Serum Insulin-like Growth Factor-I and Mammary Tumor Development in *Ad libitum*–Fed, Chronic Calorie–Restricted, and Intermittent Calorie–Restricted MMTV-TGF-α Mice

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Abstract The effect of chronic (CCR) and intermittent (ICR) caloric restriction on serum insulin-like growth factor (IGF)-I levels and mammary tumor (MT) development was investigated. Ten-week-old MMTV-TGF-α female mice were assigned to *ad libitum*–fed (AL; AIN-93M diet), ICR [3-week 50% caloric restriction using AIN-93M-mod diet, 2× protein, fat, vitamins, and minerals followed by 3 weeks of daily 100% AL consumption of AIN-93M (~75% of AL for each 6-week cycle)], and CCR (calorie and nutrient intake matched for each 6-week ICR cycle) groups. Half of the mice from each group were sacrificed at 79 (end of restriction) or 82 (end of refeeding) weeks of age. Serum was obtained at euthanasia and in cycles 1, 3, 5, 8, and 11. MT incidence was 71.0%, 35.4%, and 9.1% for AL, CCR, and ICR mice. ICR-Restricted mice had significantly lower terminal serum IGF-I and IGF-I/IGF binding protein-3 (IGFBP-3) ratio than CCR, ICR-Refed, and AL mice. There were no differences in terminal IGFBP-3. Final body, internal, and mammary fat pad weights correlated positively with IGF-I and negatively with IGFBP-3. Few changes were found for protein expression of IGF-IRα and IGFBP-3 in mammary tissue and MTs. During the study, IGF-I levels of ICR-Restricted mice were reduced, whereas refeeding allowed partial recovery. For all groups, elevated IGF-I levels preceded MT detection, although not all values were significant versus mice without MTs. However, the specific role of IGF-I in the protective effect of calorie restriction remains to be determined. These results confirm that ICR prevents MT development to a greater extent than CCR.

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Worldwide, each year, approximately 1 million women are newly diagnosed with breast cancer (1). This malignancy is second only to lung cancer as a cause of cancer death in women (2). Searching for new ways of prevention and early detection and for physiologic and (or) pathophysiologic markers of breast malignances is a prevalent area of current medical-biological studies.

Insulin-like growth factor (IGF)-I is a peptide growth factor with demonstrated roles in mammary gland development (3, 4). Both IGF-I and its primary signaling receptor, IGF-IR, are present in the developing mammary gland (5–8). Additionally, the high-affinity IGF binding protein-3 (IGFBP-3; ref. 9) is also found in this tissue and its expression correlates with specific stages of the progression of mammary epithelial growth and maturation (10). In particular, IGF-I and its signaling pathway (s) have important roles in regulating cellular proliferation, growth, and apoptosis, resulting in interest of this regulatory system to mammary tumorigenesis (11–13).

It is interesting to note that laboratory investigation of the relation of IGF-I to mammary/breast cancer was motivated by epidemiologic/clinical research (14). These studies have generally supported an increased risk of breast cancer in association with elevated serum levels of IGF-I in premenopausal women but not in those women who are postmenopausal as summarized in several recent meta-analyses (i.e., refs. 15–18). However, in one case-control study, higher levels of serum IGF-I were found in breast cancer cases versus controls in women both under and over the age of 50 (19). The evidence about risk associated with IGFBP-3 is much less clear. For example, a nonsignificant inverse association between IGFBP-3 and breast cancer risk in premenopausal women was noted by Hankinson et al. (20) and Bohlke et al. (21) but not by Toniolo et al. (22) who found no association, whereas Del Giudice et al. (23) and Vadgama et al. (24) found a positive association of IGFBP-3 with premenopausal breast cancer risk. Recently, the European Prospective Investigation into Cancer and Nutrition published data from 1,081 breast cancer cases and 2,098 matched controls, approximately equal to the total number of incident cases in all previous prospective studies

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combined, which indicated that women with the highest circulating levels of either total IGF-I or IGFBP-3 had a 40% increased risk for breast cancer diagnosed after age 50, but there was no evidence of increased risk in younger women (25). This was confirmed when analysis was restricted to postmenopausal women at the time of blood collection but not for women who were premenopausal. Another recent report from the prospective Nurses' Health Study II showed that IGF-I and IGFBP-3 were not associated with breast cancer risk in a large group of primarily premenopausal women (26).

In rodent models of breast cancer, IGF-I has also been implicated as a growth factor. Models in which circulating IGF-I levels are depleted by either expression of a growth hormone antagonist (27) or liver-specific deletion of IGF-I (LID mice; refs. 28–31) have provided evidence that lower IGF-I was associated with a significant reduction in incidence of mammary tumors (MT) following exposure to a chemical carcinogen. In contrast, transgenic mice that overexpress IGF-I in a variety of organs, including the mammary gland, exhibited enhanced susceptibility to mammary carcinogenesis (32). In another study, 53% of transgenic mice that overexpress either human IGF-I or des(1-3)IGF-I (a potent IGF-I analogue with reduced affinity for IGF binding proteins) in mammary glands developed spontaneous MTs compared with no MTs in control mice (33). In diabetic mice, IGF-I that was overproduced by tumor cells seemed to act as an autocrine stimulator of malignant cell division through binding with and stimulating the activity of IGF-IR (34). In support of evidence for the role of IGF-IR in the development of MTs, Carboni et al. (35) have shown that transgenic mice with activated intracellular IGF-IR developed mammary adenocarcinoma as early as 6 weeks after birth, with palpable MT mass at 8 weeks of age.

