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Growth Differentiation Factor 11 does not Mitigate the Lethal Effects of Total-Abdominal Irradiation

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Abstract

Total-body exposure to radiation causes widespread tissue injury. Damage to the hematopoietic and intestinal stem cell compartments is particularly lethal and mitigators of this damage are critical in providing effective treatment. Parabiosis radiation experiments, in which the vasculatures of two rodents are anastomosed prior to irradiation of one of the animals, have shown that there is a circulating factor that protects mice from radiation-induced intestinal death. Recently reported studies have suggested that growth differentiation factor 11 (GDF11) is responsible for the rejuvenation of stem cells observed in parabiosis experiments involving aging mice. In this study, we investigated the efficacy of GDF11 as a potential mitigator of radiation-induced damage to intestinal stem cells. In *ex vivo* cultures of intestinal organoids, the number of cells expressing the stem cell marker Lgr5 was increased after irradiation and GDF11 supplementation. Further *ex vivo* studies to assess stem cell function, measured by the ability to grow new crypt-like structures, did not show increased stem cell activity in response to GDF11 treatment. In addition, GDF11 was unable to improve survival of mice subjected to total-abdominal irradiation. These data demonstrate that GDF11 does not mitigate radiation damage to intestinal stem cells.

INTRODUCTION

Radiation exposure scenarios involving large groups of the general population, either after nuclear accidents or through terrorist attacks with “dirty bombs”, pose major medical and organizational challenges to first responders (1, 2). Potential victims have to be triaged based on radiation exposure levels and treated appropriately. However, in these scenarios the number of bone marrow transplant units required would far exceed the number of available units. Thus, there is an ongoing effort to develop drugs that mitigate the lethal effects of radiation to critical organ systems, even if administered 24 h or later postirradiation (2).

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Attempts to develop radiation mitigation strategies date back to the cold war era, and one strategy that successfully prevented the development of a gastrointestinal acute radiation syndrome (ARS) was parabiosis in rodents (3–6). In parabiosis radiation experiments the blood vessels of two animals are anastomosed with one animal shielded from radiation. Rescue of the hematopoietic system in these experiments can be explained by the exchange of blood cells and hematopoietic stem and progenitor cells. However, the mechanism behind the protection of the gastrointestinal system is less apparent.

Recently reported studies on the reversal of aging by parabiosis suggested that growth differentiation factor 11 (GDF11) mediates the effects of parabiosis through expansion of normal tissue stem cell pools (7, 8). GDF11 was also shown to remodel cerebral vasculature leading to increased neurogenesis in older mice (9). An independent group showed increased endothelial sprouting in *in vitro* experiments with peripheral blood endothelial progenitor cells (9). Conversely, independent laboratories were unable to reproduce the muscular regeneration results and instead showed that GDF11 actually inhibited muscle regeneration (10, 11). There is still controversy surrounding the ability of GDF11 to rejuvenate stem cells (12–14). At this time, the literature does not show a clear consensus on whether GDF11 is able to rejuvenate adult stem cells, and there are no reports attempting to treat radiation-induced damage to stem cells using GDF11.

We therefore tested the hypothesis that GDF11 treatment of mice, exposed to a lethal dose of total abdominal radiation, would mitigate the effect of radiation.

MATERIALS AND METHODS

Animals

Six-week-old female C57Bl/6 and C3H mice were originally obtained from Jackson Laboratories (Bar Harbor, ME). Lgr5-EGFP-IRES-creERT2 mice [heterozygous mice harboring a “knock-in” allele for EGFP driven by Lgr5 promoter (15)] were purchased from Jackson Laboratories, and were bred with C57Bl/6 mice for maintaining the breeding colony. All animals were re-derived, bred and maintained in a pathogen-free environment in the American Association of Laboratory Animal Care-Accredited Animal Facilities of Department of Radiation Oncology, University of California (Los Angeles, CA) in accordance with all local and national guidelines for the care of animals.

All progeny from Lgr5-EGFP-IRES-creERT2 mice were genotyped by TransnetYX® (Cordova, TN). Animals that were heterozygous for the EGFP transgene were used for experiments, while those lacking the transgene were used as wild-type controls.

