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Phenotypic Effects of Calorie Restriction and Insulin-like Growth Factor-1 Treatment on Body Composition and Bone Mineral Density of C57BL/6 Mice: Implications for Cancer Prevention*

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Abstract. *Background: Calorie restriction (CR) inhibits carcinogenesis and delays aging. Some anti-carcinogenic effects of CR are mediated by decreased circulating insulin-like growth factor-1 (IGF-1); however, IGF-1 also plays an important role in regulating growth and bone density. Materials and Methods: We quantified tradeoffs involving the CR/IGF-1 axis in C57BL/6 mice by examining body composition and bone characteristics in ad libitum fed, 20, 30 or 40% CR mice that received placebo or recombinant murine IGF-1 delivered with a time-release pellet. After 26 days, carcasses were scanned with a PIXImus II dual-energy X-ray absorptiometer. Results: CR reduced body weight and percent body fat and had non-linear effects on bone density. IGF-1 restored bone density to control levels or greater in the CR mice. Conclusion: Cancer prevention efforts based on CR and down-regulation of the IGF-1 pathway will require consideration of deleterious effects on bone.*

Calorie restriction (CR) is known to delay aging and carcinogenesis in rodents as well as in several other animal models (1-3). These results have led to intense interest in identifying mechanisms underlying the beneficial effects of CR and in developing preventive and treatment strategies based on these mechanisms (4, 5). Several lines of evidence show that modulation of the insulin-like growth factor-1 (IGF-1) pathway influences carcinogenesis and is a mediator of at least some of the cancer preventive effects of CR. CR animals have lower serum levels of IGF-1 (6) and increased latency and decreased number and size of tumors (7). Compellingly, restoration of IGF-1 levels in CR animals by exogenous administration of IGF-1 accelerates their otherwise delayed tumor development (7). *In vitro* studies have demonstrated that IGF-1 stimulates the growth of numerous cancer cell lines (8-10), and a number of epidemiological studies indicate that serum IGF-1 levels are positively associated with risk of colon, breast and prostate cancer (11-13).

Calorie restriction and changes in body weight are also known to influence bone characteristics. In humans, bone density is positively correlated with body weight (14, 15), and weight loss results in decreased bone density (14). In rodents, experimentally imposed CR reduces bone density (16, 17). IGF-1 is believed to be part of the mechanistic pathway linking body weight and bone density (18, 19). For example, MIDI mice have circulating IGF-1 levels diminished by 60% and have reduced bone density; IGF-1 treatment increases their bone density (20). Together, these observations suggest that efforts to slow aging or prevent cancer *via* CR or modulation of the IGF-1 axis must take into account the effects of reduced levels of IGF-1 on bone.

In the present study, we examined the effects of several levels of CR with and without treatment with recombinant murine IGF-1 on body composition, bone characteristics and serum IGF-1 levels, in order to further characterize

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Table I. *Diet composition.*

Component (g/kg)	*Ad Libitum* (AL) & 20% CR[1]	30% CR[2]	40% CR[2]
Dextrose	320.00	289.40	224.90
Sucrose	175.00	110.00	112.90
Starch	155.00	150.00	82.70
Casein	200.00	255.60	335.00
Corn oil	50.00	65.00	84.00
Cellulose	45.00	65.00	79.00
Salt Mix, AIN-76A	35.00	45.50	56.50
Vitamin Mix, AIN-76A	10.00	13.00	16.50
dl-Methionine	3.00	3.90	5.00
Choline Dihydrogen Citrate	2.00	2.60	3.50

[1]The 20% CR animals received daily aliquots of the same diet consumed by AL-fed mice equal to 80% of their average daily food intake.
[2]30% and 40% CR mice received the appropriate CR diet in daily aliquots equal to 70 or 60% of the average daily food intake of AL-fed mice.

potential tradeoffs between beneficial effects of CR and reduced IGF-1 levels *versus* their potentially deleterious effects on bone characteristics.

Materials and Methods

The mice were maintained according to the guidelines of the Animal Care and Use Committee of the National Cancer Institute, and the committee approved this study. Five-week-old female C57BL/6NCr mice (Charles River, Frederick, MD, USA) were singly housed and received water *ad libitum* (AL). In the first week after receipt, mice were fed AIN-76A diet (Bio-Serv Corp., Frenchtown, NJ, USA) AL, and food consumption was measured. All mice then received a time-release pellet (Innovative Research, Sarasota, FL, USA) containing placebo, recombinant murine IGF-1 or recombinant human IGF-1 obtained from Peprotech, Inc. (Rocky Hill, NJ, USA). Pellets were implanted subcutaneously under isoflurane anesthesia along the dorsal thoracic midline about 3 cm anterior of the hip *via* a small incision made on the upper dorsal skin. The study diets were started three days after pellet implantation.

