Influence of calorie restriction on oncogene expression and DNA synthesis during liver regeneration

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ABSTRACT Controlling calorie intake (CCI) extends healthful life-span by a mechanism that may involve reduced rates of cell division without detriment to inducible cellular responses. To test whether inducible cellular proliferation is preserved by CCI and whether the mRNA expression levels of oncogenes activated by cell division can be reduced by CCI, we evaluated the effect of dietary energy on the hepatocellular proliferative burst and on oncogene and growth factor mRNA expression induced by partial hepatectomy. Eighty Fischer 344 rats were separated into two dietary groups and were fed semipurified diets for 10 weeks that differed only in calories by 40%. Mean hepatic levels of [3H]thymidine incorporation were greater among CCI animals at 18, 24, 28, and 36 hr after partial hepatectomy. The expression of c-fos and c-Ki-ras mRNAs, activated during hepatic regeneration, was reduced by CCI. Peak expression of c-fos among ad libitum fed controls to levels 4–6 times greater than prehepatectomy levels was not detected among CCI animals. Protracted elevated expression of c-Ki-ras among ad libitum fed animals was foreshortened by CCI. These findings demonstrate that inducible cellular proliferative responses are preserved by CCI and that the mRNA expression levels of c-fos and c-Ki-ras activated during cell division are reduced by controlling dietary energy. Preserved inducible cellular responses and lowered oncogene expression during cell division may be attributes of the healthful protective effect of CCI.

Cancer cells contain multiple genetic lesions, including mutations, translocations, and oncogene amplifications (1). All such procarcinogenic events require cell division, and increased cell division may increase cancer risk. Cell division allows for mitotic recombination, the fixation of mutations, and the activation of oncogenes (2–4). Increased cell proliferation may increase deteriorative aging, reduce adaptability to environmental stimuli (5), and accelerate the accumulation of genetic lesions and altered genetic expression, which leads to the development of a neoplastic phenotype.

Controlled calorie intake (CCI) to a level 20–40% less than ad libitum consumption extends the latency to onset and reduces the frequency of neoplastic and nonneoplastic disease, delays aging, reproductive senescence, and immunologic involution, and greatly extends the healthful life-span of rodents (5). CCI abrogates the development of malignant and autoimmune disease in short-lived inbred mouse strains and in some strains is accompanied by reduced proviral gene expression and oncogene activation (6–10).

The protective effect of CCI may be attributed in part to reduced rates of cellular proliferation. Cellular proliferation during peripubertal growth, manifested as peaks of hepatic, renal, cardiac, and epidermal DNA synthesis in ad libitum fed rats 21–100 days old, is reduced when feeding is restricted (11). Organ and body weights are reduced by restricted feeding, but organ to body weight ratios of restricted animals are similar to ad libitum fed controls (5, 11). Maintenance rates of epidermal, mammary, and intestinal epithelial proliferation in rodents greater than 100 days old may also be reduced by lowered dietary energy (12, 13), but others have found no such influence (11).

Reduced developmental or maintenance rates of cellular proliferation may prevent the early fixation of pathogenic molecular lesions and apparently occurs without detriment to essential, inducible cellular proliferation. Mice fed a CCI level have reduced cell numbers in spleen and lymph nodes, are leukopenic and lymphopenic, and have lower unstimulated natural killer cell activity than ad libitum fed controls (5, 14, 15). In spite of this, lectin-induced lymphocyte proliferation, poly(I-C) induced natural killer cell activity, production of and responsiveness to interleukin 2, and induced T-lymphocyte killing of tumor cells are all augmented by CCI (5, 14–16).

Liver regeneration after partial hepatectomy (PH) is characterized by coordinated waves of hepatocellular proliferation, which eventually restores the removed parenchyma (17). This compensatory proliferation is regulated by circulating mitogenic factors such as epidermal growth factor, transforming growth factor α (TGF-α), and hepatopoietin A and is accompanied by elevated expression of cell cycle–associated genes such as c-fos, c-myc, and c-Ha-ras (18–20). With restoration of the hepatic parenchyma, proliferative rates return to basal levels by an undefined mechanism that may involve expression of transforming growth factor β (TGF-β) (18, 19). Hepatocellular regeneration in response to PH represents the events associated with induced, regulated cell proliferation.

The influence of dietary energy on the PH-induced patterns and levels of hepatocellular oncogene and growth factor expression and on the hepatocellular proliferative burst has not been previously described. It has been shown that food deprivation immediately before or after PH results in delayed and reduced hepatocellular DNA synthesis during regenerative proliferation (21). In contrast, we show herein that by providing a constant source of dietary energy at levels 40% less than ad libitum intake, the levels of PH-induced hepatocellular DNA synthesis that occur during the principal wave of regenerative proliferation among CCI rats are equivalent to or even somewhat greater than that of ad libitum fed controls. We also show that hepatic expression of c-fos during regenerative proliferation is suppressed and that the expression of Ki-ras is foreshortened by CCI. These findings support the premise that the beneficial effects of CCI occur without detriment to essential, inducible cellular proliferation, such as during regeneration of removed hepatic paren...

Abbreviations: CCI, controlled calorie intake; PH, partial hepatectomy; TGF, transforming growth factor; DNA LI, DNA labeling index.

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Materials and methods

Animals. Eighty Fischer 344 male rats 4 weeks old (The Jackson Laboratory), maintained in accordance with the principles of the Animal Welfare Act and as described in U.S. Public Health Service/National Institutes of Health Publ. 86-23, were separated into two experimental groups. Rats of group A (full-fed) were housed in pairs and fed a semipurified diet ad libitum at 60 kcal/day (1 cal = 4.184 J), in which calories were derived principally from carbohydrates. Rats of group B (CCI) were housed singly, were fed a proportionally similar complete semipurified diet, but were restricted 40% in calories consumed (36 kcal/day). The lighting schedule for all animals was 12 hr on:12 hr off.