Organoid Assay

Intestinal organoids were prepared as previously described (16). Briefly, 6-week-old C3H mice were sacrificed and approximately 10 cm of the small intestine adjacent to the stomach was harvested and placed in a 10-cm dish containing ice cold PBS and 1% penicillin/streptomycin (100 U/ml penicillin, 100 μg/ml streptomycin). The intestines were flushed with PBS and opened longitudinally. The villi were then gently scraped off using an ice-cold glass slide and washed with PBS three times. The intestines were chopped in small

segments, dropped in a 50-ml tube containing 20 ml of ice cold PBS and 1% penicillin/streptomycin and washed by pipetting up and down three times using a 10-ml pipette. The segments were allowed to settle by gravity. The supernatant was discarded and the procedure was repeated approximately 15–20 more times until the supernatant was clear. The intestinal segments were resuspended in 25 ml Gentle Cell Dissociation Reagent (STEMCELL™ Technologies, Vancouver, Canada) and rocked at 20 rpm for 15 min at room temperature. The segments were allowed to settle by gravity and the supernatant was discarded. The segments were resuspended in 10 ml of ice cold PBS with 0.1% bovine serum albumin (BSA), pipetted three times and allowed to settle. The supernatant was collected, passed through a 70-μm filter into a 50-ml tube and saved as fraction 1 (F1). The procedure was repeated to obtain fractions 2–4 (F2–F4). The fractions were centrifuged at 290*g* for 5 min at 2–8°C. The supernatant was discarded and the pellet was resuspended in 10 ml of PBS/0.1% BSA. The suspension was centrifuged at 200*g* at 2–8°C. The pellet was resuspended in 5 ml of DMEM/F12 medium (Gibco®/Thermo Fisher™ Scientific, Waltham, MA). The isolated crypts were observed under a microscope and the number of crypts isolated was determined by placing 10 μl of the crypt suspension on a glass slide. The crypt suspension was pelleted and resuspended in 50% complete Intesti-Cult™ Organoid Growth Medium (STEMCELL Technologies) and 50% Matrigel® (Corning® Inc., Corning, NY). Then, 60 μl of the crypt mixture, containing 150 crypts (for irradiated wells) or 50 crypts (for nonirradiated controls), was deposited in each well of a prewarmed 96-well plate. The Matrigel was allowed to solidify and then complete IntestiCult Organoid Medium was slowly added to cover the embedded organoids. The plate was incubated at 37°C overnight and irradiated the following day with 0 or 3 Gy. For studying the radiation mitigating effects of GDF11 in intestinal stem cells, 10 ng/ml GDF11 (R&D Systems™, Minneapolis, MN) was added 3 and 24 h postirradiation and the media was changed daily for 6 days. The number of organoids with budding crypts was determined 8–10 days later.

Irradiation

Mice were irradiated in groups of 5 using an experimental X-ray irradiator (Gulmay Medical Inc., Atlanta, GA) at a dose rate of 5.519 Gy/min for the time required to apply a prescribed dose. Control animals were sham irradiated. The X-ray beam was operated at 300 kV and hardened using a 4-mm beryllium, a 3-mm aluminum and 1.5-mm copper filter. For total-abdominal irradiation, the rest of the body was shielded with 10-mm lead blocks. Intestinal organoids were irradiated using the same irradiator setup. Control organoids were sham irradiated.

Drug Treatment

GDF11 (R&D Systems) was dissolved in 4 m*M* sterile HCl with 0.1% BSA. At 24 h postirradiation, mice received 5 daily intraperitoneal (i.p.) injections of GDF11 or solvent only. GDF11 doses are indicated in the individual experiments. To assess toxicity the mice were weighed daily.

Flow Cytometry Assay for Lgr5-pos Intestinal Stem Cells

Female, 8-to-10-week-old Lgr5-EGFP-IRES-creERT2 mice or wild-type littermates were used for analysis (15). Intestinal organoids were prepared as described above. Organoids

were passaged *in vitro* according to the protocols provided by STEMCELL Technologies. Briefly, organoid growth media was removed and cold Gentle Cell Dissociation Reagent was added to each well. Triturating with a P1000 pipette tip mechanically disrupted the Matrigel and the solution was rocked at 20 rpm at room temperature for 15 min. The disrupted crypts were pelleted by centrifugation at 290*g* for 5 min at 2–8°C. Approximately 400–500 crypts were suspended in 100 μl of IntestiCult Complete Organoid Growth Medium and Matrigel (50:50), and plated into a single well of a prewarmed 24-well plate. After the Matrigel solidified, organoid media was added to the well and the cultures were incubated overnight at 37°C. The organoids were irradiated with 0 or 3 Gy. At 24 h postirradiation, GDF11 (10 ng/ ml) or vehicle control was added and the media was changed daily for 6 days. On day 6, the organoids were dissociated using Gentle Cell Dissociation Reagent similar to the dissociation during passaging. To dissociate the organoids to single cells for flow cytometry, the solution was rocked for 45 min at room temperature and triturated every 10 min. The cell suspensions were washed once in DPBS. Flow cytometric analysis for EGFP-positive cells was performed on a MACSQuant® Analyzer (Miltenyi Biotec, San Diego, CA).