IGF-I signaling arose early in evolution as a regulator of cellular proliferation in relation to nutrient availability and energy metabolism (36), so nutrition has an important influence on circulating IGF-I levels. Calorie restriction has been reported to reduce serum IGF-I levels (37–39) and starvation reduces IGF-I levels (40) and intracellular signaling distal to IGF-IRα (41). It is well known that caloric restriction decreases MT incidence and extends latency of chemically induced and spontaneous MTs in rodents (42–47). Additionally, we have reported that lower serum IGF-I levels were associated with the prevention of MTs in transgenic MMTV-TGF-α mice after long-term chronic calorie restriction (CCR; refs. 38, 39). One aspect of our studies was to compare the effect of CCR with intermittent calorie restriction (ICR) on MT development. Interestingly, we found that ICR provided even greater protection than did CCR (38, 39). In the first study, serum IGF-I was measured at the terminal end point of 80 weeks of age following 1 week of refeeding after 3 weeks of 50% calorie restriction in ICR mice, and the value obtained was intermediate between *ad libitum*–fed (AL) and CCR mice that had been calorie restricted by 20% (38). In the second study, serum IGF-I was obtained both at the end of the last period of calorie restriction for ICR mice, 79 weeks of age, as well as after 1 week of refeeding, 80 weeks of age (39). Following the 3 weeks of 50% calorie restriction, IGF-I level of ICR mice was significantly reduced compared with AL mice and similar to the value for CCR mice, whereas after 1 week of refeeding IGF-I level of ICR mice was intermediate between AL and CCR mice similar to the result of the first study.

The primary aim of the present study was to assess the effect of ICR on prevention and development of MT when ICR mice were prevented from consuming unlimited calories during refeeding as had been allowed in the two earlier studies. Here, during refeeding periods, ICR mice had their food intake capped daily at the amount AL mice consumed during age-matched time periods. The secondary aim was to obtain IGF-I data across the study and to determine if IGF-I was involved in the protective action of either CCR or ICR. Additionally, the expression of proteins associated with the signaling of IGF-I was measured in both mammary tissue and MTs.

Materials and Methods

Animals and study design

MMTV-TGF-α female mice that overexpress human TGF-α oncogene were produced at the Hormel Institute (Austin, MN) and genotyped as previously described (38). At 8 wk of age, mice were housed individually and provided *ad libitum* access to water and powdered AIN-93M diet. At 10 wk of age, they were assigned to AL, CCR, or ICR groups. Those in the AL group (n = 75) continued to have free access to AIN-93M diet. Mice in the CCR group (n = 75) were given a diet formulated to be isocaloric with the AIN-93M diet, with 25% increases in protein, vitamin, mineral, and fat content. This diet was given at 75% of age-matched *ad libitum* consumption. Mice in the ICR group (n = 75) were provided a modified AIN-93M diet with 2-fold increases in protein, vitamin, mineral, and fat content at 50% of the consumption level of AL mice, during each 3 wk of restriction. Following each restriction period, ICR mice were provided with AIN-93M diet at 100% of age-matched AL consumption for 3 wk to prevent overeating during this time period. Food intakes were determined daily. Body weights were obtained weekly, and at that time, mice were palpated for MTs. Once MTs were detected, their growth was monitored using calipers. Half of the mice in each group were euthanized by CO_2 overdose when they reached terminal end points of 79 (end of restriction) or 82 (end of refeeding) weeks of age unless MT size exceeded 20 mm in length or a mouse lost more than 25% of body weight, at which point they were euthanized at an earlier age. When results are presented specific to the ICR mice during restriction periods, they are further classified as ICR-Restricted, and during refeeding periods, they are classified as ICR-Refed. All procedures with mice were done under the guidelines and with approval of the University of Minnesota Institutional Animal Care and Use Committee. The University of Minnesota is an Association for Assessment and Accreditation of Laboratory Animal Care–accredited institution.

Tissue sample collection and histopathologic analysis

At sacrifice, fat pads (mammary, retroperitoneal, and parametrial), mammary tissues from the back of the neck and axillary areas, livers, MTs, and any abnormalities were removed and weighed. A sample of each tissue was placed in 10% neutral buffered formalin. The remaining tissues were stored at −70°C. Left mammary fad pads, mammary tissues or MTs, and tissue samples that seemed abnormal were sent to the Department of Pathology and Laboratory Medicine of the Mayo Foundation (Rochester, MN) for histopathologic analyses to determine malignancy and/or disease status.

