Caloric Restriction and 7,12-Dimethylbenz(a)anthracene-induced Mammary Tumor Growth in Rats: Alterations in Circulating Insulin, Insulin-like Growth Factors I and II, and Epidermal Growth Factor

Bruce A. Ruggeri, David M. Klurfeld, David Kritchevsky, and Richard W. Furlanetto

The Wistar Institute of Anatomy and Biology [B. A. R., D. M. K., D. K.]; Department of Pediatrics, University of Pennsylvania School of Medicine, and Division of Endocrinology/Diabetes, Children’s Hospital of Philadelphia, Philadelphia [R. W. F.]; and the Department of Biochemistry and Biophysics, University of Pennsylvania [B. A. R.]; Philadelphia, Pennsylvania 19104

ABSTRACT

Caloric restriction (CR) inhibits many neoplastic diseases in rodents, yet the biochemical mechanism(s) for these effects are poorly understood. We have examined the effects of ad libitum (AL) feeding with 25 or 40% CR on the promotion of 7,12-dimethylbenz(a)anthracene-induced mammary tumorogenesis in virgin female Sprague-Dawley rats. Further, we have also studied the influence of chronic CR on temporal alterations in circulating insulin, insulin-like growth factor I/somatomedin C, insulin-like growth factor II/multiplication-stimulating activity, and epidermal growth factor levels at 0, 1, 3, 5, 11, and 20 weeks in carcinogen- and vehicle-treated animals. Tumor incidence and multiplicity were markedly inhibited (P < 0.05) with increasing CR. Fasting serum insulin-like growth factor I/somatomedin C levels exhibited a significant acute decline with CR at 1 and 3 weeks, but were comparable to AL-fed controls throughout the remainder of the 5-month study, despite continued differences in weight gain between AL and CR rats. Levels of insulin-like growth factor II/multiplication-stimulating activity exhibited no discernible pattern in relation to CR. Serum insulin levels showed age-dependent increases, but were affected by increasing CR at all time points. Insulin levels were significantly (P < 0.05) reduced in 40% CR rats from 3 weeks onward compared to controls, while 25% CR resulted in nonsignificant (P < 0.07) reductions throughout the study. No significant differences in growth factor levels were observed between 7,12-dimethylbenz(a)anthracene- and vehicle-treated rats. Circulating epidermal growth factor was not detectable in any treatment group regardless of the nature or duration of the dietary regimen, time of blood collection, or subsequent tumor-bearing status.

These data suggest that decreased serum insulin-like growth factor I/somatomedin C and insulin levels with CR and their complex interactions in vivo may play a role in the inhibition of mammary tumor promotion by CR.

INTRODUCTION

CR inhibits the development of a broad spectrum of neoplastic diseases in rodents (1, 2), including DMBA-induced (3) mammary carcinomas. Moreover, a strong relationship between caloric intake, body weight, and tumorigenesis at a number of plastic diseases in rodents (1,2), including DMBA-induced (3)

In the present study, we have examined alterations in the circulating levels of several peptide growth factors: insulin, IGF-I/Sm-C, IGF-II/MSA, and EGF. IGF-I is a growth hormone-dependent insulin-like polypeptide exerting a number of anabolic (12) and mitogenic effects (13) on numerous cell types in vivo and in vitro, including normal mammary epithelium (14) and a variety of human breast cancer cell lines (15, 16). Similarly, physiological levels of insulin (<5 µM) have been demonstrated to be mitogenic for numerous cell types (17) including human (18) and mouse (19) mammary carcinomas. The insulin dependence of a majority of carcinogen-induced rat mammary tumors has been demonstrated in vitro (20) and in vivo in diabetic animals (21–25).

The synthesis and circulating levels of IGF-I are strongly modulated by acute nutritional status (26–28), particularly protein and calorie nutrition, feed efficiency, and weight gain and are also reduced in diabetic rodents (29). Little is known regarding nutritional modulation of IGF-II/MSA.