Cell Culture and Western Blotting

SUM159PT cells (Asterand Bioscience™, Detroit, MI) were grown in F12 media with 5% fetal bovine serum (FBS), 1 μg/ml hydrocortisone, 5 μg/ml insulin, 10 m*M* HEPES and 1% penicillin/ streptomycin. Cells were maintained at 37°C and 5% CO_2 in a humidified atmosphere. One day prior to treatment, the media was changed to SUM159PT media containing 0.5% FBS. GDF11 (10 ng/ ml) or vehicle control was added to the cells and incubated for the indicated times. The cells were lysed in RIPA buffer containing 1× protease inhibitor cocktail and 1× phosphatase inhibitor cocktail (both from Sigma-Aldrich® LLC, St. Louis, MO). The lysates were resolved by SDS-PAGE and transferred to nitrocellulose. The membranes were incubated overnight with phosphoSer467-Smad2 antibody (1:1,000; Abcam®, Cambridge, MA) or Smad2 antibody (clone D43B4, 1:1,000; Cell Signaling Technology® Inc., Danvers, MA). The following day, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies and then ECL2 Western Blotting Substrate (Pierce™ Biotechnology, Rockford, IL) and imaged using a Typhoon fluorescence imager.

RESULTS

GDF11 Mitigates Radiation Effects in Lgr5-Positive Cells In Vitro

In the first set of experiments, we assessed the effect of GDF11 on the number of intestinal stem cells in organoids established from the small intestines of the animals based on the presence of the Lgr5-EGFP marker.

Organoids were irradiated with 0 or 3 Gy and treated with GDF11 (10 ng/ml) or solvent 24 h later. Media was replaced daily for six consecutive days and GDF11 was added on a daily basis. Flow cytometry assessment of the number of Lgr5-EGFP-positive cells revealed a 1.4-fold increase (not significant) in the number of intestinal stem cells in GDF11-treated organoids (Fig. 1). When the experiment was performed after a single 3 Gy dose of radiation, the number of intestinal stem cells increased 1.8-fold (Fig. 1; $P = 0.02$, unpaired *t*

test), thus indicating that GDF11 expands the number of surviving intestinal stem cells after irradiation *in vitro*.

GDF11 does not Mitigate the Radiation Effects on the Number of Functional Intestinal Stem Cells In Vitro

Lgr5 expression in the small intestines is restricted to the crypt base columnar cells, the putative intestinal stem cells (15). To test if the increase in Lgr5-EGFP-positive cells observed after GDF11 treatment would translate into an increase in functional intestinal stem cells, we performed an *in vitro* organoid formation assay with budding organoids counted at the end of the experiment to indicate the formation of functional crypts. GDF11 added 3 or 24 h postirradiation with 0 or 3 Gy did not lead to an increase in functional intestinal stem cells (Fig. 2A and B). To confirm the activity of our GDF11 preparation, we treated SUM159PT cells, a well-established breast cancer cell line that expresses ALK5, the GDF11 receptor (17). Receptor activation causes rapid phosphorylation of Smad2. We collected vehicle-treated and GDF11-treated cell lysates at 15, 30, 60 and 120 h after GDF11 administration and analyzed for Smad2 phosphorylation by Western blotting. Within 15 min, a marked increase in the amount of phospho-Smad2 was observed and this increase was maintained over the 2-h collection window, peaking at 3.6-fold increase after 60 min (Fig 2C).

GDF11 does not Mitigate Gastrointestinal Acute Radiation Syndrome In Vivo

In the final experiment, we tested if GDF11 would mitigate gastrointestinal ARS *in vivo*. C57Bl/6 mice received a single dose of 15, 16 or 18 Gy total-abdominal irradiation. GDF11 treatment was started 24 h postirradiation with daily injections for 5 consecutive days. None of the GDF11 applied doses prolonged survival of the animals (Fig. 3) or prevented weight loss (Fig. 4).