For the main study, 45 mice were randomized (n=5 per group) to one of nine groups: i) AIN-76A diet AL + placebo pellet, ii) AIN-76A diet AL + murine IGF-1 (20 μg/day), iii) 20% CR + placebo pellet, iv) 20% CR + murine IGF-1 (20 μg/day), v) 30% CR + placebo pellet, vi) 30% CR + murine IGF-1 (20 μg/day), vii) 30% CR + human IGF-1 (20 μg/day), viii) 40% CR + placebo pellet, ix) 40% CR + murine IGF-1 (80 μg/day). The human IGF-1 treatment group was included to determine if mouse and human IGF-1 had different effects. The AL-fed groups received AIN-76A diet AL. Mice in the 20% CR groups received a daily aliquot of AIN-76A diet equal to 80% of the mean daily AL consumption measured in the previous week. CR mice (30% and 40%) received modified versions (Bio-Serv. Corp) of the AIN-76A diet formulated such that, when provided as daily aliquots equal to 70% or 60%, respectively, of the mean daily AL consumption, the reduction in calorie intake was entirely due to carbohydrates; intakes of all other nutrients were equivalent to those in the AL group (Table I) (21). Body weight and food consumption data were recorded weekly and at study termination after 26 days on the diets. The first body weight measurement was obtained 2 days after dietary treatment began and 5 days after pellet implantation. On the final day of the experiment, mice were sacrificed under continuous CO_2/O_2 anesthesia and blood collected. Serum, selected tissues and necropsied carcasses were frozen and stored at –80˚C for subsequent analyses.

We examined the time-specific effects of IGF-1 pellets in a second cohort of 15 mice randomized (n=5 per group) to one of three groups: i) AIN-76A diet AL + placebo pellet, ii) 30% CR + placebo pellet, and iii) 30% CR + murine IGF-1 (20 μg/day). Blood was collected *via* the retroorbital sinus or saphenous vein at baseline, 24 and 72 h after pellet insertion and at days 2, 4, 7, 14, 21 and 28 of CR (72 h after pellet insertion corresponded to day 1 of CR); the serum was frozen and stored at –80˚C until assay of IGF-1.

Fat weight, lean weight, bone mineral density (BMD) and bone mineral content (BMC) were determined using dual-energy X-ray absorptiometry (DXA) (GE Lunar Piximus II, Madison, WI, USA). Replicate measurements (2-3) were made on each individual animal. In brief, necropsied carcasses were placed on the specimen tray and repositioned after each scan (22). After scanning, GE-supplied software (version 1.46) was used to extract data. Animals were weighed prior to and after necropsy and prior to scanning. An adjusted fat weight was calculated as the fat weight estimated for the necropsied carcass plus fat weight contained in tissue removed during necropsy. The fat content of necropsied tissue was estimated by assuming that these tissues contained the same average percentage fat as animals from the calorie-restricted treatment. This procedure is a conservative approach that is likely to underestimate true fat contents. Lean weight was calculated by subtracting adjusted fat weight from weight prior to necropsy. Lastly, we estimated bone density in the tibia alone and in the vertebrae alone by selecting the left tibia as the region of interest (ROI) or the vertebrae from the midpoint of the pelvis to the point where the ribs met the vertebrae for each mouse. We validated our DXA measurements of 40 necropsied carcasses (human IGF-1-treated group not included) using gravimetric and chemical (Soxhlet) extraction (22, 23).

Serum IGF-1 was measured with a rat radioimmunoassay (RIA) IGF-1 kit that recognizes both rat and mouse IGF-1 (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). Serum IGF-1 in mice from three groups (AIN-76A diet AL + placebo pellet, 30% CR + placebo pellet, and 30% CR + human IGF-1) was also measured with a human IGF-1 RIA that recognizes both rodent and human IGF-1 (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). Average values for two determinations made on a single aliquot of serum from each animal are reported. Total IGF-binding protein (IGFBP) activity was assessed using a slot-blot assay (24). In brief, 2 μl of serum was applied to a nitrocellulose membrane (0.45-μm pore size) (Schleicher and Schuell, Keene, NH, USA), by slot-blot manifold (Schleicher and Schuell); the membrane was probed with $1.5x10^6$ cpm of ^{125}I-IGF-2 (Amersham Biosciences, Piscataway, NJ, USA) and exposed to XAR film (Kodak, Rochester, NY, USA). Samples were assayed on 3 independent blots and the banding signal intensity was quantified with a densitometer. Total IGFBP activity was expressed as percent of the average value for the AIN-76A diet AL + placebo pellet group.