The metabolic and mitogenic effects of EGF are well documented (30). Physiological levels of EGF have marked stimulatory effects on the growth of normal or transformed murine and human mammary tissues in vitro (31, 32). In vivo, EGF has been shown to affect the growth and functional differentiation of normal mammary glands (33, 34), and to play a critical role in spontaneous (35) and transplantable (36) murine mammary tumor latency and growth.

In this study, we have examined the effects of long-term CR on temporal alterations in circulating insulin, IGF-I/Sm-C, IGF-II/MSA, and EGF levels in rats and their correlation to tumor growth inhibited by CR. Alterations in growth factor binding properties to carcinogen-induced mammary tumors and normal tissues as a function of CR are the subject of the subsequent report (37).

MATERIALS AND METHODS

Hormones, Growth Factors, Antibodies. Human IGF-I and IGF-II were purified from Cohn fraction IV as previously described (38). Human recombinant IGF-I/Sm-C [Thr-59] was obtained from Amgen Biologicals (Thousand Oaks, CA). IGF-I and IGF-II were iodinated to specific activities of 200–320 µCi/µg using limited quantities of chloramine-T (39). Rabbit anti-IGF-I/Sm-C antisera was used in the IGF-I/Sm-C radio-
immunoassay has been previously described (38). Bovine liver membrane fractions for use in IGF-II radioreceptor assays were prepared as detailed by Cuatrecasas (40). Porcine insulin (receptor grade) was obtained from Sigma (St. Louis, MO). 125I-Inulin (porcine, receptor grade) was obtained from New England Nuclear (Boston, MA) at a specific activity of 2200 Ci/mmol. Murine EGF (receptor grade) and rabbit anti-EGF antiserum for radioimmunoassay were purchased from Collaborative Research, Inc. (Irvinton, MA). 125I-EGF (murine, receptor grade) was obtained from New England Nuclear at a specific activity of ~170 

Caloric restriction and circulating growth factors

Results

Effects of CR on host body weight and tumor incidence and multiplicity. The mean body weights of rats fed AL and 25% or 40% calorically restricted regimens throughout the 5-month study are depicted in Fig. 1. The rate of body weight gain of rats subject to increasing CR was reduced, with the terminal 20-22% and 32-37% reductions, respectively, from the AL-fed study are depicted in Fig. 1. The rate of body weight gain of rats subject to increasing CR was reduced, with the terminal 20-22% and 32-37% reductions, respectively, from the AL-fed

Processing of Serum Samples. Rat sera were processed chromatographically by a slight modification of the method of Pfeifle et al. (42) using reversed-phase C18 Sep-Pak cartridges (Waters Associates, Milford, MA) and an acetic acid/methanol mobile phase in order to remove IGF binding proteins which interfere with quantitative IGF analyses (44). Following a 5-day incubation at 4°C, antibody bound and free 125I-IGF-I were separated using a polyethylene glycol-1% human IgG precipitation method exactly as described (44). Pellets were counted in a Micromedic automated gamma-counter.

Levels of IGF-II/MSA in serum extracts were assessed using a radioreceptor assay employing bovine liver membranes in a buffer consisting of 0.05 M Tris-HCl, 0.25% bovine serum albumin, 0.02% sodium azide, pH 8.0. Briefly, diluted serum extracts in assay buffer were incubated with approximately 40,000 cpm of 125I-II and ~75 

μg of beef liver membrane preparation in a total assay volume of 0.5 ml. A standard curve of 0.01 to 10 nM pure IGF-II was used in quantitating unknowns and extracted pooled human serum used for controls. Nonspecific binding was assessed using either 250-fold molar excess of unla beled IGF-II, or more routinely, 100 nM of an IGF preparation of 4% purity containing three parts IGF-I to two parts IGF-II. Samples were incubated overnight at 4°C and receptor bound and free 125I-IGF-II were separated using polyethylene glycol-1% human IgG and counted as outlined above for IGF-I. The assay demonstrated a lower limit of sensitivity of approximately 7.6 pg radioreceptor IGF-II/ml and an EC50 value of 3.16 ± 0.44 (SD) ng/ml. Variations in control sera were ±10% in 10 assays and the coefficient of variation in sample and standard replicates ±6%.