DISCUSSION

The effects of parabiosis on the radiation response of rodents have been extensively studied in the past (3–6, 18). In more recently reported studies, this old technique was revisited and GDF11 was identified as the mediator of the age-reversing effects of parabiosis (7, 8).

In our current study, we hypothesized that GDF11 also contributes to the radiation-mitigating effects of parabiosis in rodents. Here, we tested whether recombinant GDF11 would mitigate the effects of a lethal dose of abdominal radiation when given 24 h postirradiation.

The intestinal epithelium is compartmentalized into villi and crypts with the latter containing intestinal stem cells at their base, which are responsible for the continuous generation of differentiating epithelial cells that migrate upwards and out of the crypts to form the villi that perform the resorptive function of the intestines (19). Survival after a lethal dose of radiation to the intestines critically depends on the number of surviving intestinal stem cells that are able to form regenerating crypts (20). Therefore, compounds that mitigate gastrointestinal ARS have to alter the number of surviving intestinal stem cells and the resulting number of regenerating crypts 96 h postirradiation predicts survival of the animals.

Crypt basal columnar cells have been identified as the stem cells in the small intestine (15), and express Lgr5, a G-protein-coupled receptor for R-spondin, a regulator of Wnt signaling (15, 21–24). Although reserve intestinal stem cell populations exist and committed progenitor cells in the small intestines can revert into a stem cell state, Lgr5 is accepted as a good surrogate marker for the number of intestinal stem cells (25).

Our initial studies using GDF11 indeed indicated that its application increases the number of Lgr5-positive cells when given after a single dose of 3 Gy, thus suggesting that GDF11 affects the intestinal stem cell pool. However, when we tested the effects of GDF11 on the generation of functional intestinal stem cells, GDF11 had no effect. This result was supported by a lack of *in vivo* activity of GDF11 in mice that received lethal doses.

Our *in vivo* experiments used a range of GDF11 doses including the very high doses that had been reported to reverse aging in mice (100 μg/kg), excluding the possibility that the applied dose of GDF11 was insufficient (7). We discontinued GDF11 administration on day 6 postirradiation, a point in time at which intestinal stem cell expansion would have to be sufficient to result in regenerating crypts (5). Although we cannot completely rule out that prolonged GDF11 treatment could affect radiation survival, this seems unlikely given that animals succumb to gastrointestinal ARS within the first 10 days postirradiation.

In summary, we found that while GDF11 has some effect on Lgr5-positive cells originating from the small intestines *in vitro*, it does not increase the number of functional intestinal stem cells and does not mitigate radiation effects in the small intestines *in vivo*. Therefore, we conclude that GDF11 does not mediate the radiation mitigating effects of parabiosis in rodents.

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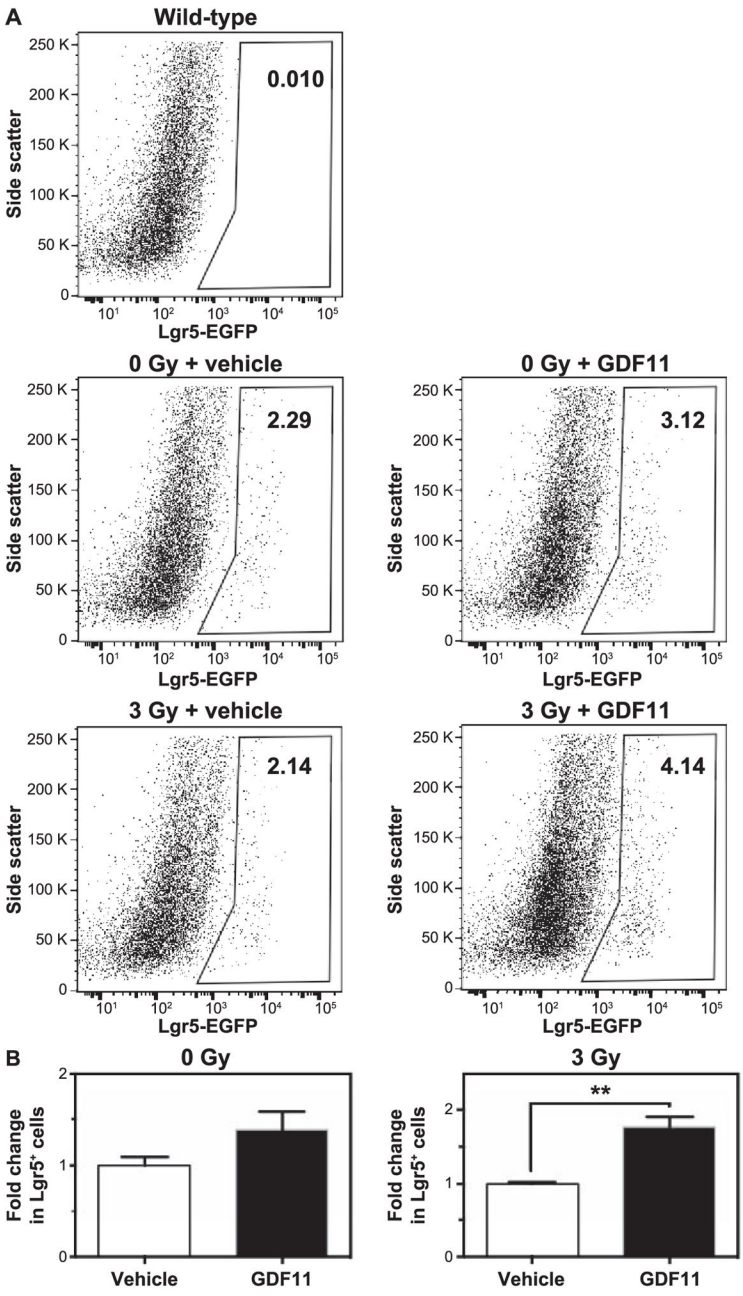

