that muscle expression of insulin and glucokinase is well-tolerated over a prolonged period. Studies confirmed the safety of the approach even under marked physical exertion, when high levels of glucose consumption increase the risk of hypoglycemic episodes. The use of a large animal model with a long life span allowed us to follow-up animals for early indicators of secondary complications. The absence of clinical findings, such as cataracts or urinary tract infection, and the reduction of biomarkers, such as glycosylated proteins (fructosamine), suggest that Ins and Gck gene transfer may prevent diabetes complications. Hence, normalization of glycemia with a one-time intervention

muscle. Results are shown as mean ± SEM (*n* = 23–56 measurements per dog). *F*: Time course of insulin secretion after a meal 4 years after AAV1 treatment of Dog1, Dog2, and DogIns. Basal levels correspond to a 24-h fasting period, after which animals were fed a 30-g/kg serving of standard diet. Only the healthy dogs showed the first peak of insulin release, which corresponds to pancreatic secretion of the insulin stored in secretory granules. *G*: Normal circulating glucagon levels were observed in AAV1-Ins– and AAV1-Gck–treated dogs indicating preservation of α-cell function. Results are shown as mean ± SEM (*n* = 5–6 measurements per dog). H, healthy; Db, diabetic; Con, control; ND, nondetected.

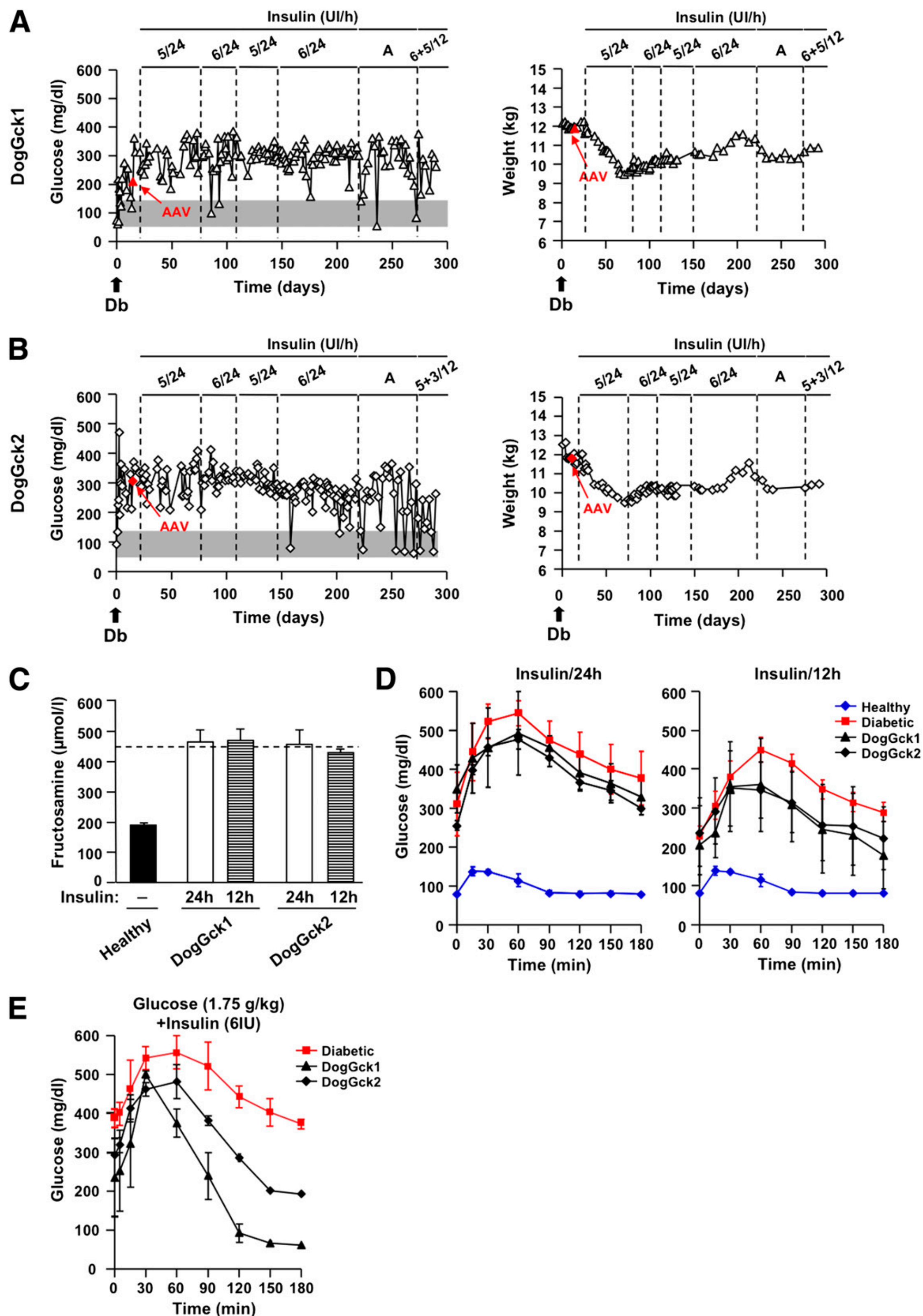

FIG. 8. Diabetic dogs treated with AAV1-oGck alone. Follow-up of glycemia and body weight of diabetic dogs treated with AAV1-oGck vectors (2×10^{12} vg/kg) in DogGck1 (*A*) and DogGck2 (*B*). DogGck remained hyperglycemic during fast and required daily administration of exogenous insulin (dosage [IU] and timing [24 h vs. 12 h] are shown). *C*: Fructosamine levels were altered in DogGck1 and DogGck2 receiving exogenous insulin every 24 or 12 h. Results are shown as mean ± SEM of 2–4 determinations. *D*: DogGck1 and DogGck2 showed similar OGTT profiles at 1.75 g/kg as diabetic untreated dogs when administered with exogenous insulin once daily. On implementation of the optimized treatment, OGTT improved. *E*: The synergistic effect of glucokinase and insulin. DogGck1 and DogGck2 and diabetic control dogs were administered a glucose load (1.75 g/kg) together with a subcutaneous injection of insulin (6 IU). Data are represented as mean ± SEM of 3 OGTTs. Db indicates dog treatment with streptozotocin (STZ) plus alloxan. Gray bars indicate fasting normoglycemia range in dogs (20).

could result in a substantial improvement in patient quality of life, particularly in populations with difficulties in diabetes management, such as brittle diabetes (35).

One possible limitation of the results presented here is that the dog model of diabetes used in this study does not fully mimic the immunological state of type 1 diabetic patients. However, although future studies in autoimmune models of diabetes are warranted, studies of mice (36), dogs (37), and humans (38) would suggest that targeting muscle with AAV vectors may at least partially escape immune recognition. This may be the result of lower levels of major histocompatibility complex class I presentation in this tissue, or the result of the induction of apoptosis of reactive T cells (36,38).