Serum insulin levels were determined by a nonequilibrium single antibody radioimmunoassay using both rat and porcine insulin standards (0.125 to 6 ng/ml) at a final antibody dilution of 1:500,000.

Serum EGF levels were measured by the nonequilibrium double antibody radioimmunoassay exactly as detailed by Kurachi and Oka (45) at a final antibody dilution of 1:500,000. Antibody bound and free 125I-EGF were separated by the addition of 7% goat anti-rabbit serum as outlined (45). This assay measures rat EGF.

Data Processing and Statistics. Radioimmunoassays and radioreceptor binding data was processed using Micromedic Assay Computer software (Micromedic Systems, Inc., Horsham, PA). Statistical analyses were performed using NWA Statpac software (Northwest Analytical, Inc., Portland, OR). Tumor incidence was analyzed by the χ2 test on a contingency table. Tumors per tumor-bearing rat were compared using a one-way analysis of variance. Circulating levels of growth factors were compared using a completely randomized two-way analysis of variance in which time and dietary treatment were the two variables. Dunnett’s multiple-range test was used to make individual pairwise comparisons of the calorie restricted groups to the controls fed ad libitum when the analysis of variance resulted in a rejection of the null hypothesis.

Table 1 Semipurified diet composition (g/100 g)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Ad libitum</th>
<th>25% restricted</th>
<th>40% restricted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>48.0</td>
<td>30.7</td>
<td>34.7</td>
</tr>
<tr>
<td>Casein</td>
<td>21.6</td>
<td>28.8</td>
<td>26.5</td>
</tr>
<tr>
<td>α-Methionine</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Corn oil</td>
<td>15.0</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>10.1</td>
<td>13.5</td>
<td>10.1</td>
</tr>
<tr>
<td>AIN-76A vitamins</td>
<td>1.0</td>
<td>1.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Bernhart-Tomarelli minerals</td>
<td>3.8</td>
<td>5.1</td>
<td>6.2</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* The 25%-restricted diet provides identical nutrient intake as that of ad libitum, except for carbohydrate. The 25%- and 40%-restricted diets were pair fed at 75% and 60% that of the ad libitum-fed rats, respectively.

* The 40%-restricted diet provides identical micro- and similar macronutrient intake, since adjustment of all ingredients would have required reducing sucrose to 13%.

Results

Effects of CR on host body weight and tumor incidence and multiplicity. The mean body weights of rats fed AL and 25% or 40% calorically restricted regimens throughout the 5-month study are depicted in Fig. 1. The rate of body weight gain of rats subject to increasing CR was reduced, with the terminal body weights of 25% CR and 40% CR animals representing 20-22% and 32-37% reductions, respectively, from the AL-fed group. No significant difference was observed between terminal

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CALORIC RESTRICTION AND CIRCULATING GROWTH FACTORS

Fig. 1. Body weight of female Sprague-Dawley rat given DMBA (○, △, ■) or vehicle treatment (●, ▲, ▼) and fed ad libitum (□) and 25% (△) or 40% (■) calorie-restricted diets. Animals were 57 days of age at 0 weeks.

Table 2 DMBA-induced mammary tumors in ad libitum-fed and calorically restricted female Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Dietary regimen</th>
<th>Tumor incidence</th>
<th>% of LP tumors</th>
<th>% of SNP tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad libitum</td>
<td>18/20 90%</td>
<td>5.6 ± 1.7</td>
<td>87</td>
</tr>
<tr>
<td>25% Restriction</td>
<td>49/80 61%</td>
<td>3.4 ± 0.6</td>
<td>77</td>
</tr>
<tr>
<td>40% Restriction</td>
<td>2/10 20%</td>
<td>1.5 ± 0.5</td>
<td>33</td>
</tr>
</tbody>
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*LP, large, palpable tumors; SNP, small, nonpalpable tumors ≤100 mg.

body weights of DMBA- and vehicle-treated animals within a given dietary regimen (Fig. 1).