FIG. 1.

GDF11 mitigates the effect of radiation on Lgr5-positive cells *in vitro*. Intestinal organoids from Lgr5-EGFP-IRES-creERT2 mice were cultured *in vitro*. Organoids were irradiated with 0 or 3 Gy and then treated with 10 ng/ml GDF11 24 h postirradiation. GDF11 was replenished daily for 6 days. The organoids were dissociated and the single cell solution was analyzed by flow cytometry for EGFP-positive cells. Panel A: Representative flow cytometric plots show side scatter versus EGFP fluorescence. Wild-type control organoids were used to establish a suitable gate. Panel B: Bar graphs show the average fold change in the percentage of EGFP-positive cells (n = 4) ± SEM. $^{**}P < 0.01$.

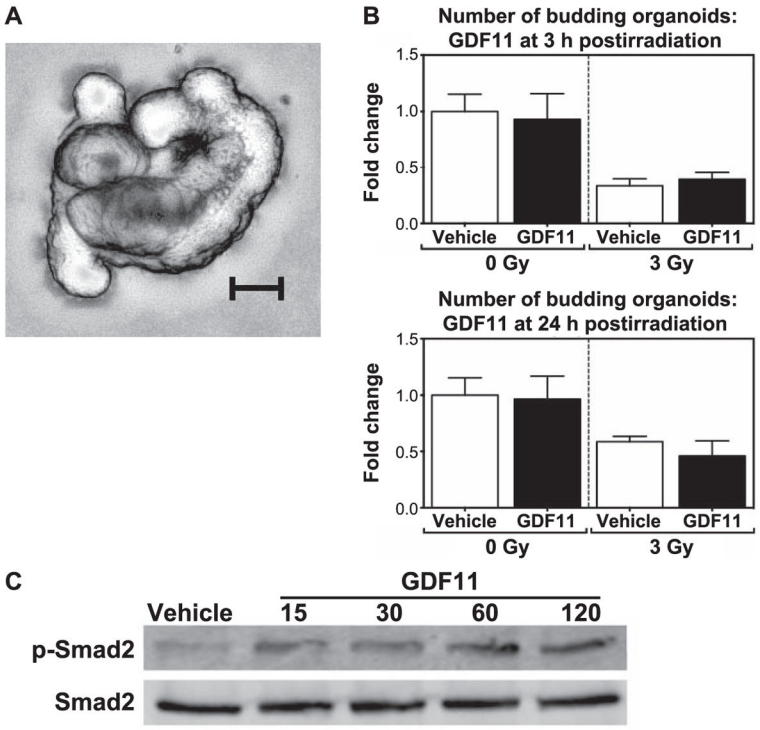

FIG. 2.

GDF11 does not mitigate the effect of radiation on functional stem cells *in vitro*. Intestinal organoids from C3H mice were cultured *in vitro*. Organoids were irradiated with 0 or 3 Gy and then treated with 10 ng/ml GDF11 at 3 or 24 h postirradiation. GDF11 was replenished daily for 6 days. After 8–10 days the number of budding organoids was counted. Panel A: Representative image of a budding organoid. Panel B: Bar graphs show the average fold change in the number of budding organoids (n = 4) ± SEM. Panel C: Western blot for phospho-Smad2 and total Smad2 after treatment with vehicle control or 10 μg/ml GDF11 for the indicated time period (in minutes).