In summary, our data represent the first demonstration of long-term correction of diabetes in a large animal model using gene transfer. Future safety and efficacy studies will help to determine the range of Ins and Gck vector doses that are therapeutic, as well as the glucokinase/insulin expression ratio that is optimal for a tight control of glycemia. These studies will provide the basis for the initiation of a clinical veterinary study in companion animals with diabetes, a strategy also proposed for the clinical development of cancer therapeutics (39). The proposed clinical trial of diabetic dogs that are pets will greatly help define the safety and efficacy profiles of our approach in humans. One added advantage of this strategy is also related to the fact that large experimental animal models of autoimmune diabetes do not exist; thus, companion animals with naturally occurring diabetes constitute an extremely valuable and stringent model. In conclusion, this study lays the foundation for the clinical translation of this approach to veterinary medicine and possibly to humans.

ACKNOWLEDGMENTS

This work was supported by grants from Ministerio de Ciencia e Innovación, Plan Nacional I+D+I (SAF2008-00962 and SAF2011-24698), Generalitat de Catalunya (2009 SGR-224), Spain and European Commission (EUGENE2, LSHM-CT-2004-512013 and CLINIGENE, LSHB-CT-2006-018933), the National Institutes of Health grants 2P01-HL64190 and N01-HV78203, and the Howard Hughes Medical Institute.

D.C. and C.J.M. are recipients of predoctoral and postdoctoral fellowships from Ministerio de Ciencia e Innovación, Spain, respectively. F.B., D.C., and E.A. are inventors of a patent application regarding the use of insulin and glucokinase treatment of diabetes. F.M. and K.A.H. are inventors of a patent application of the AAV vector technology. No other potential conflicts of interest relevant to this article were reported.

D.C., C.J.M., and E.A. designed experiments, generated reagents, performed experiments, and wrote and edited the manuscript. R.L., I.G., C.R., A.A., R.R.-d.G., J.M., S.M., T.F., V.H., S.Z., J.R., F.M., and K.A.H. generated reagents and performed experiments. V.H., F.M., and K.A.H. wrote and edited the manuscript. F.G. designed experiments, generated reagents, and performed experiments. F.B. designed experiments and wrote and edited the manuscript. F.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Malcolm Watford (Rutgers University), Sylvie Franckhauser (Universitat Autonoma de Barcelona, Spain), and Judith Storch (Rutgers University), for helpful discussions, and Juan Benitez (Universitat Autonoma de Barcelona, Spain), Marta Moya (Universitat Autonoma de Barcelona, Spain), and Luca Maggioni (Universitat Autonoma de Barcelona, Spain) for technical assistance.

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