Long-Acting FGF21 Has Enhanced Efficacy in Diet-Induced Obese Mice and in Obese Rhesus Monkeys

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Fibroblast growth factor 21 (FGF21), a hormone with short half-life, has consistently shown strong pharmacological efficacy. We first assessed the efficacy of murine recombinant FGF21 in C57BL6 lean mice for 5 wk. We then generated a long-acting FGF21 molecule by fusing a Fc to a variant of human recombinant FGF21 (hrFGF21) that contained two engineered mutations [L98R, P171G; Fc-FGF21(RG)] and tested it in C57BL6 diet-induced obese mice and obese rhesus monkeys. We compared its metabolic properties with those of the hrFGF21. Groups of diet-induced obese mice were treated for 36 d with different doses of hrFGF21 (01, 0.3, and 1 mg/kg twice daily) and with Fc-FGF21(RG) (2.3 mg/kg, every 5 d). Body weight, glucose, insulin, cholesterol, and triglyceride levels were decreased after treatment with either compound. A glucose tolerance test (GTT) was also improved. Obese rhesus monkeys were treated with hrFGF21 (once a day) and Fc-FGF21(RG) (once a week) in a dose-escalation fashion. Doses started at 0.1 and 0.3 mg/kg and ended at 3 and 5 mg/kg for hrFGF21 and Fc-FGF21(RG), respectively. Doses were escalated every 2 wk, and animals were followed up for a washout period of 3 wk. Body weight, glucose, insulin, cholesterol, and triglyceride levels and the GTT profile were decreased to a greater extent with Fc-FGF21(RG) than with hrFGF21. The PK-PD relationship of Fc-FGF21(RG) exposure and triglyceride reduction was also conducted with a maximum response model. In conclusion, in more than one species, Fc-FGF21(RG) chronically administered once a week showed similar or greater efficacy than hrFGF21 administered daily. (***Endocrinology*** **153: 4192–4203, 2012)**

The worldwide incidence of type 2 diabetes increased dramatically over the last 20 yr (1, 2). Although overproduction and underutilization of glucose are hallmarks of diabetes (3), most patients develop additional impairments that lead to hyperglycemia and/or hyperinsulinemia (4). Generally, these patients present other complications, such as increased body weight, elevated lipid levels [combined hyperlipidemia (5, 6)], and/or other abnormalities (7). Most currently available therapies control glucose levels and/or improve insulin resistance, but multiple factors make the development of new therapies more challenging. First, recent clinical trials have shown that tight regulation of glycemia may not significantly affect cardiovascular outcome (8), whereas other trials have shown the opposite (9, 10). Second, some existing therapies are efficacious for only a limited period of time and may be accompanied by a number of side effects such as hypoglycemia [insulin, sulfonylureas, (11)], increased body weight [insulin, thiazolidinediones (12, 13)], or edema [thiazolidinediones (14)]. Lastly, patients do not always comply with the offered therapy because of the number of injections (15), the side effects (12, 14), or other reasons. New therapies for the treatment of type 2 diabetes that would eliminate or further reduce the challenges described above or that would provide additional advantages to lowering blood glucose are highly desirable.

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Abbreviations: AUC, Area under the curve; BID, twice daily; DIO, diet-induced obese; E_{max}, maximum response; FGF21, fibroblast growth factor 21; GTT, glucose tolerance test; hrFGF21, human recombinant FGF21; LDL, low-density lipoprotein; mrFGF21, murine recombinant FGF21 protein; OGTT, oral glucose tolerance test; PK-PD, pharmacokinetic-pharmacodynamic; Q5D, every 5 d.

Fibroblast growth factor 21 (FGF21) is a novel hormone involved in the regulation of glucose, lipids, and energy homeostasis. Both pharmacological and genetic data indicate that FGF21 has therapeutic potentials for the treatment of type 2 diabetes, obesity, and other comorbidities (16–18). Administration of recombinant FGF21 into rodent or monkey models taken together with the phenotype of transgenic mice overexpressing FGF21 suggest that this hormone is associated with overwhelming improvements in many metabolic parameters including normalized blood glucose and triglyceride levels, ameliorated lipoprotein profiles, enhanced insulin sensitivity, increased energy expenditure, and decreased hepatic steatosis and body weight (16–18). Also, recent characterizations of FGF21 germline knockout mice and mice with adenovirus-mediated knockdown of hepatic FGF21 suggest that FGF21 plays an essential role in normal physiology (19–21). Deficiency in FGF21 led to mild weight gain, an increase in hepatic fat content, and dyslipidemia when mice were exposed to a ketogenic diet (19).

In rodents and primates, the half-life of recombinant FGF21 was determined to be approximately 1–2 h (17). As a result, daily or twice-daily administration was required in preclinical studies to achieve desired pharmacological effects (16–18). Such a dose regimen would pose a significant challenge for clinical application; therefore, we generated a long-acting FGF21 analog [Fc-FGF21(RG)], which is potent and efficacious and exhibits markedly improved pharmacokinetics over the native molecule.

In this manuscript, we assessed the efficacy of murine recombinant FGF21 protein (mrFGF21) in C57BL6 lean mice. We then compared the efficacy of the human recombinant FGF21 (hrFGF21) to Fc-FGF21(RG) in different species and animal models, diet-induced obese (DIO) mice, and obese rhesus monkeys, when administered chronically. We then investigated the relationship of Fc-FGF21(RG) exposure and triglyceride reduction in obese rhesus monkeys with a maximum response (E_{max}) model. In both species, Fc-FGF21(RG) administered once every 5–7 d showed a similar or a greater efficacy than hrFGF21 administered once or twice daily.

Materials and Methods

Reagents

mrFGF21, hrFGF21 (amino acids 1-181 or 2-181) and Fc-FGF21(RG) were prepared as described previously (18). hrFGF21 (1-181) and hrFGF21 (2-181) showed similar *in vitro* and *in vivo* bioactivity (Yie, J., unpublished data). hrFGF21 (2-181) was used for the mouse DIO experiment only and hrFGF21 (1-181) for all the other experiments.

Animals

All mouse studies were conducted at Amgen Inc. (Thousand Oaks, CA) and approved by the Institutional Animal Care and Use Committee. The rhesus monkey study was conducted at Yunnan Laboratory Primate Inc. (Yunnan, China). Research protocol and animal housing were approved by the Yunnan Laboratory Primate Inc., China Institutional Animal Care and Use Committee. Conditions in which animals are maintained are described in Supplemental Material, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Metabolic effects of mrFGF21 in C57BL6 lean mice

First, mrFGF21 was tested chronically in C57BL6 lean mice. Nine-week-old C57BL6 male mice were randomized into groups on the basis of body weight and blood glucose levels. mrFGF21 was injected ip twice daily (BID) for a total daily dose of 0 (vehicle, PBS), 1, and 10 mg/kg. The study was conducted for 36 d. Body weight was monitored throughout the study. Blood from mice that had been fed *ad libutum* was collected at treatment d 17, 1 h after the morning injection, and metabolic parameters were measured (Supplemental Material). A glucose tolerance test (GTT) using a 2-g/kg glucose dose (ip) was performed at treatment d 24 1 h after the morning injection. Animals were fasted for 12 h before the GTT was started.