Assessment of serum IGF-I and IGFBP-3 concentrations

Blood samples were collected from orbital sinus from all mice at the terminal age points and over the study (cycles 1, 3, 5, 8, and 11 from three cohorts corresponding to the first, second, and third weeks of restriction and refeeding of ICR mice). For each mouse, two samples were obtained per cycle such that for cohort one samples were obtained corresponding to the first week of restriction and the first week of refeeding (weeks 1 and 4 of the cycle), for cohort two after 2 wk of

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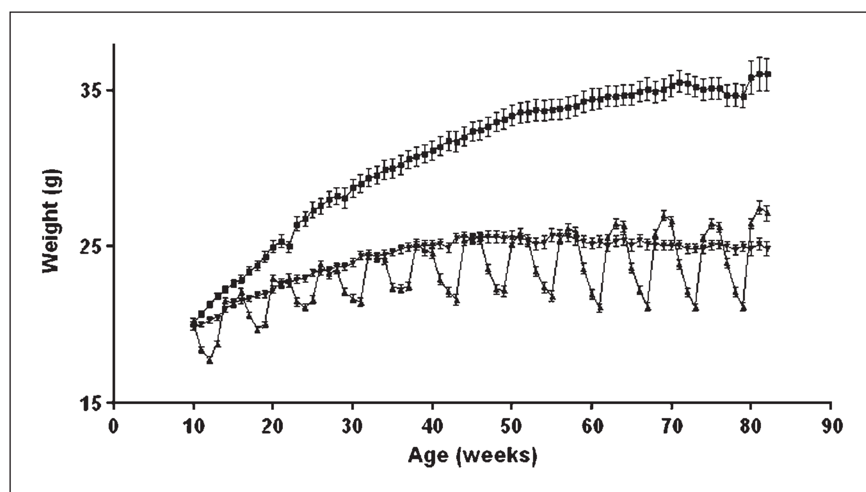

Fig. 1. Body weight curves of TGF-α female mice over the course of the experiment. ■, AL (n = 25-73 depending on age); ▲, ICR (n = 29-73 depending on age); ▼, CCR (n = 31-74 depending on age). $P < 0.0001$, ANOVA; AL group was significantly different ($P < 0.001$) from ICR and CCR groups, whereas there was no difference between the ICR and CCR groups.

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restriction and 2 wk of refeeding (weeks 2 and 5 of the cycle), and cohort three after 3 wk of restriction and 3 wk of refeeding (weeks 3 and 6 of the cycle). Serum samples were stored in −20°C until used. IGF-I was measured initially using mouse/rat IGF-I EIA (DSL-10-2900, Diagnostic Systems Laboratory) and then by mouse/rat IGF-I ELISA (DSL-10-29200, Diagnostic Systems Laboratory) kits. Identical results were obtained with both kits. The change was necessitated by the discontinuation of production of the former kits by the manufacturer. Serum IGFBP-3 levels were determined using a commercial human IGFBP-3 ELISA kit (DSL-10-6600, Diagnostic Systems Laboratory).

Western blot analysis

Tissue samples were homogenized in extraction buffer with protease inhibitors. Total protein was extracted using a Total Protein Extraction kit (Chemicon International) and quantitated by the Bradford assay. Extracted proteins were electrophoresed on 4% to 15% polyacrylamide gradient gels and then transferred to a Immobilon membranes (Millipore), then blocked in PBS solution containing 0.5% milk concentrate and 1% Tween 20, and probed with antibodies against IGF-IRα (Santa Cruz Biotechnology) and IGFBP-3 (Santa Cruz Biotechnology) proteins in mammary tissue and MT samples. Anti-rabbit immunoglobulin G (Cell Signaling) was used as a secondary antibody. Antibody-bound proteins were detected by enhanced chemifluorescence (ECF substrate; Amersham Pharmacia Biotech) and analyzed with the Storm 840 Machine Imaging System (Molecular Dynamics). Standard molecular weight markers were run simultaneously for comparing molecular weights of the visualized proteins. The intensity of Western blot bands was quantified by densitometric analysis using the ImageJ program. Results were expressed as the ratio of intensity of the protein of interest to that of β-actin (Delta Biolabs LLC) from the same sample.

Statistical analysis

Results are presented as mean ± SE. Serum data were analyzed by ANOVA followed by Neuman-Keuls test or t test and are presented as mean ± SE. Number of MTs per mouse, MT incidence, and grade were analyzed by the χ^2 and two-group log-rank test.

Results

Food intake

As expected based on the experimental design, CCR (1,616.9 ± 11.4 g) and ICR (1,656.4 ± 10.2 g) mice had significantly lower cumulative food intakes over the course of the study in comparison with AL mice (2,203.4 ± 18.8 g; $P = 0.0001$, ANOVA, ICR and CCR; $P < 0.001$ versus AL). During each weight loss/regain cycle, food intake for both restricted groups was 22.9% to 28.7% lower than that of AL mice (data not shown) and food intakes of the CCR and ICR groups were almost identical during each food restriction/refeeding cycle (data not shown).

Body and fat pad weights

Body weights over the study are presented in Fig. 1. The body weight curve for AL mice was significantly higher than for CCR and ICR mice. ICR mice exhibited a pattern of weight loss during each caloric restriction period followed by rapid weight regain during the first week of refeeding. A summary of terminal comparisons for the mice in the three dietary groups for body and fat pad weights is presented in Table 1. At euthanasia, AL mice had body and fat pad weights significantly heavier than CCR ($P < 0.001$), ICR-Refed ($P < 0.001$), and ICR-Restricted ($P < 0.001$) mice. In addition, ICR-Restricted mice had significantly lower body and fat pad weights than CCR ($P < 0.01$) and ICR-Refed ($P < 0.05$). There were no significant differences between ICR-Refed and CCR mice for any of these measurements.