Table II. *Body composition and bone characteristics of ad libitum (AL)-fed, CR and IGF-1 treated C57BL/6 mice.*

Treatment[1,2]	Body weight g (S.E.)	Fat weight g (S.E.)	Lean weight g (S.E.)	Bone mineral content g (S.E.)	Bone mineral density g/cm^2 (S.E.)	Tibial bone density g/cm^2 (S.E.)	Vertebral bone density g/cm^2 (S.E.)
Ad libitum (AL)	23.8 (1.0)	5.8 (1.0)	18.0 (0.5)	0.43 (0.01)	0.0480 (0.0010)	0.051 (0.002)	0.053 (0.001)
AL + IGF-1 (20 μg/day)	23.5 (1.0)	6.1 (0.8)	17.5 (0.4)	0.44 (0.02)	0.0476 (0.0017)	0.050 (0.002)	0.057 (0.004)
20% CR	20.4 (0.2)	4.9 (0.3)	15.4 (0.5)	0.40 (0.01)	0.0450 (0.0007)	0.049 (0.001)	0.050 (0.001)
20% CR + IGF-1 (20 μg/day)	18.5 (0.6)	4.1 (0.2)	14.4 (0.4)	0.40 (0.01)	0.0459 (0.0004)	0.046 (0.001)	0.054 (0.002)
30% CR	19.4 (0.3)	4.3 (0.4)	15.1 (0.5)	0.35 (0.01)	0.0427 (0.0003)	0.046 (0.0003)	0.049 (0.001)
30% CR + IGF-1 (20 μg/day)	19.1 (0.3)	3.8 (0.5)	15.3 (0.7)	0.38 (0.01)	0.0449 (0.0003)	0.047 (0.0003)	0.058 (0.004)
40% CR	14.8 (0.2)	2.7 (0.1)	12.1 (0.2)	0.39 (0.01)	0.0445 (0.0008)	0.047 (0.0003)	0.053 (0.002)
40% CR + IGF-1 (80 μg/day)	13.8 (0.5)	2.4 (0.1)	11.4 (0.3)	0.40 (0.01)	0.0464 (0.0007)	0.047 (0.0004)	0.054 (0.003)
p	<0.0001	<0.0001	<0.0001	0.003	0.003	0.007	0.173
r^2	0.89	0.58	0.85	0.47	0.47	0.43	0.26

[1]Means and standard errors obtained from univariate analyses, n=5 for all treatments.
[2]*p* and r^2 values obtained from Analysis of Variance.

Analysis of variance and analysis of covariance were used to assess CR and IGF-1 treatment effects. Means from univariate analyses were compared using Tukey's HSD test. Analysis of covariance allows the inclusion of weight as a covariate in the statistical analysis of the association between bone density, fat content and treatment effects (25). All analyses were performed using SAS JMP Version 5.0 (SAS Institute Inc., Cary, NC, USA). These tests were followed by standardized linear contrasts comparing CR to the AL-fed controls, IGF-1-treated groups to their respective CR controls, and IGF-1-treated groups to the AL-fed controls. These linear contrasts use a *t*-test to determine whether control and CR groups differ, without regard to the level of CR. Presence or absence of dose-response relationships between CR and body composition were tested with linear regression models. All tests were two-sided; probability values less than 0.05 were considered significant. Lastly, IGF-binding protein activity was analyzed using ordinal logistic regression because the data are reported as ratios.

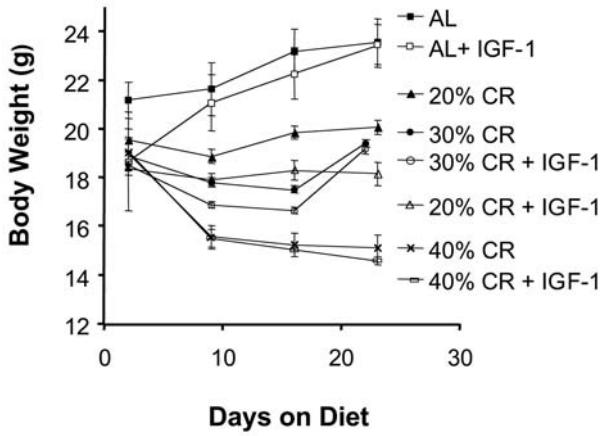

Figure 1. *Effects of calorie restriction and IGF-1 on body weight of C57BL/6 mice. Error bars are one standard error.*