Metabolic effects of recombinant human FGF21 (hrFGF21) and Fc-FGF21(RG) in C57BL6 DIO mice

Four-week-old C57BL6 male mice were fed a high-fat diet (60% of calories from fat) enriched with saturated fatty acids (D12492; Research Diets, Inc., New Brunswick, NJ). After 12 wk of high-fat diet feeding, DIO mice were sorted into groups on the basis of body weight and blood glucose levels. hrFGF21 was injected ip BID at doses of 0 (vehicle), 0.1, 0.3, and 1 mg/kg. Fc-FGF21(RG) was administered ip every 5 d (Q5D) at doses of 0 (vehicle) and 2.3 mg/kg. The determination of Q5D for Fc-FGF21(RG) and BID hrFGF21was made on the basis of previous studies (data not shown) that suggested a diminished *in vivo* efficacy after 5 d or 6 h after a single injection of Fc-FGF21(RG) or hrFGF21, respectively. Such a dose regimen was selected to achieve a sustained glucose-lowering effect throughout the study period. Fc-FGF21(RG) at 2.3 mg/kg is molar equivalent to hrFGF21 at 1 mg/kg. The study was conducted for 36 d. Body weight was monitored throughout the study. Blood was collected from mice fed *ad libutum* at treatment d 20 [approximately 16 or 120 h after the last hrFGF21or Fc-FGF21(RG) injection, respectively], and metabolic parameters were measured as described in the Supplemental Material. A GTT was performed at treatment d 30.

Metabolic effects of hrFGF21 and Fc-FGF21(RG) in obese male rhesus monkeys

Fc-FGF21(RG), hrFGF21, or vehicle were administered chronically and sc to obese male rhesus monkeys. The rhesus monkeys were 5–18 yr old. Their body weight ranged from 6.4 to 11.7 kg with a body mass index greater than 35 kg/m^2. Animals were acclimated for 23 d before the administration of any test compound (Supplemental Material). After 4 wk of training, baseline oral glucose tolerance tests (OGTT) and plasma metabolic parameters were measured. Rhesus monkeys were then randomized into vehicle or treatment groups to achieve compa-

rable average baseline levels among the groups for OGTT area under the curve (AUC) and body weight (Supplemental Material). Rhesus monkeys were then divided into groups of 10 and administered multiple sc injections of test compounds or control article in a blinded fashion (as depicted graphically elsewhere; see Fig. 3). Administration of vehicle or hrFGF21 was performed daily, whereas Fc-FGF21(RG) was administered weekly. Animals treated with Fc-FGF21(RG) received vehicle injection on the days they were not injected with the compound. Fc-FGF21(RG) and hrFGF21 doses were escalated every 2 wk (as shown elsewhere; see Fig. 3). Three different dose levels were selected: the low dose was 0.1 and 0.3 mg/kg, the middle dose was 0.3 and 1 mg/kg, and the high dose was 1 and 5 mg/kg for hrFGF21 and Fc-FGF21(RG), respectively (doses justification are described in the Supplemental Material). Body weight was monitored throughout the study. Baseline body weights of animals from each group are shown in Supplemental Table 1. Two OGTT were performed before the start of the treatment (Supplemental Material). OGTT 3, 4, and 5 were performed every 2 wk at the end of each dose treatment of low, middle, and high doses. Blood samples were collected from fasted animals weekly and were used to measure glucose, insulin, total cholesterol, high-density lipoprotein, and low-density lipoprotein (LDL) cholesterol and triglyceride levels and the test compound levels (Supplemental Material). Blood samples were also collected weekly during the 3-wk washout period.

FGF21 enzyme-linked immunosorbent assay

The concentrations of immunoreactive hrFGF21 analogs in rhesus monkey plasma samples were determined by a sandwich ELISA that used hrFGF21-specific antibodies developed in-house (Supplemental Material).

Terminal levels of hrFGF21 and Fc-FGF21(RG) were also measured in plasma samples of DIO mice using a sandwich ELISA hrFGF21-specific antibodies also developed in-house (Supplemental Material).

Pharmacokinetic-pharmacodynamic (PK-PD) modeling in obese male rhesus monkeys

Fc-FGF21(RG) plasma concentration and PD response (triglyceride concentrations) were measured at a predose and 5 d after the dose (Supplemental Material). [For the Fc-FGF21(RG) dosing regimen and detailed study design, see shown in Fig. 3].

The population PK-PD analysis was performed using NONMEM, version VII, level 2.0 (ICON Development Solutions, Ellicott City, MD), a nonlinear mixed-effects modeling software. The first-order conditional estimation method with interaction was used.

Statistical analysis

For the rodent experiments, analyses were performed for each metabolic end point at each time point independently (for a given end point, a separate analysis for each time point after the first administration of the test articles). Statview software (SAS Institute, Inc., Cary, NC) was used to perform statistical analyses. Statistical comparison of the means among the groups was made using one-way ANOVA model with a Fisher's *post hoc* test. Reported $P < 0.05$ was considered statistically significant.

For the rhesus monkey experiment, analyses were performed for each metabolic end point and for low dose, middle dose, high dose, and washout period independently (for a given end point, a separate analysis per dosing period). SAS version 9.1 (SAS Institute) was used to perform statistical analyses (Supplemental Material for more details).

Results

Metabolic effects of mrFGF21 in C57BL6 lean mice

The efficacy of mrFGF21 in normal C57BL6 lean mice after chronic administration was determined. C57BL6 lean mice treated with mrFGF21 showed a dose-dependent reduction in body weight gain (Fig. 1A). Blood glucose levels were minimally decreased with the high dose of mrFGF21 (10 mg/kg; Fig. 1B), whereas the insulin levels were decreased with both doses of mrFGF21 (Fig. 1C). Triglyceride levels were significantly decreased at both doses (1 and 10 mg/kg; Fig. 1D), but cholesterol levels were decreased only at 10 mg/kg (Fig. 1E). Both doses of mrFGF21 significantly improved GTT, and the chronic administration of mrFGF21 did not cause hypoglycemia after an overnight fast as shown at baseline (Fig. 1F). Plasma β-hydroxybutyrate levels trended toward lower levels after 17 d of mrFGF21 treatment with both doses (1 and 10 mg/kg; Fig. 1G).

Metabolic effects of short- and long-acting FGF21 in C57BL6 DIO mice

The efficacy of BID short-acting hrFGF21 and Q5D long-acting Fc-FGF21(RG) was compared in DIO mice when administered chronically. Metabolic parameters were measured in blood samples collected 16 h after the last dose of hrFGF21 and 5 d after Fc-FGF21(RG) administration on d 20. DIO mice treated with BID hrFGF21 showed a dose-dependent reduction in body weight (Fig. 2A), plasma glucose (Fig. 2B), insulin (Fig. 2C), and triglyceride and cholesterol levels (Fig. 2, D and E) and improved glucose tolerance (Fig. 2F). Near maximal reductions in plasma insulin, triglyceride, and cholesterol levels were obtained when hrFGF21 was administered at 0.3 mg/kg BID. Fc-FGF21(RG) at Q5D 2.3 mg/kg achieved similar metabolic effects to hrFGF21 administered BID at 0.3 mg/kg (Fig. 2). Both compounds caused similar reductions in circulating levels of glucose, insulin, triglycerides, and cholesterol levels as well as reductions in body weight (Fig. 2, A–E). They also similarly improved glucose tolerance in DIO mice (Fig. 2F). Plasma β-hydroxybutyrate levels were trended toward a decrease after hrFGF21 or Fc-FGF21(RG) treatment (only significantly decreased at the 0.3 mg/kg·d dose of hrFGF21 group; Fig. 2G). Terminal blood samples at d 36 were also collected 16 h after the last dose of hrFGF21 and 5 d after Fc-FGF21(RG) admin-