Pathology

Twenty-five mice (6 AL, 10 CCR, and 9 ICR) died before the age when MTs usually develop (i.e., 39 weeks of age) and were removed from further consideration. The majority (6 AL, 9 CCR, and 7 ICR) died during orbital blood collection. One CCR and one ICR mouse died from unknown illnesses, and one ICR mouse died from myeloproliferative disease. Results related to MT development for mice that completed the study are presented in Table 1. Tumor incidence of 71% for AL mice (49 of 69) was the highest followed by 35.4% for CCR mice (23 of 65), and tumor incidence of 9.1% for ICR mice (6 of 66) was the lowest. All groups were significantly different from each other. Tumor-bearing AL mice developed one to six MTs per mouse, whereas CCR mice had one to four MTs and ICR mice only had one MT per mouse. All values were significantly different among the three groups. AL mice had the highest MT weight followed by an intermediate tumor weight for ICR mice and CCR mice had the lightest tumor weight; however, this did not reach statistical significance. CCR mice

developed MTs on average significantly later, than did AL mice, whereas there was no difference in age of tumor detection between the two restricted groups and also between AL and ICR mice.

A summary of the histopathologic properties of the MTs is also presented in Table 1. A total of 89 MTs were detected in 49 AL mice followed by 32 MTs in 23 CCR mice and 6 ICR mice developed 6 tumors. Most of the MTs regardless of dietary group were classified as grade 2 adenocarcinomas with additional MTs classified as grade 3 or low-grade adenocarcinomas/carcinomas *in situ*. Tumor grade was significantly different among the three groups.

In 13 TGF-α mice, additional benign or malignant pathologies were noted. Four tumor-bearing (two AL, one CCR, and one ICR) mice and one tumor-free CCR mouse had myeloproliferative disorder. In one AL mouse, the MT contained kerathoacanthoma cells. There were three mice with liver tumors (one AL with MT had hemangioma, one tumor-free AL mouse had adenoma in a liver, and one ICR without MT had a hematopoietic malignancy). In addition, one tumor-free ICR mouse had a high-grade angiosarcoma in a mammary fad pad.

Terminal IGF-I and IGFBP-3 serum levels

At euthanasia, AL mice had serum IGF-I levels (Fig. 2A) significantly higher than CCR ($P < 0.01$), ICR-Refed ($P < 0.01$), and ICR-Restricted ($P < 0.001$) mice. ICR-Restricted mice had significantly lower values than CCR ($P < 0.05$) and ICR-Refed ($P < 0.05$) mice. There were no significant differences between ICR-Refed and CCR mice. There was no statistical difference ($P > 0.05$) for terminal IGFBP-3 among the groups (Fig. 2B). At euthanasia, ICR-Restricted mice had serum IGF-I/IGFBP-3 ratio (Fig. 2C) significantly lower than CCR ($P < 0.01$), ICR-Refed ($P < 0.01$), and AL ($P < 0.001$) mice. There were no significant differences between ICR-Refed, CCR, and AL mice.

Terminal IGF-I serum concentration of AL, CCR, and ICR mice positively correlated with terminal body weight ($P < 0.0001$, $r = 0.404$, number of pairs = 128) and mammary fad pad weights ($P < 0.0001$, $r = 0.364$, number of pairs = 128). In contrast, terminal IGFBP-3 serum concentration of MMTV-TGF-α mice was negatively correlated with terminal body weight ($P < 0.01$, $r = -0.309$, number of pairs = 78) but was not correlated with terminal mammary fad pad weights.

Interestingly, when all mice that developed MTs were considered together, they had significantly higher ($P < 0.05$) terminal IGF-I (731.8 ± 44.1 ng/mL) and lower ($P < 0.01$) IGFBP-3 (1.46 ± 0.12 ng/mL) serum levels compared with mice without tumors, whose values were 614.8 ± 28.8 ng/mL and 1.67 ± 0.17 ng/mL, respectively.

Longitudinal IGF-I serum levels

As indicated, one of the goals of this study was to evaluate IGF-I serum concentrations in a prospective manner. Serum samples were obtained during cycles 1, 3, 5, 8, and 11. During each cycle, cohorts of mice had samples obtained at weeks 1 and 4, 2 and 5, and 3 and 6. For AL and CCR mice, there were little differences in values obtained over the 6-week periods and these results were combined and data were presented for mice with and without MTs. As shown in Fig. 3A, IGF-I serum concentrations were higher for AL mice with MTs versus those without MTs from cycle 3 (23-28 weeks of age), although the difference in cycle 8 did not reach statistical significance. CCR mice that eventually developed MTs had significantly higher IGF-I values measured in all cycles compared with those that were MT-free at 79 to 82 weeks of age (Fig. 3B).