Results

Validation of DXA on necropsied animals. Precision was determined by calculating the mean intra-individual coefficient of variation (CV) for our repeated DXA measurements. Mean % coefficients of variation for fat weight, bone mineral content and bone mineral density were 6.2%, 4.6% and 1.6%, respectively. Accuracy was characterized by performing analyses of covariance with DXA-estimated parameters as dependent variables, chemical carcass analysis estimates as independent parameters and experimental treatments as covariates. We report regression coefficients for the association between DXA and chemical methods (CHEM) for estimates of body fat (g) and total body ash. Here, we used the uncorrected estimate of fat weight from the necropsied carcasses in order to match the results from chemical extraction. The following equations are presented as: DXA measurement=a + b* Chemical Measurement; 95% confidence intervals are given in parentheses. For fat weight (g), DXA Fat Weight=1.74 (1.2-2.2) + 0.93 (0.7-1.1) * CHEM Fat Weight (n=40, r=0.98). For bone mineral content, DXA BMC=0.17 (0.0-0.3) + 0.61 (0.2-1.1) * CHEM Bone Ash (n=40, r=0.96). These r values are comparable to results

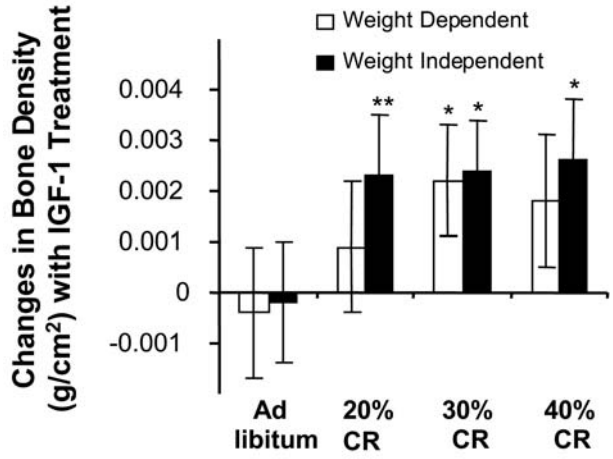

Figure 2. *Effects of IGF-1 treatment on bone density of C57BL/6 mice. The histograms represent differences in mean bone density of study groups with and without IGF-1 treatment. Error bars are one standard error, and asterisks denote significance levels, with *p<0.05; ** p<0.01. A significant p value indicates that IGF-1 treatment increased bone density compared to untreated animals at the same level of CR.*

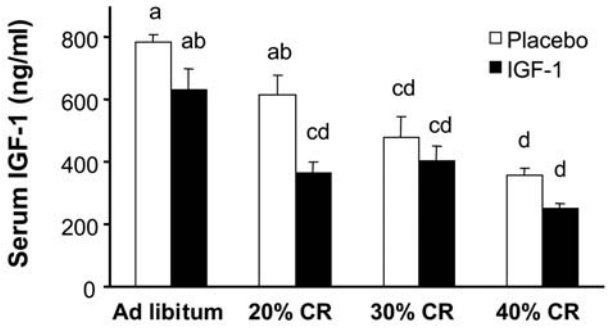

Figure 3. *Effects of calorie restriction and IGF-1 treatment on serum IGF-1 levels in C57BL/6 mice at study termination (26 days). AL-fed mice, 20% CR and 30% CR mice received murine IGF-1 (20 μg/day) or placebo; 40% CR mice received 80 μg/day murine IGF-1 or placebo. Error bars are one standard error. Values with different superscripts are significantly different (p<0.05).*

from similar validation studies (22, 26). In the remainder of the paper, untransformed DXA measurements are reported for ease of comparison with past studies.

Growth, body composition and bone characteristics. Calorie restriction resulted in significant changes in growth characteristics (Figure 1). AL-fed animals gained weight continuously, 20% and 30% CR animals maintained approximately stable weights, and 40% CR animals showed rapid weight loss followed by weight stabilization at about 25% lower weight than at study onset. Food consumption data (not shown) indicated that CR-treated mice consumed their entire ration. There were significant treatment effects on all body composition and bone characteristics except vertebral bone density (Table II). CR resulted in dose-dependent decreases in body weight and fat weight (Table II). CR also influenced lean weight and bone density, but these responses were non-linear. Lean weights of animals from both the 20% and 30% CR treatments were about 15% lower than in the AL treatment and were 28% lower in the 40% CR group. Compared to AL-fed animals, overall bone density was lower in CR animals, with the largest reduction in bone density in the 30% CR group (Table II). We contrasted CR and IGF-1 effects on total bone density with bone density in the tibia and in vertebrae. Examination of regional differences in rodent bone density is near the limits of DXA resolution (26). Univariate analysis indicated significant effects of treatments on tibial bone density (Table II, $p=0.007$), but no effect on vertebral bone density (Table II, $p=0.173$). Note that all three measurements of bone were correlated (Total Bone Density *vs.* Tibia; r=0.74, $p<0.001$; Total Bone Density *vs.* Vertebrae; r=0.47, $p=0.002$; Tibia *vs.* Vertebrae; r=0.39, $p=0.013$).