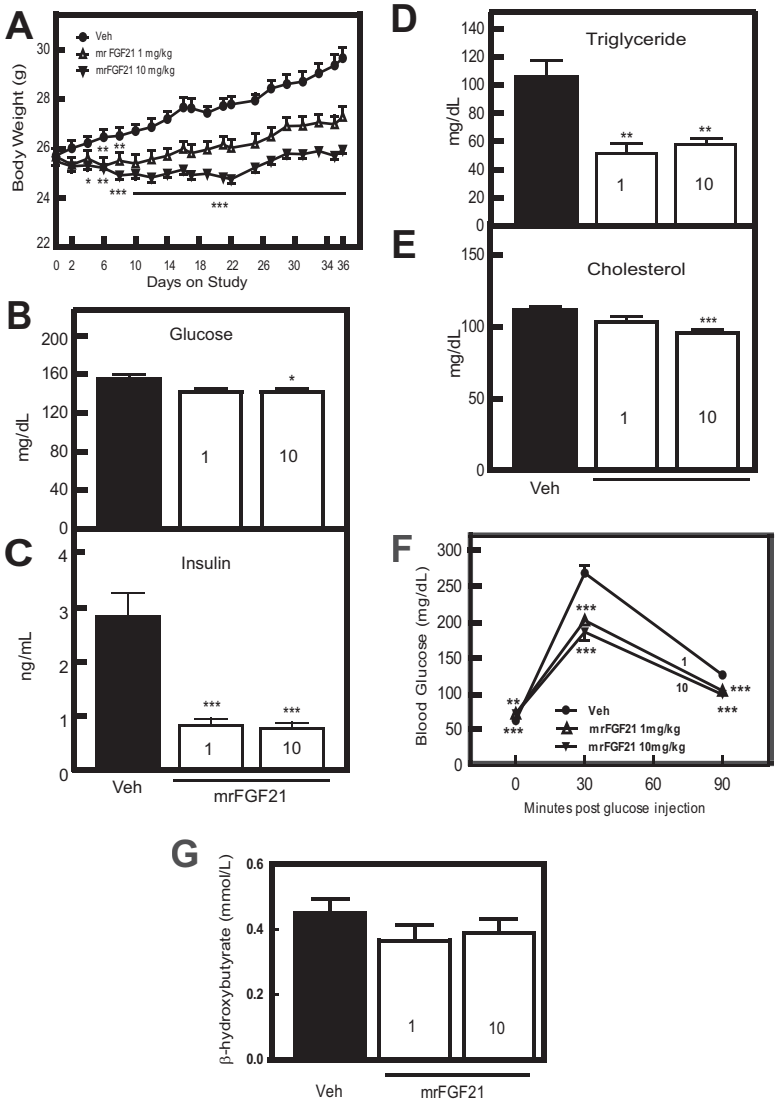

FIG. 1. Chronic metabolic effects of mrFGF21 in C57BL6 lean mice. Mice were injected ip with mrFGF21 BID at the indicated doses. A, Body weight was measured every other day up to the end of the treatment on d 36. Veh, Vehicle. B, Blood glucose levels. C, Insulin levels. D, Triglyceride levels. E, Cholesterol levels were measured at treatment d 17. F, Glucose tolerance test was performed at treatment d 24. A 2-g/kg dose of glucose was injected ip and at 30 and 90 min, blood glucose levels were measured. G, β-hydroxybutyrate levels. All data represent mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* vehicle control.

istration. Plasma concentration of hrFGF21 was detected only in the highest dose group (1 mg/kg) with a mean value of 0.05 nM, whereas Fc-FGF(RG) concentration reached a mean value of 1.8 nM (Fig. 2H).

Compound exposure in obese male rhesus monkeys

The exposure of hrFGF21 and Fc-FGF21(RG) on chronic sc administration to obese male rhesus monkeys was evaluated by FGF21 ELISA. Briefly, each animal was injected once a day with compound or vehicle. Administration of hrFGF21 was performed daily, whereas Fc-FGF21(RG) was administered weekly (Fig. 3). Compound levels were measured in fasting plasma samples collected weekly during the treatment phase and washout period, at approximately 5 d after the last Fc-FGF21(RG) injection and approximately 21 h after the last hrFGF21 injection.

The majority of the animals in the hrFGF21-treated group had concentrations below the quantification limit, particularly for those in the lowest- and middle-dose groups (Fig. 4A). In the highest hrFGF21-treated group, plasma concentrations of hrFGF21 were detected in about half of the animals (Fig. 4A). Fc-FGF21(RG) showed detectable levels during each dosing phase with a tight range (two weekly doses at the same dose strength, Fig. 4). The average concentration from each dosing phase increased approximately dose proportionally from 0.3 to 5 mg/kg for Fc-FGF21(RG) (Fig. 4B). There was minimal accumulation, as demonstrated by the steady concentrations after the first and second weekly dose within each dose-escalation phase. During the washout period, the Fc-FGF21(RG) levels were detectable up to approximately d 48 (13 d after the last dose) and were below the quantification limit afterward (Fig. 4B).

Body weight effects in obese male rhesus monkeys

Baseline body weight values for each group are included in Supplemental Table 1. Body weight was followed up throughout the study, both before and after administration of FGF21 compounds. As shown in Fig. 5A, the body weight of the animals treated with Fc-FGF21(RG) and hrFGF21 decreased over the course of the 6-wk treatment period. The body weight reduction was observed at the dose level of 1 mg/kg for both compounds; however, Fc-FGF21(RG) was administered weekly, whereas hrFGF21 was administered daily. In addition, Fc-FGF21(RG) resulted in a more pronounced body weight decrease than hrFGF21 did (Fig. 5A).

Effect of test compounds on OGTT (glucose and insulin) in obese male rhesus monkeys

Baseline OGTT1 and OGTT2 presented the expected glucose profile as seen in normal animals, with a maxi-