Table 2 summarizes the data on mammary tumor incidence and multiplicity in animals exposed to carcinogen. Tumor incidence and multiplicity were reduced significantly with increasing CR as demonstrated previously (1, 3, 41). Moreover, we have consistently observed in this and other tumorigenesis studies (3, 41) that with increasing CR the incidence of larger, palpable tumors is reduced, and an increasing proportion of the tumors developing in CR animals are extremely small, ≤100 mg in mass, and are not palpable through the skin, even at necropsy. Presumably these tumors are growth inhibited. We have examined the properties of these tumors with regard to growth factor binding in the subsequent report (37) and previously (41) with regard to tumor carbohydrate metabolism.

Circulating Growth Factor Levels in AL-fed and CR Rats. Shown in Fig. 2a are mean serum IGF-I/Sm-C levels in carcinogen and vehicle-treated AL, 25% CR and 40% CR rats throughout the 5-month study period. Fasting serum IGF-I/Sm-C levels showed a significant acute decline with increasing CR at 1 and 3 weeks; by 5 weeks on CR regimens and throughout the remainder of the 5-month study, IGF-I/Sm-C levels were comparable to those of control animals fed AL. Therefore, over a longer term, there appears to be either an adaptation of fasting IGF-I/Sm-C levels to chronic CR or attainment of constitutive levels, despite the observed differences in body weight gain between AL-fed and CR groups of rats (Fig. 1). No significant differences were found between vehicle- and carcinogen-treated animals within each group.

In contrast to IGF-I/Sm-C, levels of IGF-II/MSA (Fig. 2b) demonstrated wide variation and showed no significant pattern as a function of CR. To our knowledge, this is the first specific examination of IGF-II/MSA levels (rather than total IGFs and IGF-I/Sm-C levels) as a function of CR, and suggests little, if any, observable nutritional modulation of circulating IGF-II/MSA levels. No significant differences in IGF-II/MSA levels were observed between DMBA- and vehicle-treated rats.

There are significant effects of CR and the duration of feeding on fasting serum insulin levels (Fig. 2c). Serum insulin levels exhibited an age-dependent progressive increase, the magnitude of which was reduced, however, with increasing CR at all time points examined in both vehicle- and carcinogen-treated rats. Serum insulin levels were significantly reduced by 40% CR from 3 weeks onward compared to controls, while 25% CR resulted in reductions throughout the study, albeit nonsignifi-
Circulating EGF levels were below detection limits of the assay (<100 pg/ml) in these experimental animals regardless of nutritional status, duration on the dietary regimens, time of blood collection, or subsequent tumor-bearing status of the animals.

DISCUSSION

In this study, we have observed differential effects of chronic CR on the circulating levels of several peptide growth factors. Changes in serum levels of two of these growth factors (insulin and IGF-I/Sm-C) with CR are correlated with observed reductions in tumor incidence and multiplicity.

A number of reports have demonstrated that circulating IGF-I/Sm-C levels are correlated with acute alterations in dietary protein and energy intake or weight gain (26-28). The acute alterations in IGF-I/Sm-C levels reported here confirm previous reports demonstrating that a 24% reduction in calories produced significant reductions in serum IGF-I/Sm-C and total serum IGF activity over a 3-week period in female rats, with concomitant growth retardation. Serum IGF-I/Sm-C and total serum IGF activity demonstrated no correlation with serum GH levels. These findings suggested that “anabolic restriction” reduces circulating IGF levels, without consistently altering levels of GH, thyroid hormones, or corticosterone. It was concluded that, in rats, GH may serve a permissive rather than regulatory role on serum IGF-I/Sm-C homeostasis in situations of nutritional deficiency (28).

The studies reported here are the first to examine circulating IGF-I/Sm-C levels in rats as a function of long-term CR. The mechanism responsible for these effects is not known, but may result from increased GH secretion, or enhanced tissue sensitivity to GH as a result of up-regulation of GH receptors. It has been observed (46) that circulating IGF-I/Sm-C levels are not necessarily correlated with increased growth rate, as paracrine and autocrine production of IGF-I/Sm-C at multiple sites in vivo may be responsible for these growth-promoting effects (47). These findings are of potential significance in our tumorigenesis studies given the observed effects of IGF-I/Sm-C on mammary epithelial cells (14-16), and mammary tumor growth in mice (48).