Table 1. Final body, fat pad weights, and MT characteristic for AL, CCR, and ICR TGF-α female mice

	AL (n = 69)	CCR (n = 65)	ICR (n = 59)	
			ICR-Refed (n = 29)	ICR-Restricted (n = 30)
Final body weight (g)	33.79 ± 0.71*	27.19 ± 0.36†	27.38 ± 0.50†	24.27 ± 0.50‡
Mammary fad pad weight, g (combined right and left mammary fat pads)	1.77 ± 0.11*	0.87 ± 0.04†	0.86 ± 0.07†	0.35 ± 0.06‡
Internal fat pad weight, g (combined parametrial and retroperitoneal fat pads)	2.27 ± 1.37*	0.93 ± 0.47*	1.01 ± 0.0.08†	0.48 ± 0.03‡

	AL (n = 69)	CCR (n = 65)	ICR (n = 66)
Age of MT detection (wk)	61.0 ± 1.1*	69.0 ± 1.6†	67.0 ± 4.2*,†
% of mice in group with MT (number with MT/total mice)	71.0% (69/49)*	35.4% (65/23)†	9.1% (66/6)‡
MT weight (g)	0.93 ± 0.11	0.43 ± 0.04	0.66 ± 0.38
No. MTs/mouse (average in tumor-bearing mice)	1.82 ± 0.17*	1.39 ± 0.18†	1.00
Total (no. MTs)	89	32	6
Low grade and a carcinoma *in situ* (number/%)	5/5.6%	2/6.3%	1/16.7%
Grade 2 (number/%)	76/84.3%	27/84.3%	4/66.6%
Grade 3 (number/%)	9/10.1%	3/9.4%	1/16.7%

NOTE: Values are mean ± SE.

*Values within a row with different superscript symbol are significantly different by ANOVA (final weights) or by χ^2 analysis (MT characteristics).

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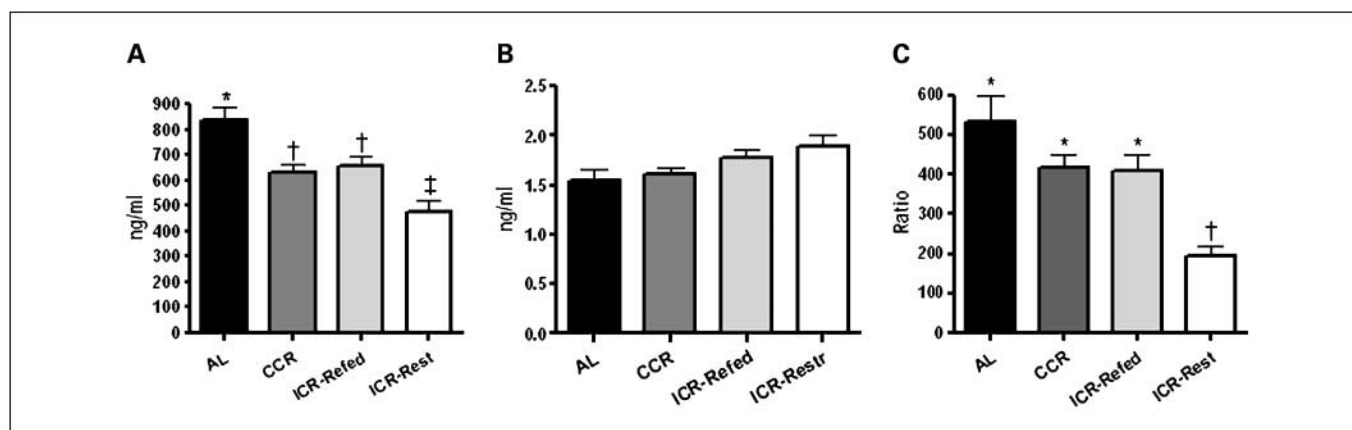

Fig. 2. Terminal IGF-I and IGFBP-3 serum level and ratio of IGF-I:IGFBP-3 of TGF-α female mice. *A*, terminal IGF-I serum levels. $P = 0.0001$, ANOVA. AL ($n = 38$), CCR ($n = 37$), ICR-Refed ($n = 19$), and ICR-Restricted ($n = 30$) mice. *B*, terminal IGFBP-3 serum levels. $P = 0.0001$, ANOVA. AL ($n = 25$), CCR ($n = 26$), ICR-Refed ($n = 12$), and ICR-Restricted ($n = 13$) mice. *C*, ratio of IGF-I to IGFBP-3 levels. $P = 0.01$, ANOVA. AL ($n = 18$), CCR ($n = 26$), ICR-Refed ($n = 12$), and ICR-Restricted ($n = 12$) mice. In *A* to *C*, ICR-Refed mice were euthanized during a refeeding period and ICR-Restricted mice were euthanized during a restriction period. Columns with different superscripts are significantly different from each other.

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Results of the terminal measurements of IGF-I in this and an earlier study (39) indicated that IGF-I values were different when samples from ICR mice were obtained after restriction or refeeding. Therefore, the longitudinal data for this group were presented separately for restriction and refeeding periods. As shown in Fig. 3C throughout the study, ICR-Restricted mice without MTs had consistently lower IGF-I levels than when they were refed, although results were not always significantly different. As the study progressed, there was a less dramatic change in IGF-I concentrations between restriction and refeeding periods for ICR mice without MTs. Above each column is presented the mean IGF-I value for those ICR mice that developed MTs and for whom results were available ($n = 4$). From cycle 3, ICR mice with MTs had higher IGF-I serum levels after refeeding than after restriction, although the only significant value ($P < 0.01$) was in cycle 8. Across the study at most ages, tumor-bearing ICR mice had higher IGF-I concentrations than did ICR tumor-free mice and it was statistically different ($P < 0.01$) after refeeding in cycle 8. For comparison, we have included lines on Fig. 3C that represent corresponding mean values for the same ages for AL or CCR mice without MTs. As can be seen from cycle 5, ICR mice had IGF-I serum levels similar to AL during refeeding and similar to CCR during restriction.