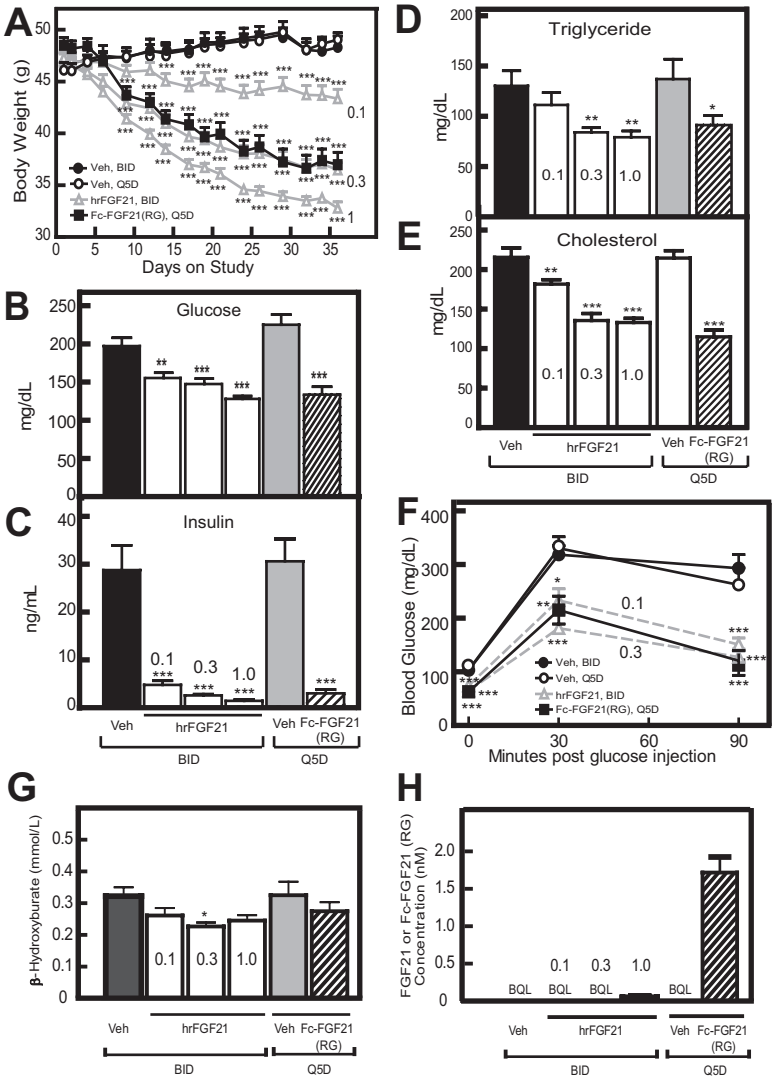

FIG. 2. Chronic metabolic effects of hrFGF21 and Fc-FGF21(RG) in C57BL6 DIO mice. DIO mice were injected ip with Fc-FGF21(RG) Q5D and hrFGF21 BID at the indicated doses. A, Body weight was measured daily up to the end of the treatment on d 36. Blood metabolic parameters shown were measured at treatment d 20. Veh, Vehicle. B, Blood glucose levels. C, Insulin levels. D, Triglyceride levels. E, Cholesterol levels. F, A GTT was performed at treatment d 30. A 2-g/kg dose of glucose was injected ip and at 30 and 90 min, blood glucose levels were measured. G, β-Hydroxybutyrate levels. H, FGF21 or Fc-FGF21(RG) concentration. All data represent mean ± SEM. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ *vs.* vehicle control.

mum plasma glucose obtained at 30 min. Stable AUC for the three different groups were demonstrated (data not shown). Fasting baseline values for plasma chemistry measurements performed on blood samples collected before the start of treatment are shown in Supplemental Table 1.

Three OGTT (OGTT 3, 4, and 5) were performed after the treatments were initiated (Fig. 3). The OGTT5 glucose and insulin profiles are shown in Fig. 5, B and C, respectively. OGTT5 glucose and insulin level profiles were measured in animals treated with vehicle, hrFGF21, or Fc-FGF21(RG) for 6 wk, which corresponded to the last 2 wk of the high-dose escalation regimen. OGTT5 was conducted approximately 7 d after the last Fc-FGF21(RG) injection and approximately 21 h after the last hrFGF21 injection. Relative to vehicle-treated animals, those treated with Fc-FGF21(RG) showed improvement in glucose clearance compared with hrFGF21 or vehicle at 120 and 180 min (Fig. 5B). No improvement in glucose clearance was observed in hrFGF21 compared with vehicle (Fig. 5B). Insulin levels during OGTT5 were slightly decreased in both hrFGF21 and Fc-FGF21(RG) treatment groups; however, statistical significance was observed only at the last time point measured in animals treated with Fc-FGF21(RG) relative to animals treated with vehicle or hrFGF21 (Fig. 5C).

Glucose and insulin AUC expressed as percent change from baseline were calculated for the three OGTT (OGTT 3, 4, and 5) performed at the end of each of the low, middle, and high doses in the three different groups of rhesus monkeys as shown in Fig. 5, D and E, respectively. Glucose AUC during the OGTT in animals treated with Fc-FGF21(RG) were significantly improved compared with the vehicle group for OGTT 4 and 5 at the middle and high doses and to the hrFGF21 group for OGTT 3, 4, and 5 at all tested doses. Insulin AUC during the OGTT in animals treated with Fc-FGF21(RG) were significantly improved compared with the vehicle group for OGTT 4 and 5 at the middle and high tested doses.

FGF21 decreased insulin levels in obese male rhesus monkeys

Insulin levels were measured in blood samples that had been collected after an overnight fast or after an afternoon meal. Fasting plasma insulin levels were measured every week in animals treated with vehicle, hrFGF21, or Fc-FGF21(RG) and during the 3-wk washout period. Fasted blood samples were drawn approximately 5 d after the last Fc-FGF21(RG) injection and approximately 21 h after the last hrFGF21 injection. Fed plasma insulin levels were measured in rhesus monkeys during the fifth and sixth week of treatment. Fed blood samples were drawn approximately 3 d after the Fc-FGF21(RG) injection and approximately 1 h after the last hrFGF21 injection. Although Fc-FGF21(RG) showed a

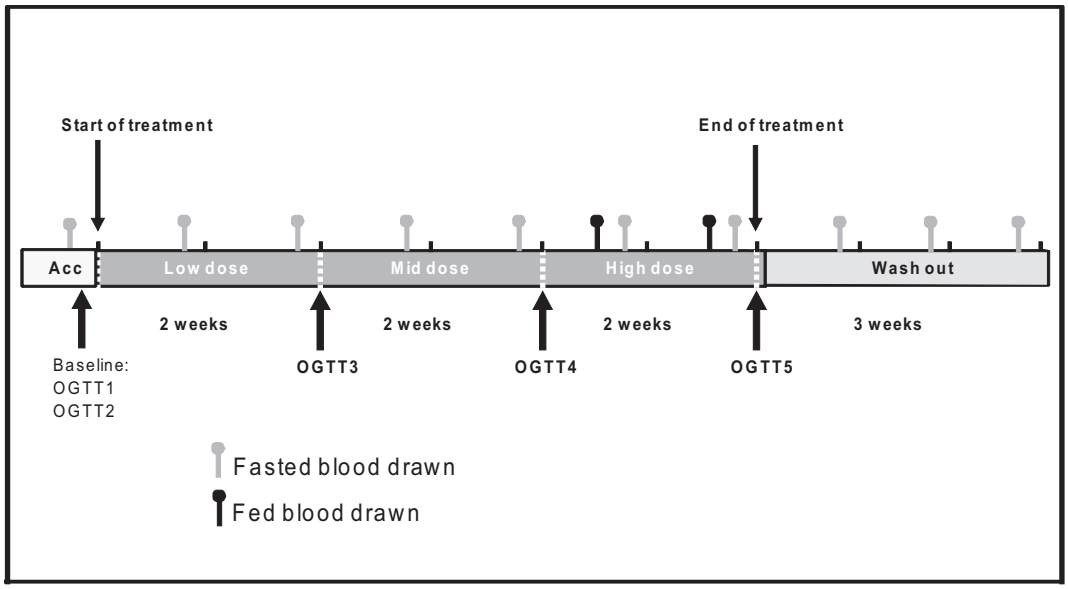

FIG. 3. Study design of the rhesus monkey efficacy study. The diagram graphically depicts the study design for a 6-wk dose-escalation study performed in obese rhesus monkeys. *Shaded symbols* indicate blood draws in the fasted state, and *stippled symbols* indicate blood draws in the fed state. Body weight was measured weekly and two OGTT (OGTT1 and OGTT2) were performed during the acclimation period and for randomization purposes. OGTT3, OGTT4, and OGTT5 were performed at the end of each dose level with low (0.1 and 0.3 mg/kg·wk), middle (0.3 mg/kg·d and 1 mg/kg·wk), and high (1 and 5 mg/kg·d) for hrFGF21 and Fc-FGF21(RG), respectively. A washout period of 3 wk was observed.

trend in decreasing fasting and fed plasma insulin levels, only hrFGF21 statistically significantly decreased fasted and fed plasma insulin levels at the two highest tested doses (Fig. 6, A and B, respectively).