We further analyzed the effects of CR and IGF-1 treatment by comparing CR *versus* AL-fed animals and IGF-1-treated *versus* CR animals with and without adjustment for body weight by analysis of covariance (ANCOVA). In these tests, we pooled different treatments to increase statistical power. Calorie restriction significantly reduced bone mineral density ($p<0.001$), but this effect was due entirely to the influence of CR on body weight; after adjustment for body weight, the effect of CR on bone mineral density was not significant ($p=0.91$). IGF-1 treatment increased bone mineral density whether or not results were adjusted for body weight ($p<0.001$ unadjusted for weight; $p=0.002$ with weight adjustment). This result is also illustrated in Figure 2. Fat content was reduced by CR with ($p<0.001$) and without ($p<0.001$) adjustment for weight, but IGF-1 treatment did not influence fat content.

Serum IGF-1 and IGFBPs. After 26 days on treatment, total serum IGF-1 concentrations were significantly reduced by CR ($p<0.0001$), and there was a dose-response relationship between the level of CR and the magnitude of this decrease (Figure 3; $p<0.001$). Serum IGF-1 levels in AL-fed animals averaged 785 ng/ml and were reduced to 616, 479 and 357 ng/ml in the 20, 30 and 40% CR treatment groups. IGF-1 treatment did not increase serum IGF-1, but instead resulted in small decreases in serum IGF-1 levels (Figures 3 and 4A); this decrease averaged 26% for all four groups receiving IGF-1 treatment and was significantly different from zero ($p<0.001$). Total IGF-binding protein activity

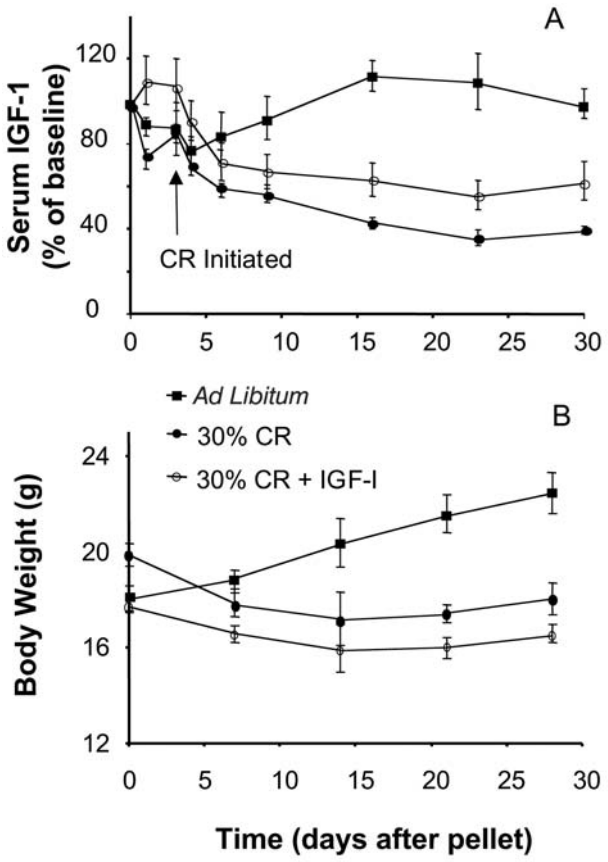

Figure 4. *Time course effects of CR and IGF-1 treatment on IGF-1 and body weight. Error bars are one standard error. A. Serum IGF-1 levels in AL-fed, 30% CR and 30% CR mice treated with IGF-1 via a time-release pellet. B. Body weights.*

(expressed as a percentage of the AL-fed + placebo pellet group, mean ± S.E.) decreased in 30 and 40% CR-treated animals (20% CR=106.2±6.8; 30% CR=85.7±10.1; 40% CR=62.6±4.0). The effect of CR was significant ($p=0.002$, but total IGF-binding was not influenced by IGF treatment ($p=0.872$).

To further characterize serum levels of IGF-1 in response to CR and implantation of time-release IGF-1 pellets, we made repeated measures of IGF-1 over a period of 28 days on mice from three treatment groups; AL fed, 30% CR and 30% CR + 20 μg/day IGF-1. CR reduced serum IGF-1 levels, and in this experiment IGF-1 treatment partially restored these levels (Figure 4A). However, the increase in serum IGF-1 compared to AL-fed controls was transient, persisting for only about one week after pellet implantation. AL-fed animals were larger than CR animals, and IGF-1-treated CR animals were smaller than CR animals ($p<0.001$; Figure 4B).