IGFBP-3 and IGF-IRα expression in mammary tissue and MTs

MT expression of IGFBP-3 was not detected for AL mice and was not different for CCR and ICR mice (Fig. 4A and B). IGFBP-3 expression was significantly higher in mammary tissue in CCR mice ($P < 0.05$) compared with all other groups (Fig. 4A and C). Expression of IGF-IRα was not different in MTs and in mammary tissue for mice from different diet groups (Fig. 4A, D, and E).

Discussion

In the present investigation, we used an experimental design that differed from that used in our previous studies (38, 39). To avoid overeating during refeeding, food intake of ICR mice was capped at what the AL mice consumed during age-matched time periods. The findings in the current study confirm that multiple cycles of ICR/refeeding protect MMTV-TGF-α mice from developing MTs to a greater extent than does the same degree of restriction implemented in a chronic fashion. ICR mice had the lowest tumor incidence and number of MTs per tumor-bearing mouse. Furthermore, these findings are consistent with several earlier publications, which reported that intermittent fasting/refeeding interventions prevented spontaneous MT development in rodents (46, 48–50). Somewhat different results have been reported for the consequences of ICR interventions using chemical carcinogen models when the feeding regimen is initiated at the time of carcinogen administration. This has been discussed in detail in our previous publications (38, 39). The finding that refeeding may enhance tumor development seems to be unique to timing of the administration of the carcinogen and would have limited application to most human studies. This is supported by a study reporting that, when ICR was initiated 2 months after carcinogen administration, there was a 100% reduction in MT incidence as well as an 80% reduction in tumor burden in rats administered 7,12-dimethylbenz(*a*)anthracene in comparison with AL rats (51).

Despite the fact that the percentage of ICR tumor-bearing mice in our three studies is somewhat different (i.e., 3% in the first and 15% in the second versus 9.1%, here), results from all three studies clearly indicate that ICR provides a greater protective effect against oncogene-induced mammary tumorigenesis than does the same degree of CCR. Based on studies where different levels of CCR have been compared, it had been concluded that the degree of caloric restriction determines levels of tumor preventing protection in rodents (44, 52). However, the results of our three studies have shown that not only the degree of restriction but also the manner in which calorie restriction is implemented affects MT development. Additionally, the use of the capped refeeding protocol in the present study suggests that it is the periods of calorie restriction, which are important for the protective effect of this type of intervention.

The IGF signaling pathway has been proposed to play a crucial role in mammary tumorigenesis as evidenced by epidemiologic (15, 19, 24, 53) and rodent (32–34) studies, which have

shown that increased levels of IGF-I are associated with increased risk of breast/mammary cancer. In support of these data, our results indicate that mice with MTs had higher terminal IGF-I serum levels than mice without MTs regardless of dietary intervention. Additionally, AL and CCR mice that eventually developed MTs had consistently higher IGF-I serum concentrations early in a life (from cycle 1 for CCR mice and from cycle 3 for AL mice) even before tumor detection. To the best of our knowledge, this is the first longitudinal study in rodents indicating that IGF-I levels were elevated before MT detection. The observed association between higher levels of IGF-I and increased tumor incidence in our study or cancer risk in general may be explained by stepwise accumulation of genetic damage (14). As a growth factor, IGF-I enhances the rate of cell division and thus increases the probability of genetic mutations or hits. Even a slight increase in IGF-I–activated proliferation could increase availability for second and subsequent hits expanding the pool of damaged cells. In addition, higher levels of IGF-I would be expected to activate the IGF-IR survival pathway decreasing apoptosis.