FGF21 decreased triglyceride levels in obese male rhesus monkeys

Fasting and fed plasma cholesterol and triglyceride levels were measured in rhesus monkeys treated with vehicle, hrFGF21, or Fc-FGF21(RG) and during the 3-wk washout period (fasted only). Fasted blood samples were drawn weekly approximately 5 d after last Fc-FGF21(RG) injection and approximately 21 h after the last hrFGF21 injection. Fasting baseline values are shown in Supplemental Table 1. Fed blood samples were drawn during the fifth and sixth week of treatment approximately 3 d after the last Fc-FGF21(RG) injection and approximately 2 h after the last FGF21 injection.

Fasted and fed plasma total cholesterol as well as high-density lipoprotein and LDL cholesterol levels were not changed with any FGF21 treatments (Fig. 7, A and B, and Supplemental Table 2, respectively). However, animals treated with either test compound showed a decrease in fasting triglyceride levels, with Fc-FGF21(RG) having a greater lowering effect than hrFGF21 (Fig. 7C). Animals treated with hrFGF21 and Fc-FGF21(RG) also showed

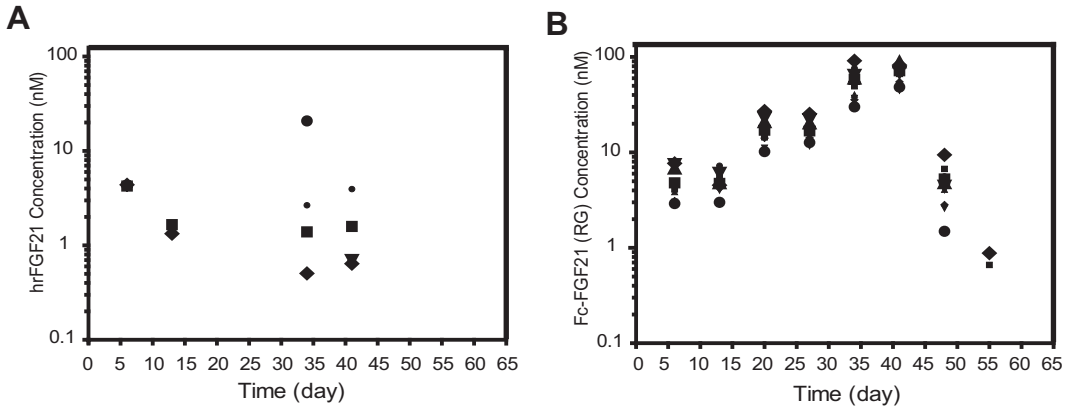

FIG. 4. Plasma concentrations of hrFGF21 (A) and Fc-FGF21(RG) (B) in the obese rhesus monkey efficacy study. The concentrations of immunoreactive hrFGF21 analogs in fasting plasma samples were determined by a sandwich ELISA using hrFGF21-specific antibodies developed in-house. Test compound levels (nanomoles) in individual monkeys were measured at 6, 13, 20, 27, 34, 41, 48, 55, and 62 d, with samples acquired at approximately 21 h after the last hrFGF21 injection and approximately 5 d after the last Fc-FGF21(RG) injection. The *individual symbols* represent the FGF21 levels for each animal.

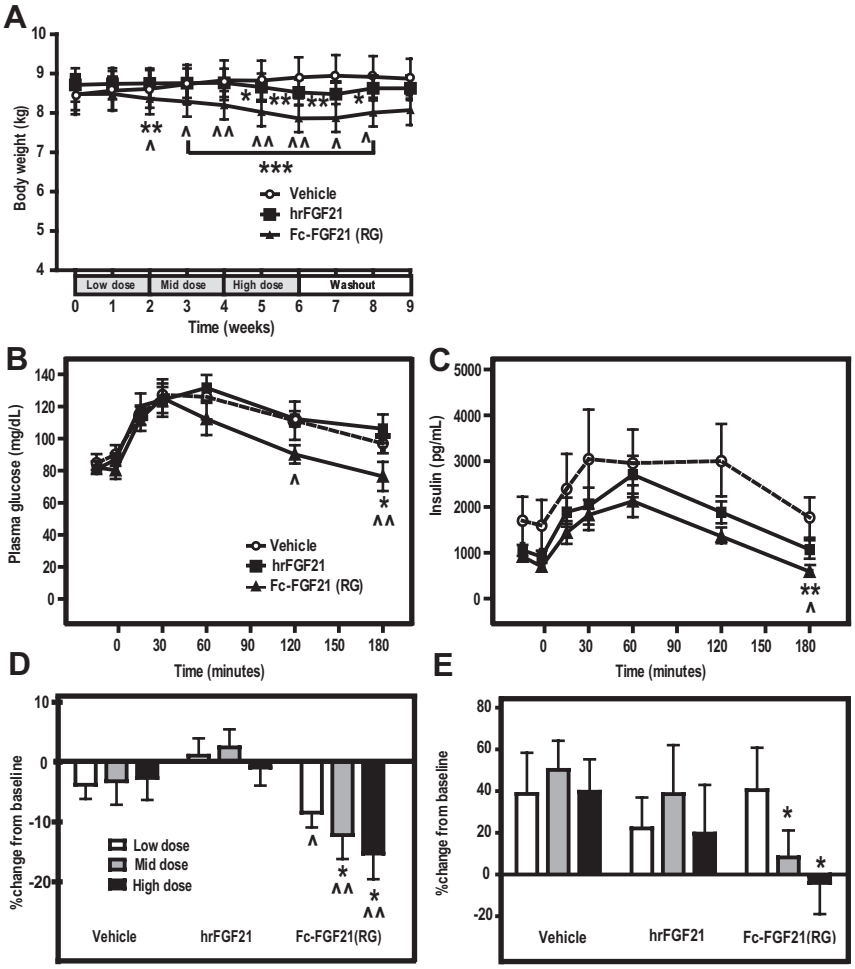

FIG. 5. Chronic effects of hrFGF21 and Fc-FGF21(RG) on body weight, glucose, and insulin levels during OGTT in obese rhesus monkeys. A, Effects of vehicle (*open circles*), hrFGF21 (*filled squares*), and Fc-FGF21(RG) (*filled triangles*) on body weight in rhesus monkeys. B, Glucose profiles of OGTT5 performed at the end of the 2-wk high-dose treatment with Fc-FGF21(RG); *open circle* and *dotted line* correspond to vehicle; *filled square* and *solid line* correspond to hrFGF21; and *solid triangle* and *solid line* correspond to Fc-FGF21(RG). C, Insulin profiles of OGTT5 performed at the end of the 2-wk high-dose treatment with Fc-FGF21(RG); *open circle* and *dotted line* correspond to vehicle; *filled square* and *solid line* correspond to hrFGF21; and *solid triangle* and *solid line* correspond to Fc-FGF21(RG). D, Glucose OGTT AUC during the 6-wk treatment period of the obese rhesus monkeys; *open bars* correspond to the glucose AUC measurements calculated from OGTT3; *shaded bars* correspond to the glucose AUC measurements calculated from OGTT4; and *solid bars* correspond to the glucose AUC measurements calculated from OGTT5. E, Insulin OGTT AUC during the 6-wk treatment period of the obese rhesus monkeys; *open bars* correspond to insulin AUC measurements calculated from OGTT3, *shaded bars* correspond to insulin AUC measurements calculated from OGTT4, and *solid bars* correspond to insulin AUC measurements calculated from OGTT5. All data represent mean ± SEM. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ *vs.* vehicle treatment; ^, $P < 0.05$, ^^, $P < 0.01$ *vs.* hrFGF21.

statistically significantly lower fed plasma triglyceride levels than animals treated with vehicle (Fig. 7D). Plasma β-hydroxybutyrate levels were not changed after either test compound treatment measured in the blood samples collected in the fasted conditions (Supplemental Table 2).