Discussion

Some evidence suggests that calorie restriction (CR) or, alternatively, targeting CR-related pathways, could contribute to cancer prevention and control (4). One of the main pathways through which CR acts to prevent cancer is the IGF-1 pathway (4, 7, 27). CR animals have significant reductions in serum concentrations of IGF-1 and are, therefore, thought to have reduced signaling through the IGF-1 receptor, with subsequent reduction in pro-cell survival pathways. Past studies have shown that CR reduces body weight and fat contents, and may influence bone characteristics (2). Our results extend this work by characterizing the dose-response relationship between CR and body composition/bone characteristics. We also demonstrate experimentally that reductions in bone density caused by CR can be reversed by administration of IGF-1 and establish that time-release capsules provide a minimally invasive approach to exploring the physiological and molecular effects of IGF-1 on mice. Overall, our results support the argument that efforts to use CR or energy balance-related mechanistic targets to ameliorate the effects of aging and/or to delay carcinogenesis will require attention to potential effects of the IGF-1 pathway on bone (4, 20, 27-30).

Increased body weight is associated with increased bone density in humans and rodents (15, 31, 32). Furthermore, reduction of bone density in rhesus monkeys caused by 30% CR appears to be mediated through CR effects on body size (30). Our study demonstrates that experimental reduction in weight loss *via* CR also reduces bone mineral density, but these effects are non-linear. For example, body weights of 40% CR animals were reduced by 38% compared to AL-fed animals, but bone mineral density was reduced by only 9.3%. In contrast, the body weight of 30% CR animals was reduced only 18.5% and bone density was reduced 10.4%. Thus, the effects of CR on bone characteristics are non-linear, and 40% CR has body weight-independent effects on bone density.

Treatment with IGF-1 increased bone density overall and in the vertebrae, but not in the tibia. Effects of CR and IGF-1 on bone characteristics are also dependent on bone type and genetic differences between strains and species (19). One study reports that IGF-1 treatment in growing rats does not influence tibial bone density (33). Other studies of mice suggest the effects of CR and IGF-1 on bone characteristics are dependent on mouse strain (34-36). For example, C57BL/6, DBA/2 and SENCAR mice differ in their response to CR, and 40% CR in SENCAR mice increases tibial bone density (35). Comparison of our findings with those of mice congenitally deficient in IGF-1, such as MIDI, LID and ALSKO (20, 28) may be informative regarding the role of IGF-1 in determining bone characteristics. MIDI mice have serum IGF-1 levels reduced

by ~60% compared to those in wild-type controls, and LID mice show a 75% reduction in IGF-1 levels. Similarly, CR also reduces IGF-1 levels, with 40% CR resulting in an almost 50% decrease in IGF-1 levels. Each of the congenitally-deficient mouse strains also have significantly reduced bone density, with changes in both trabecular and cortical bone characteristics (20). Thus, not only are there strain and species differences in bone responses to CR and IGF-I, but the response of bone may differ based on whether reductions in serum IGF-1 concentrations are caused by CR-induced *versus* congenital perturbations.

Serum IGF-1 levels were significantly decreased by calorie restriction. This is consistent with a number of past studies (6, 7, 37). However, in the current study IGF-1 treatment resulted in long-term decreases in serum IGF-1. This result contrasts with other studies that report increased serum IGF-1 in mice receiving IGF-1 (7, 20). Previous studies have largely used injection or osmotic mini-pumps for IGF-1 delivery. Stress levels of mice in past studies could be increased due to implantation of osmotic pumps or use of injections. However, serum urinary corticosterone measurements output did not differ between animals receiving pellets *vs.* osmotic pumps in our study, nor did we observe elevated serum IGF-1 in AL-fed mice receiving IGF-1 from pumps (data not shown). Almost all past studies in rodent models have used human IGF-1, but our results do not suggest that the use of murine *vs.* human IGF-1 accounts for differences between this study and others. Mice in our study that received human rather than murine IGF-1 did not show elevated serum levels of IGF-1 (data not shown). Treatment with IGF-1 did not influence total IGF-binding protein activity in serum. Twice daily injections, such as those used in some past studies (27), could result in elevated serum levels of IGF-1 if serum is collected shortly after injections, even if the kinetics of IGF-binding proteins are changed by treatment. Further experiments to explore these possibilities are underway. Repeated measurements of IGF-1 levels in treated mice indicated that serum IGF-1 did increase for the first week on study (Figure 4A); measurements of IGF-1 receptor over time could be informative.