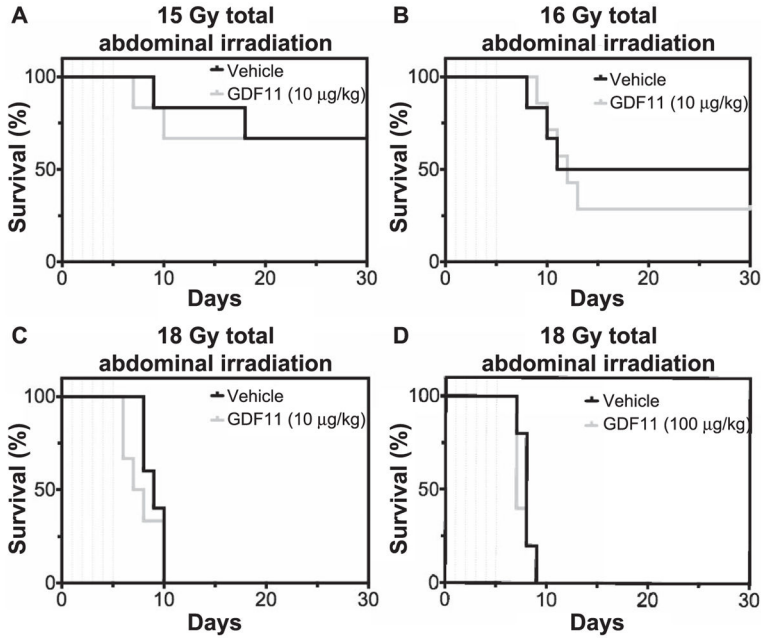

FIG. 3.

GDF11 does not mitigate gastrointestinal ARS *in vivo*. C57Bl/6 mice received 15, 16 or 18 Gy total-abdominal irradiation. Twenty-four hours later, the indicated dose of GDF11 or vehicle control was administered by i.p. injection. GDF11 or vehicle was administered daily for 5 consecutive days, indicated by the vertical gray lines. Kaplan-Meier survival curves are shown for vehicle-treated animals (black lines) versus GDF11-treated animals (gray lines).

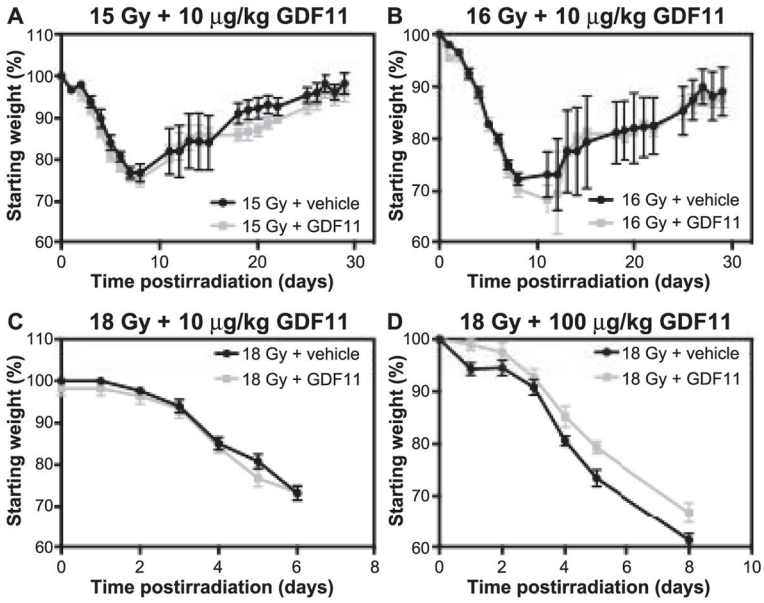

FIG. 4.

GDF11 does not prevent weight loss during gastrointestinal ARS. C57Bl/6 mice received 15, 16 or 18 Gy total-abdominal irradiation. Treatment with GDF11 at 10 μg/kg on 5 consecutive days starting 24 h postirradiation did not improve radiation-induced weight loss after a sublethal dose of 15 or 16 Gy (panels A and B). GDF11 at 10 μg/kg or 100 μg/kg failed to improve radiation-induced weight loss after lethal 18 Gy irradiation (panels C and D).