PK-PD modeling in obese male rhesus monkeys

The objective of the PK-PD modeling analysis was to characterize the exposure response relationship and help guide future study designs. Before the population PK-PD analysis, the relationship of Fc-FGF21(RG) PK concentration and PD responses (glucose, insulin, and triglyceride lowering) were visually examined. The most significant exposure-response relationship was observed in triglyceride reduction. Therefore, the triglyceride level was selected for further PK-PD investigation. As shown in Fig. 8, triglyceride levels appear to have reached a plateau when PK concentration increases. Thus, a direct response, inhibitory E_{max} model was developed to characterize this PK-PD relationship. Sigmoidicity of the model was also investigated but did not show improvement (Akaike's information criterion increased by 0.2). The parameter estimates are shown in Supplemental Table 3. The mean maximum triglyceride reduction relative to baseline was 62%. In the concentration range (0–4000 ng/ml), Fc-FGF21(RG) decreased triglyceride levels with the population mean EC_{50} of 378 ng/ml (Supplemental Table 3). The mean vehicle effect was 0.936. The goodness-of-fit plots of the final model are shown and explained in the Supplemental Material (Supplemental Fig. 1). Our modeling analysis demonstrated that the inhibitory E_{max} model was appropriate to describe our observed data and the derived PK-PD parameters could be further used to perform simulation under various dosing scenario.

Discussion

The occurrence of type 2 diabetes is a worldwide epidemic and threatens the health of many individuals (22). Although multiple therapeutic options are available to treat type 2 diabetes, a large number of patients do not achieve adequate glycemic control for many different reasons. Many therapies present a number of side effects, such as hypoglycemia (11), gain in body weight (5), and/or loss of efficacy after long-term administration (23). Recently marketed therapies such as glucagon-like peptide-1 analogs decrease body weight in addition to controlling hy-

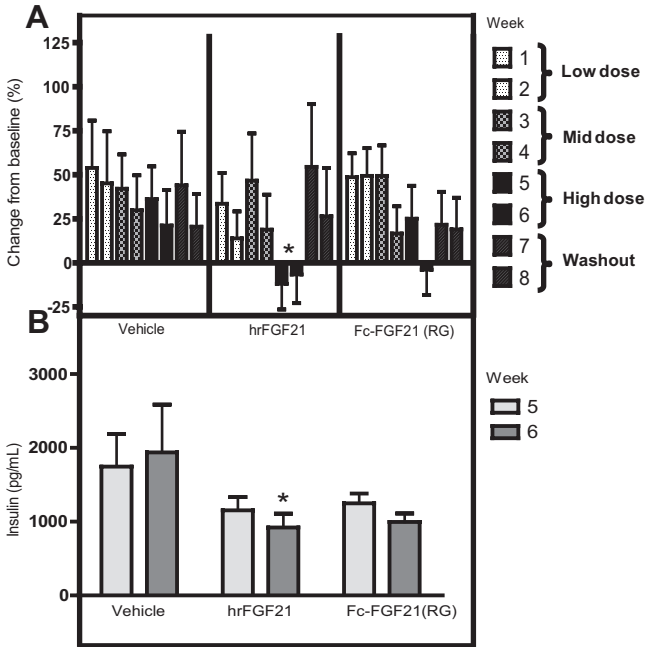

FIG. 6. Chronic effects of hrFGF21 and Fc-FGF21(RG) on fasted and fed insulin levels in obese rhesus monkeys. A, Percent change in fasted insulin relative to baseline in vehicle-, hrFGF21-, and Fc-FGF21(RG)-treated obese rhesus monkeys; *light shaded bars 1 and 2* correspond to wk 1 and 2 at the low dose, *stippled bars 3 and 4* correspond to wk 3 and 4 at the middle dose, *solid bars 5 and 6* correspond to wk 5 and 6 at the high dose, and *solid bars 7 and 8* correspond to wk 7 and 8 during the washout period. B, Effects of vehicle, hrFGF21, and Fc-FGF21(RG), given at the highest tested dose, on fed insulin levels of obese rhesus monkeys acquired during wk 5 and 6 of the study; *light bars* correspond to wk 5 and *dark bars* correspond to wk 6. All data represent mean ± SEM. *, $P < 0.05$ *vs.* vehicle treatment.

perglycemia (24). Frequency of injection (15) and side effects (12, 14), however, cause challenges with patient compliance. New therapies that improve multiple metabolic parameters are highly desirable. FGF21 could be such a therapy because it not only lowers glucose and insulin levels but also decreases triglyceride levels and body weight. Yet native FGF21 protein has a short half-life and may need daily injection to accomplish the expected outcome. We generated a Fc fusion protein with an engineered FGF21 analog containing two point mutations (L98R, P171G). The two mutations reduced FGF21 aggregation and *in vivo* degradation, respectively. The Fc-FGF21(RG) variant demonstrated a dramatic PK improvement with a half-life of approximately 30 h in monkeys compared with 1–2 h for native FGF21.

The goals of our study were to determine whether mrFGF21 showed efficacy in C57BL6 lean mice without inducing hypoglycemia and whether a once-a-week injection of a long-acting FGF21 analog could demonstrate efficacy either similar to or greater than hrFGF21. DIO mice and obese rhesus monkeys were selected. DIO mice are frequently used for studying type 2 diabetes because they are obese and present mild hyperglycemia and hyperinsulinemia accompanied by insulin resistance. Also, this model has already been used to test a large number of compounds for the treatment of diabetes or obesity. Because it was previously shown (18), hrFGF21 significantly decreased body weight, glucose, insulin, and lipid levels, and improved glucose tolerance in a dose-dependent fashion. In the current study, Fc-FGF21(RG) administered at a dose of 2.3 mg/kg to DIO mice was as efficacious as hrFGF21 in all the parameters measured. However, it had the advantage of only needing one injection of Fc-FGF21(RG) every 5 d to accomplish what hrFGF21 did when administered BID.

Demonstration of efficacy in a mouse model was an important accomplishment with Fc-FGF21(RG); however, translation of that into higher species was of even greater interest. Rhesus monkeys with type 2 diabetes have previously been used to test FGF21 (17), but the availability of these monkeys is limited and the risk of immunogenicity restricts the use in chronic testing of human proteins. Obese rhesus monkeys were selected to compare our FGF21 molecules because they are more abundant than diabetic monkeys. Since these obese rhesus monkeys are normoglycemic, normoinsulinemic, and normolipidemic, we anticipated to observe a narrow pharmacological window in the metabolic parameters we measured. Nevertheless, it was reasonable to expect some beneficial effects on glucose and lipid measurements on the basis of results seen when mrFGF21 administered to C57BL6 lean mice showed improved glucose tolerance and decreased insulin and triglyceride levels (Fig. 1). Our expectations were fulfilled since body weight was decreased with both tested molecules (Fig. 5A), glucose tolerance was improved with Fc-FGF21(RG) (Fig. 5, B–E), and insulin levels were decreased with hrFGF21 (Fig. 6B). As we had observed in the past in a normoglycemic mouse model (Fig. 1B), plasma glucose was minimally affected. We also confirmed that no hypoglycemia was observed with any of the molecules in either species [data not shown (18)]. The effects of FGF21 compounds on food intake, energy expenditure and body composition including lean and fat mass were not measured in the current studies as the data from DIO mice were published previously (18). FGF21 had no significant effects on daily absolute food intake but caused a significant decrease in fat mass and a slight decrease in lean mass as a result of a significant increase in energy expenditure (18).