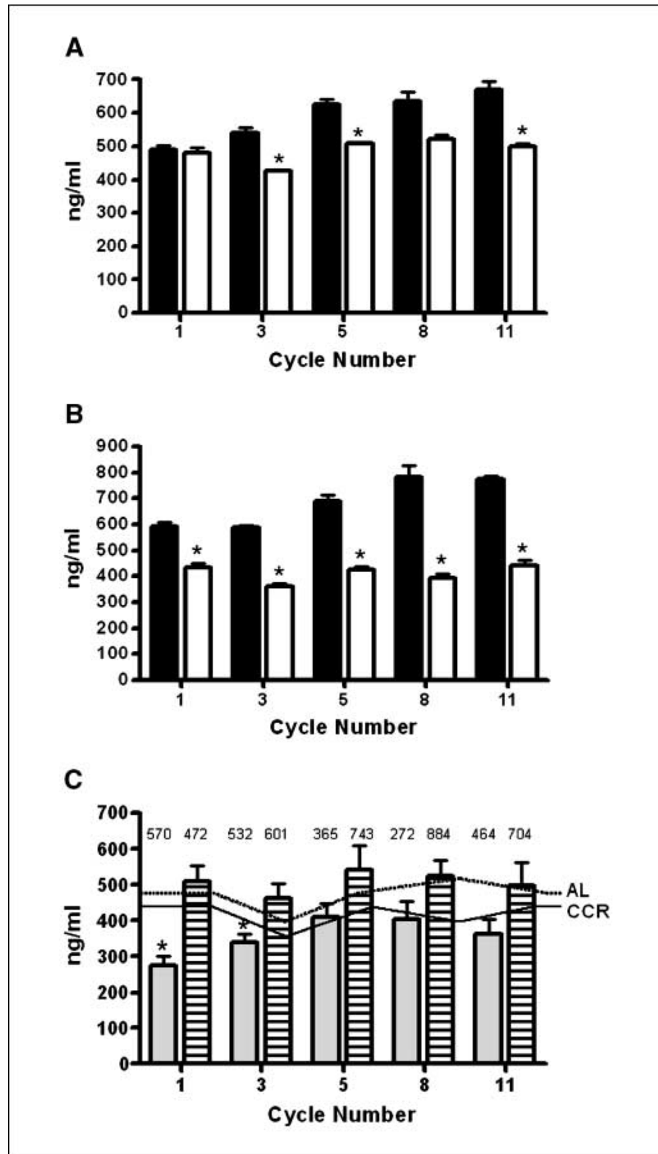

Fig. 3. Serum IGF-I levels for AL, CCR, and ICR TGF-α mice with and without MTs over the course of the study. *A,* serum IGF-I serum levels for AL mice during cycles 1, 3, 5, 8, and 11 for mice that never developed MTs (*white columns*) compared with those that did (*black columns*). Columns, mean of IGF-I concentrations. $P < 0.05$, ANOVA. AL without MTs ($n = 24$) and AL with MTs ($n = 24$). *B,* serum IGF-I serum levels for CCR mice during cycles 1, 3, 5, 8, and 11 for mice that never developed MTs (*white columns*) compared with those that did (*black columns*). $P < 0.01$, ANOVA. CCR without MTs ($n = 24$) and AL with MTs ($n = 42$). *, significant difference by χ^2 analysis between IGF-I serum levels for mice with versus without MTs in *A* and *B*. *C,* serum IGF-I serum levels for ICR-Restricted (*gray columns*) versus ICR-Refed (*hatched columns*). $P < 0.01$, ANOVA. Columns, mean of IGF-I concentrations for ICR mice without MTs. ICR after restriction ($n = 20$) and ICR after refeeding ($n = 20$). Numbers above the columns, means of IGF-I for ICR mice with MTs ($n = 4$). Lines, IGF-I serum levels for AL and CCR mice without MTs. *, significant difference by χ^2 analysis between IGF-I serum levels for ICR mice in Restriction versus Refeeding.

It has been shown that energy-restricted diets significantly reduce levels of circulating IGF-I in rodents (37, 39, 54). Confirming this, ICR-Restricted mice had significantly lower terminal IGF-I than did the mice in the other dietary groups. IGF-I serum levels of ICR-Restricted mice over the study were consistently lower during restriction than in refeeding; however, as the study progressed, there was a less dramatic change in IGF-I concentrations between restriction and refeeding periods. The mechanisms of IGF-I serum level reduction by dietary restriction are unknown. Most of the circulating IGF-I is produced in the liver (55). Recently, Powolny et al. (54) reported that 40% dietary restriction reduced IGF-I mRNA expression in the liver and this reduction correlated with decreased IGF-I serum concentration. However, white adipose tissue (56, 57) also produces IGF-I and autocrine IGF-I action in adipocytes controls systemic IGF-I concentration (58). Surprisingly, IGF-I mRNA level in white adipose tissue has been reported to be similar to that in liver and is far greater than the level in other organs (59). In our study, final body weights and mammary and internal fat pad weights of AL mice were heaviest followed by CCR and ICR-Refed mice with similar weights, whereas ICR-Restricted mice weights were the lowest. Terminal IGF-I serum concentration of mice in the different dietary groups positively correlated with terminal body weight and mammary fad pad weights. Thus, it would seem possible that circulating levels of IGF-I during calorie restriction were mediated by both effects on the liver and by the amount of adipose tissue.

Nearly 90% of IGF-I circulating in serum is bound to IGFBP-3 (60), but it is not clear how dietary restriction influences plasma IGFBP-3 levels (54, 61). In the present study, calorie restriction did not significantly increase IGFBP-3 serum levels; however, there was a reduced IGF-I/IGFBP-3 ratio in the ICR-Restricted group. Despite the fact that lower serum concentration of IGFBP-3 has been associated with higher risk of some cancers (15, 62), there is not conclusive evidence that there is an inverse association between IGFBP-3 and breast cancer risk (20). Here, mice with MTs had significantly lower IGFBP-3 serum concentrations than those without MTs regardless of dietary interventions. A possible explanation for this is competition between IGFBP-3 and IGF-IRα to bind IGF-I such that higher IGFBP-3 levels would decrease availability of IGF-I and subsequently IGF-IRα signaling activation (14). IGFBP-3 in addition directly affects IGF-I in cancer cells in an independent manner (9, 63, 64). In support of these data, mice from both restricted groups, with low tumor incidence