Translation of discoveries concerning cancer prevention *via* nutritional interventions requires renewed focus on costs as well as benefits of candidate interventions. The major focus of our research is the role of energy balance in cancer prevention and control (4, 7, 21, 37), and we are particularly concerned about the potential for deleterious side-effects on bone characteristics that might be associated with preventive interventions targeting energy balance-related pathways, including the IGF-1 pathway. The present results indicate that this pathway could mediate a tradeoff between the beneficial results of CR in relation to cancer and negative effects of weight loss on bone characteristics. In this paper, we establish that treatment with murine recombinant IGF-1 delivered by time-release pellets reverses some of the effects of calorie restriction on bone characteristics. We are now exploring the effects of IGF treatment on gene expression. Preliminary results suggest that IGF-1 treatment reverses some of the gene expression changes induced by calorie restriction (J. Lavigne, personal communication). Identifying and characterizing the best systems for studying the combined effects of CR on carcinogenesis and bone is an ongoing challenge (38); C57BL\6 mice appear to be a useful model for further studies of this topic.

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References

1 Tannenbaum A: The genesis and growth of tumors. III. Effects of a high-fat diet. Cancer Res 468-475, 1942.

2 Weindruch R and Walford RL: The retardation of aging and disease by dietary restriction. Springfield, IL, Charles C. Thomas Publisher, pp. 436, 1988.

3 Hursting SD and Kari FW: The anti-carcinogenic effects of dietary restriction: mechanisms and future directions. Mutat Res *443*: 235-249, 1999.

4 Hursting SD, Lavigne JA, Berrigan D, Perkins SN and Barrett JC: Calorie restriction, aging, and cancer prevention: mechanisms of action and applicability to humans. Annu Rev Med *54*: 131-152, 2003.

5 Roth GS, Lane MA, Ingram DK, Mattison JA, Elahi D, Tobin JD, Muller D and Metter EJ: Biomarkers of caloric restriction may predict longevity in humans. Science *297*: 811, 2002.

6 Berrigan D, Perkins SN, Haines DC and Hursting SD: Adult onset calorie restriction and fasting delay spontaneous tumorigenesis in p-53 deficient mice. Carcinogenesis *23*: 817-822, 2002.

7 Dunn SE, Kari FW, French J, Leininger JR, Travlos G, Wilson R and Barrett JC: Dietary restriction reduces insulin-like growth factor I levels, which modulates apoptosis, cell proliferation, and tumor progression in p53-deficient mice. Cancer Res *57*: 4667-4672, 1997.