We previously reported that in a fed state, the insulin levels of mice treated with FGF21 significantly decreased 1 h after a single injection (25). We wanted to determine whether FGF21 molecules would decrease insulin levels in

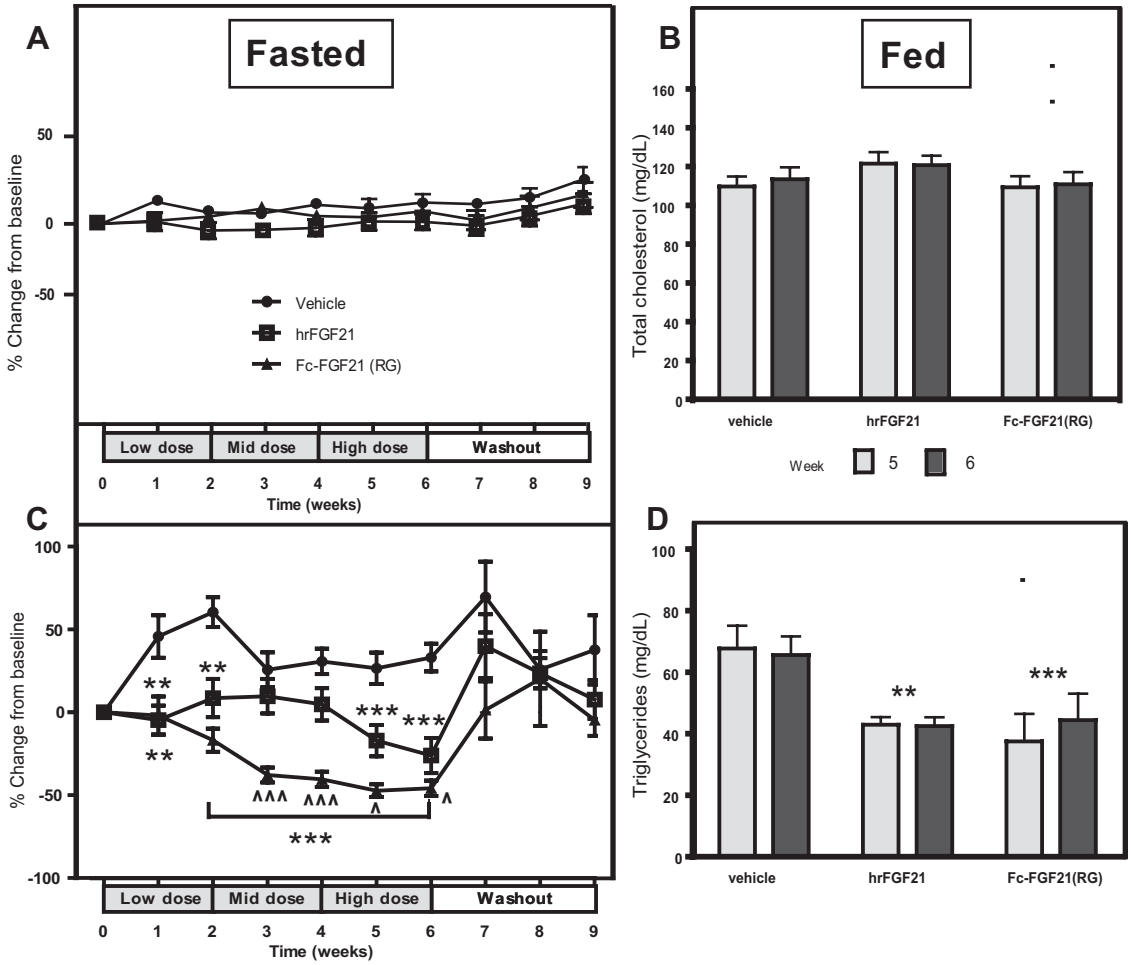

FIG. 7. Chronic effects of hrFGF21 and Fc-FGF21(RG) on fasted (*left panels*) and fed (*right panels*) rhesus monkeys of total cholesterol (A and B) and triglyceride (C and D) levels. A, Effects of vehicle, hrFGF21, and Fc-FGF21(RG) on percent change from baseline of the fasted cholesterol levels in obese rhesus monkeys. B, Fed cholesterol levels of the rhesus monkeys as measured during the fifth and sixth weeks of treatment with vehicle, hrFGF21, or Fc-FGF21(RG) at the high dose; *light bars* correspond to wk 5 and *dark bars* correspond to wk 6. C, Effects of vehicle, hrFGF21, and Fc-FGF21(RG) on percent change from baseline of the fasted plasma triglyceride levels of the rhesus monkeys. D, Fed plasma triglyceride levels of the rhesus monkeys as measured during the fifth and sixth weeks of treatment with vehicle, hrFGF21, or Fc-FGF21(RG) at the high dose; *light bars* correspond to wk 5 and *dark bars* correspond to wk 6. All data represent mean ± SEM. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ *vs.* vehicle treatment; ^ $P < 0.05$, ^^ $P < 0.01$, ^^^ $P < 0.01$ *vs.* hrFGF21.

rhesus monkeys in a fed state as well. Also, we hypothesized that the effect on insulin levels may be stronger in an animal that is in a fed state than in a fasted state. Rhesus monkeys were bled 2 h after their afternoon snack on the third day after administration of Fc-FGF21(RG) or 1 h after administration of hrFGF21. As expected, hrFGF21 showed a statistically significant decrease in fed insulin levels (Fig. 6B) whereas Fc-FGF21(RG) showed only a trend in decreasing fed insulin levels ($P = 0.07$). These effects were similar when the animals were fasted; however, variability was increased (Fig. 6A). This was not a surprise because the pharmacological window is narrower in the fasted state than in the fed state. Also, when fasted blood samples had been collected, hrFGF21 had been administered 21 h before the collection and Fc-FGF21(RG) 5 d before the collection.