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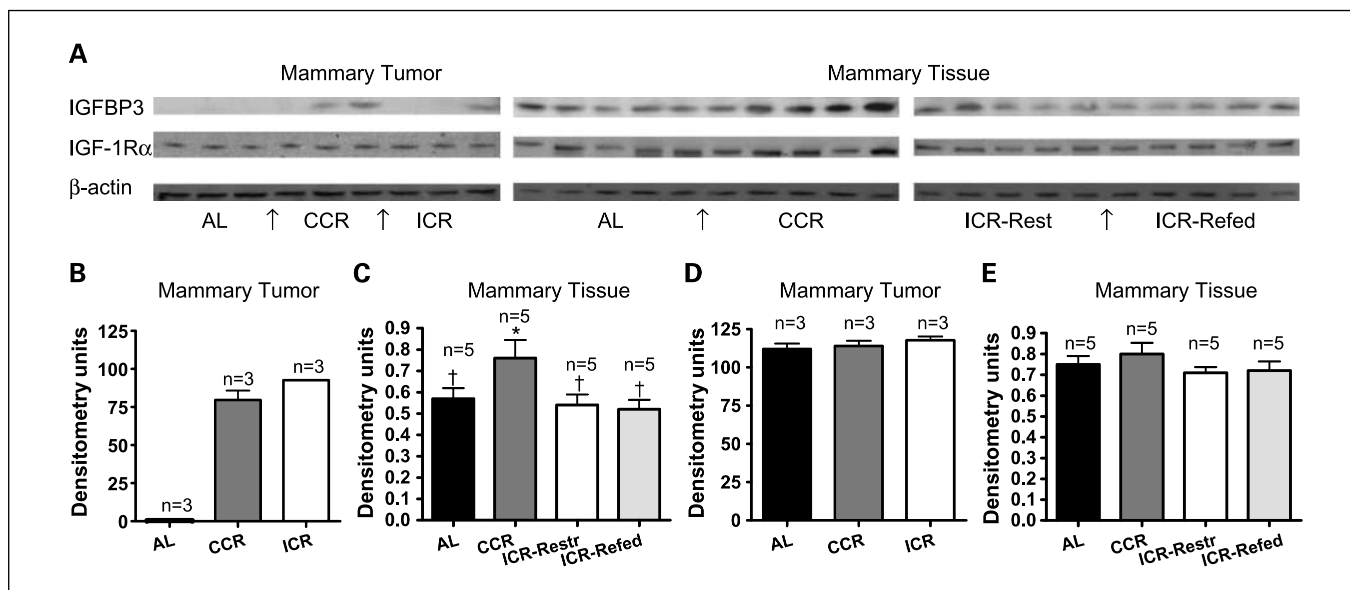

Fig. 4. Protein expression of IGFBP-3 and IGF-IRα in MT and mammary tissue. *A,* IGFBP-3 and IGF-IRα protein expression levels were detected by Western blot analysis. β-Actin protein was probed as a loading control. *B,* expression of IGFBP-3 in MTs of TGF-α mice. *C,* expression of IGFBP-3 in mammary tissue of TGF-α mice. ICR-Refed mice were euthanized during a refeeding period, and ICR-Restricted mice were euthanized during a restriction period. *, values within a column with different superscript symbol are significantly different by χ^2 analysis. *D,* expression of IGF-IRα in MTs of TGF-α mice. *E,* expression of IGF-IRα in mammary tissues of TGF-α mice. ICR-Refed mice were euthanized during a refeeding period, and ICR-Restricted mice were euthanized during a restriction period.

compared with AL mice, had higher IGFBP-3 expression: ICR in MTs and CCR in both MTs and mammary tissues.

The critical role of IGF-IR in breast cancer growth, survival, and transformation has been well documented *in vitro* and in animal models as summarized by Surmacz et al. (65). IGF-IR pathway is especially important at early stages of mammary tumorigenesis (66). There are no published data about influence of calorie restriction on IGF-IR expression in MTs. In the present study, expression of IGF-Rα was not different in MTs and in mammary tissue for mice from the different dietary groups.

In conclusion, these results confirm that caloric restriction provides a protective effect resulting in prevention of MT development. Additionally, it is noteworthy that ICR provided greater protection than CCR as evidenced by reduced MT incidence and reduced tumor burden despite identical caloric and nutrient intakes. The IGF-I axis seems to be involved in MT development and in the protective effect of calorie restriction. Lowest IGF-I serum levels as well as lowest IGF-I/IGFBP-3 ratio of ICR-Restricted mice support involvement of the IGF-I axis in the greater protective effect of ICR versus CCR. We hypothesize the reduced IGF-I during the periods of 50% restriction are sufficient to suppress cell proliferation. It is noteworthy that ICR and CCR affected fat pad weights differently, so fat tissue metabolism could also be involved in mammary tumorigenesis. There is increasing evidence that factors produced directly in adipose tissue, adipokines, specifically adiponectin and leptin, affect breast cancer development. Leptin may promote mammary tumorigenesis. In contrast, adiponectin is lower in women with breast cancer and seems to prevent proliferation of breast cancer cell lines. Ongoing analysis of these factors in serum, mammary tissue, and MT will help to answer why ICR is superior to CCR with respect to prevention of mammary tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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