IGF-II/MSA is considered a fetal or embryonic somatomedin (47). In this study we have shown that circulating levels of IGF-II/MSA appear to be minimally influenced by acute and chronic CR. Levels of IGF-II/MSA in adult rats have not been previously examined as a function of age or chronic CR. Based on our data, a role for IGF-II/MSA as a potential mediator of the effects of CR on tumor growth seems unlikely.

Serum insulin levels demonstrated a progressive increase with increasing age, particularly AL-fed rats, confirming the findings of others (49, 50). The magnitude of this progressive increase was reduced with increasing CR, in agreement with previous observations (49, 50) of the influence of 30-35% calorie restriction in ameliorating age-related increases in pancreatic islet size and volume, plasma insulin levels, and insulin resistance in female rats.

Consistent alterations in insulin levels with increasing CR are of interest in view of the insulin dependency of a majority of carcinogen-induced rat mammary carcinomas in vitro (20) and in vivo (21-25). Several reports have demonstrated that alloxan- (21) or streptozotocin-induced (23, 25) diabetes in rats caused a regression of 60% to 90% of DMBA-induced mammary tumors similar to that observed following oophorectomy and hypophysectomy (22). Tumor growth was restored and tumor latency reduced in these studies upon insulin administration to diabetic rats (21-25).

The data obtained for insulin and IGF-I/Sm-C are suggestive that IGF-I/Sm-C plays a role in the early phases of tumor promotion inhibited by CR, that interactions with insulin are likely, and that insulin may act independently during latter stages of tumor development inhibited by CR. Paradoxically, there were no consistent patterns between tumor-bearing (or subsequent tumor bearing) status and the serum levels of any of the growth factors examined within each dietary group (data not shown).

There is evidence for complex interactions between insulin and IGF-I/Sm-C homeostasis. Some reports (28, 29) suggest a permissive role of insulin in IGF-I/Sm-C growth regulation in vivo. Also, nanomolar levels of insulin promote IGF-I/Sm-C secretion in isolated hepatocytes, liver tissue explants, and perfused livers (17). In addition to these direct insulin-IGF-I/Sm-C interactions, insulin has been shown to play a role in the regulation of at least one low molecular weight IGF binding protein in vivo (51).

Prolactin levels are reduced by caloric or dietary restriction (10, 11) and prolactin has acute somatogenic effects in vivo, causing marked elevations in hepatic IGF-I/Sm-C mRNAs and serum IGF-I/Sm-C levels but less pronounced effects chronically (52). Thus, alterations in prolactin levels with CR may be of importance in mammary tumorigenesis both for their acute somatogenic activity and their mammotropic effects as well, although the latter would not be a likely influence on other tissues affected by CR.

The interactions of EGF and a variety of mammogenic hormones on the growth and differentiation of mammary tissue have been well documented (31, 32). The inability to measure circulating EGF levels in our experimental system is probably caused by the reported concentration of blood EGF in adult male rats (53) being below the limit of detection for the assay used. In view of the androgen dependence of EGF synthesis and secretion, plasma EGF levels are several-fold higher in male than in female mice (54, 55). In female mice, EGF levels increase markedly during pregnancy, lactation, and shortly thereafter, but are 5- to 13-fold lower in virgin mice (45).

In conclusion, we have observed marked reductions in mammary tumor incidence and multiplicity with increasing chronic CR. Moreover, circulating levels of the peptide growth factors examined were differentially affected by chronic CR, with acute reductions in IGF-I/Sm-C, both acute and long term reductions in serum insulin levels, and no discernible pattern of alterations in circulating IGF-II/MSA levels. Epidermal growth factor could not be detected in the serum of animals regardless of age, dietary state, or bleeding time. Alterations in circulating IGF-I/Sm-C and insulin, and their complex interactions in vivo, may contribute to the inhibition of tumor growth by CR. Specifically, such alterations in conjunction with previously observed influences of CR on circulating prolactin and estrogen levels or estrous cycles (10, 11) may explain the inhibitory effects of CR on mammary tumorigenesis per se.

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