8 Macaulay VM: Insulin-like growth factors and cancer. Br J Cancer *65*: 311-320, 1992.
9 LeRoith D, Baserga R, Helman L and Roberts CT Jr: Insulin-like growth factors and cancer. Ann Intern Med *122*: 54-59, 1995.
10 Singh P, Dai B, Yallampalli U, Lu X and Schroy PC: Proliferation and differentiation of a human colon cancer cell line (CaCo2) is associated with significant changes in the expression and secretion of insulin-like growth factor (IGF) IGF-II and IGF binding protein-4: role of IGF-II. Endocrinology *137*: 1764-1774, 1996.
11 Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, Rosner B, Speizer FE and Pollak M: Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. Lancet *351*: 1393-1396, 1998.
12 Giovannucci E: Insulin, insulin-like growth factors and colon cancer: a review of the evidence. J Nutr *131*: 3109S-3120S, 2001.
13 Chokkalingam AP, Pollak M, Fillmore CM, Gao YT, Stanczyk FZ, Deng J, Sesterhenn IA, Mostofi FK, Fears TR, Madigan MP, Ziegler RG, Fraumeni JF Jr and Hsing AW: Insulin-like growth factors and prostate cancer: a population-based case-control study in China. Cancer Epidemiol Biomarkers Prev *10*: 421-427, 2001.
14 Holbrook TL and Barrett-Connor E: The association of lifetime weight and weight control patterns with bone mineral density in an adult community. Bone Miner *20*: 141-149, 1993.
15 Reid IR: Relationships among body mass, its components, and bone. Bone *31*: 547-555, 2002.
16 Lee CJ, Panemangalore M and Wilson K: Effect of dietary energy restriction on bone-mineral content of mature rats. Nutr Res *6*: 51-59, 1986.
17 Talbott SM, Rothkopf MM and Shapses SA: Dietary restriction of energy and calcium alters bone turnover and density in younger and older female rats. J Nutr *128*: 640-645, 1998.
18 Jones JI and Clemmons DR: Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev *16*: 3-34, 1995.
19 Rosen CJ, Beamer WG and Donahue LR: Defining the genetics of osteoporosis: using the mouse to understand man. Osteoporos Int *12*: 803-810, 2001.
20 Stabnov L, Kasukawa Y, Guo R, Amaar Y, Wergedal JE, Baylink DJ and Mohan S: Effect of insulin-like growth factor-1 (IGF-1) plus alendronate on bone density during puberty in IGF-1-deficient MIDI mice. Bone *30*: 909-916, 2002.
21 Hursting SD, Perkins SN and Phang JM: Calorie restriction delays spontaneous tumorigenesis in p53-knockout transgenic mice. Proc Natl Acad Sci USA *91*: 7036-7040, 1994.
22 Nagy TR and Clair AL: Precision and accuracy of dual-energy X-ray absorptiometry for determining *in vivo* body composition of mice. Obes Res *8*: 392-398, 2000.
23 Dobush GR, Ankney CD and Krementz DG: The effect of apparatus, extraction time, and solvent type on lipid extractions of snow geese. Can J Zool *63*: 1917-1920, 1985.
24 Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S and Binoux M: Analysis of serum insulin-like growth factor binding proteins using Western blotting: use of the method for titration of the binding proteins and competitive binding studies. Anal Biochem *154*: 138-143, 1986.
25 Packard GC and Boardman TJ: The misuse of ratios, indices, and percentages in ecophysiological research. Physiol Zool *61*: 1-9, 1988.
26 Nagy TR, Prince CW and Li J: Validation of peripheral dual-energy X-ray absorptiometry for the measurement of bone mineral in intact and excised long bones of rats. J Bone Mineral Res *16*: 1682-1687, 2003.
27 Wu Y, Yakar S, Zhao L, Hennighausen L and LeRoith D: Circulating insulin-like growth factor-1 levels regulate colon cancer growth and metastasis. Cancer Res *62*: 1030-1035, 2002.
28 Yakar S, Rosen CJ, Beamer WG, Ackert-Bicknell CL, Wu Y, Liu JL, Ooi GT, Setser J, Frystyk J, Boisclair YR and LeRoith D: Circulating levels of IGF-1 directly regulate bone growth and density. J Clin Invest *110*: 771-781, 2002.
29 Bikle D, Majumdar S, Laib A, Powell-Braxton L, Rosen C, Beamer W, Nauman E, Leary C and Halloran B: The skeletal structure of insulin-like growth factor 1-deficient mice. J Bone Miner Res *16*: 2320-2329, 2001.
30 Black A, Allison DB, Shapses SA, Tilmont EM, Handy AM, Ingram DK, Roth GS and Lane MA: Calorie restriction and skeletal mass in rhesus monkeys *(Macaca mulatta)*: evidence for an effect mediated through changes in body size. J Gerontol A Biol Sci Med Sci *56*: B98-107, 2001.
31 Hemenway D, Colditz GA, Willett WC, Stampfer MJ and Speizer FE: Fractures and lifestyle: effect of cigarette smoking, alcohol intake, and relative weight on the risk of hip and forearm fractures in middle-aged women. Am J Public Health *78*: 1554-1558, 1988.
32 Wronski TJ, Schenck PA, Cintron M and Walsh CC: Effect of body weight on osteopenia in ovariectomized rats. Calcif Tissue Int *40*: 155-159, 1987.
33 Rosen HN, Chen V, Cittadini A, Greenspan SL, Douglas PS, Moses AC and Beamer WG: Treatment with growth hormone and IGF-1 in growing rats increases bone mineral content but not bone mineral density. J Bone Miner Res *10*: 1352-1358, 1995.
34 Brochmann-Murray EJ, Beamer WG, Duarte ME, Behnam K, Grisanti MS and Murray SS: Effects of dietary restriction on appendicular bone in the SENCAR mouse. Metabolism *50*: 436-442, 2001.
35 Brochmann EJ, Duarte ME, Zaidi HA and Murray SS: Effects of dietary restriction on total body, femoral, and vertebral bone in SENCAR, C57BL/6, and DBA/2 mice. Metabolism *52*: 1265-1273, 2003.
36 Murray SS, Duarte ME and Brochmann EJ: The effects of dietary restriction on humeral and mandibular bone in SENCAR, C57BL/6, and DBA/2 mice. Metabolism *52*: 970-977, 2003.
37 Hursting SD, Perkins SN, Brown CC, Haines DC and Phang JM: Calorie restriction induces a p53-independent delay of spontaneous carcinogenesis in p53-deficient and wild-type mice. Cancer Res *57*: 2843-2846, 1997.
38 Rosen CJ, Dimai HP, Vereault D, Donahue LR, Beamer WG, Farley J, Linkhart S, Linkhart T, Mohan S and Baylink DJ: Circulating and skeletal insulin-like growth factor-1 (IGF-1) concentrations in two inbred strains of mice with different bone mineral densities. Bone *21*: 217-223, 1997.

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