Interestingly, even in a monkey model that did not present any abnormal lipid profiles, hrFGF21 and Fc-FGF21(RG) significantly decreased plasma triglyceride levels during both the fasted and fed states (Fig. 7, C and D, respectively). When FGF21 was administered, plasma triglyceride levels were reduced to near normal levels in both *ob/ob* and *db/db* mice (16) as well as in diabetic rhesus monkeys (17). Triglyceride levels of C57BL6 mice treated for 5 wk with mrFGF21 were decreased (Fig. 1D). We have consistently observed these triglyceride level decreases in normal and diseased animal models. Kharitonenkov *et al.* (17) attributed the triglyceride level decreases in their diabetic rhesus monkeys to apolipoprotein CIII circulating levels. The fact that FGF21 is downstream from peroxisomal proliferator-activated receptor-α (26) and that FGF21 decreased sterol regulatory element-bind-

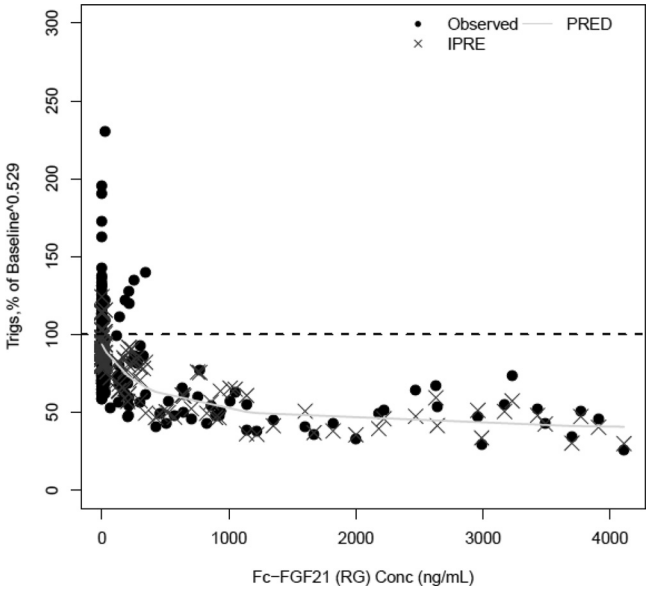

FIG. 8. Final population PK-PD model fit. Plot of observed triglyceride levels (●, observed) and final PK-PD model predicted [*grey* ×, individual predicted (IPRE); *grey line*, population predicted (PRED)] *vs.* Fc-FGF21(RG) concentrations.

ing protein-1c maturation (27) may also explain the strong pharmacological properties of FGF21 in decreasing triglyceride levels. Total cholesterol levels in obese rhesus monkeys were not significantly changed in either FGF21 groups during fed or fasted conditions; we found only a small significant reduction in C57BL6 mice at 10 mg/kg (Fig. 1E). The data from our monkey study is different from what was reported (17). We attribute this difference to the fact that our obese rhesus monkeys did not present lipid impairments. Additionally, the blood samples collected at the trough drug levels may also preclude the findings of some moderate effects. The diabetic monkeys from that report exhibited frank hyperlipidemia with mean total cholesterol and LDL levels of approximately 100 and 30% higher than those observed in our obese rhesus monkeys. The plasma β-hydroxybutyrate levels were mainly unchanged or slightly reduced (Fig. 2G), which is consistent with our previous report (18) and argue against an increase in ketogenesis after FGF21 treatment as it was previously reported (28). Our data agree with the observation that FGF21 is not required for ketogenesis as shown in FGF21 knockout mice and human study that indicated a lack of correlation between plasma FGF21 concentration and ketone bodies, (29). Taken together, a weak cause-effect relationship between FGF21 and ketogenesis was found in both mice and humans.

When Kharitonenkov *et al.* (17) tested FGF21 in diabetic rhesus monkeys, FGF21 was administered in a dose-escalation fashion and starting at doses lower than the lowest dose we selected (0.03 *vs.* 0.1 mg/kg). However, the efficacy observed in their diabetic monkeys was greater than that observed in our obese monkeys. As we have shown, animal models with normal metabolic parameters respond to FGF21 pharmacologically to a lesser extent than animal models with metabolic impairments (Ref. 31 and Fig. 1). The only abnormal metabolic parameter our monkeys presented was increased body weight. Surprisingly, our obese monkeys showed a greater body weight reduction than the diabetic monkeys (17). The latter monkeys were heavier than our obese monkeys (19 ± 1.4 kg *vs.* 8.5 ± 0.5) and may also have been older. One possible explanation for the difference between the two sets of data is that the FGF21 dose levels may have been too low in the diabetic monkeys or that the length of treatment may not have been sufficient in the same monkeys. Statistical significance was only observed at wk 5 in our obese monkeys with 3 mg/kg·d of hrFGF21. Lastly, the OGTT was not conducted in the diabetic monkeys. In the obese monkeys, the OGTT was performed before initiation of the treatment and at the end of each dose period. Statistical significance was only seen at a few time points (for glucose and insulin levels during OGTT; Fig. 5, B and C) and only in animals treated with Fc-FGF21(RG) except for one time point for the insulin levels being reduced in animals treated with hrFGf21 (Fig. 5C). Some of the effects may have been masked because OGTT were conducted at the trough levels of drug concentration instead of the peak levels of drug concentrations. In addition, stress (nonanesthetized animals) may also have masked the effects, even though the animals had been acclimated for 5 wk and stress during the OGTT was kept to a minimal level. Binding antibodies were detected in eight of 10 obese monkeys treated with hrFGF21, and one animal was positive for neutralizing antibodies. In the Fc-FGF21(RG)-treated group, six of 10 animals were positive for binding antibodies, but no neutralizing antibodies were detected. Despite the presence of binding antibodies, there was no apparent effect on the exposure of hrFGF21 or Fc-FGF21(RG).

The strong PD effect of triglyceride lowering in obese monkeys was further investigated by conducting PK-PD modeling of vehicle and Fc-FGF21(RG)-treated animals. An inhibitory E_{max} model incorporating baseline and time-independent vehicle effect was developed to characterize drug exposure-response relationship. The mean maximum triglyceride reduction was 62%. In the concentration range (0–4000 ng/ml), Fc-FGF21(RG) caused decrease in triglyceride levels with population mean EC_{50} of 378 ng/ml (Supplemental Table 3). In this study, 20 rhesus monkeys were assigned in either treatment or vehicle group. We applied a population-based PK-PD modeling approach to simultaneously fit all these animal data, which resulted in reasonable estimates of model parame-

ters as well as between/within subject variability. The variability of EC_{50} (ω_{EC50}) between the individual monkeys was mild (68%), whereas the variability of E_{max} (ω_{Emax}) or vehicle effect ($\omega_{vehicle}$) was small (14%). This PK-PD modeling exercise quantifies the maximum triglyceride reduction and the potency of Fc-FGF21(RG) and could be further used for predicting triglyceride lowering.

In general, the acute and chronic effects of FGF21 are similar and consistent, although changes of some metabolic parameters require chronic FGF21 exposure to become evident. These include lipid-lowering and body weight reduction. Changes of these parameters may be minimal or slow to respond and therefore need longer exposure to reach steady state. Despite that, insulin sensitization effect can be observed acutely and sustained chronically (18, 25). The effects of FGF21 examined in our different animal models, including lean, DIO, and obese rhesus monkeys, are also consistent. However, due to the sensitivity and/or metabolic state of the animal models studied, the magnitude of the response varied with different parameters. For example, the lean mice or nondiabetic obese monkeys are less responsive to FGF21 treatment on lowering glucose levels than the insulin-resistant DIO mice. Interestingly, a decrease in triglyceride levels and body weight observed during FGF21 treatment are generally similar across different animal models.

In summary we demonstrated that in DIO mice, body weight, glucose, insulin, cholesterol, and triglyceride levels were decreased following treatment with either hrFGF21 or Fc-FGF21(RG), and GTT were also improved with either compound. Subsequently, in obese monkeys, body weight, glucose, insulin, cholesterol, and triglyceride levels as well as OGTT results were decreased/improved to a greater extent with Fc-FGF21(RG) than with hrFGF21. In conclusion, in more than one species, Fc-FGF21(RG) chronically administered once a week showed similar or greater efficacy than hrFGF21 administered daily.

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