Semipurified Diets. The preparation and composition of the two semipurified diets used have been described in detail (6, 7) (Table 1). All dietary constituents were obtained from ICN. Both diets were low in dietary fat (~6% of total calories) but differed by 40% in the level of total caloric energy available; the diets were otherwise comparable. The amounts of essential dietary constituents added to each diet were determined with regard to the gram and caloric consumption of rats fed ad libitum. As shown in Table 1, the diet prepared for CCI rats (group B) was further enriched so that although the CCI diet contained 40% fewer calories, equivalent amounts of vitamins, minerals, essential fatty acids, and 30% of calories as protein were consumed by all rats. Approximately 60% of total calories in both diets were derived from carbohydrates. All rats were fed twice weekly and weighed weekly.

PH. PH was performed by removal of the median and left lateral hepatic lobes comprising two-thirds of the parenchyma as described by Higgins and Anderson (17). All operations were done during the light phase of the 24-hr cycle, between 0900 and 1200 hr.

Purification and Analysis of RNA. Total RNA was isolated from regenerating liver by an acid guanidinium isothiocyanate method (22), with tissues mechanically disrupted in a tissue homogenizer. RNA purity and quantity were ascertained from scanning spectrophotometric analysis.

RNA was denatured in a loading premixture containing 8.0% formaldehyde, 60% deionized formamide, 0.12% ethidium bromide, and 1.2× Mops buffer (1× Mops buffer is 20 mM 4-morpholinepropanesulfonic acid/50 mM sodium acetate/1 mM EDTA). Samples were heated for 15 min at 55°C and chilled on ice prior to electrophoresis in formaldehyde-denaturing gels (2.2% formaldehyde/1% agarose/1× Mops buffer). RNA sizes were estimated by semilogarithmic graph comparison with RNA bands and with an RNA ladder on the same gel (GIBCO/BRL). RNA was transferred from gels to Hybond-N nylon hybridization membranes (Amer sham) with a vacuum blotting apparatus (1996 Vacugene, Pharmacia LKB) and then was bound to the membranes by exposure to UV light (UV Stratalinker 1800, Stratagene).

Prehybridization and hybridization were at 42°C in buffer containing 5× SSC (1× SSC is 0.15 M NaCl/15 mM trisodium citrate), 50% formamide (deionized), denatured salmon sperm DNA (60 μg/ml), yeast tRNA (37 μg/ml), 0.4% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate (pH 7; prepared from 1 M stocks of mono- and dibasic sodium phosphate), 0.1% sodium pyrophosphate, and 2× Denhardt’s solution (1× is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin).

Probes were radiolabeled with [α-32P]dCTP by use of a random-priming kit (Boehringer Mannheim). Each 11× 14 cm filter was incubated with 107 cpm of radiolabeled probe in 10 ml of hybridization buffer in a small (14 cm) hybridization chamber tube (Robbins Scientific, Sunnyvale, CA). Probes for oncogenes c-fos (23), c-myc (24), c-Ha-ras (25), c-Ki-ras (26), c-sis (27), c-erbA (28), and c-erbB (29) were obtained from Oncor, and TGf-α (30), TGf-B (31), and c-jun (32) were from the American Type Culture Collection.

After hybridization, filters were washed at 55°C three times for 20 min each in 2× SSC/0.1% SDS and then once for 20 min in 0.1× SSC/0.1% SDS. Filters were sealed in plastic bags and then exposed to Kodak X-Omat RP x-ray film at −80°C with spectroline L Plus intensifying screens (Kodak; Sigma). The density of transcript bands was semiquantified by laser densitometry (Ultrascan XL, Pharmacia LKB).

Analysis of DNA Synthesis. Rats that had undergone PH were given 15 μCi (1 Ci = 37 GBq) of [3H]thymidine per g of body weight intraperitoneally 1 hr prior to euthanasia. Two to four animals each of group A or B were euthanized 12, 18, 24, and 28 hr after PH. Hepatic DNA was extracted by proteinase K digestion followed by phenol/chloroform extraction. DNA quantity was ascertained from scanning spectrophotometric analysis. Radiolabeled DNA was quantified by scintillation spectroscopy of 100-μg samples of total hepatic DNA.

Samples of liver, spleen, thymus, duodenum, colon, and urinary bladder were fixed in 10% buffered formalin, dehydrated, embedded in paraffin, and serially sectioned at 3- to 5-μm thickness. Each section was deparaffinized, coated with NTB-2 nuclear track emulsion (Kodak), and stored for 10 days in lightproof boxes at 4°C. Slides were developed with D-19 developer (Kodak) and processed in Kodak acid fixer. Autoradiographs were stained with hematoxylin/eosin. The DNA labeling index (DNA LI) of four sections per organ per animal was determined by counting the number of labeled nuclei and the total number of cells and expressed as a percentage of labeled nuclei per 100 cells.

Experimental Plan. Animals were fed semipurified diets for 10 weeks. Two to four rats each of group A or B were euthanized 15, 30, and 45 min, 1, 3, 8, and 24 hr, and 2, 5, 7, 14, 21, and 28 days after PH for analyses of oncogene and growth factor mRNA expression patterns and levels as influenced by dietary energy. Rats were also euthanized at 12, 18, 24, 28, and 36 hr after PH as described above for assessment of the patterns of DNA synthesis, as influenced by dietary energy.

Results

Physical Parameters. Male Fischer 344 rats were fed semipurified diets ad libitum (group A) or restricted 40% in calories (group B, CCI) for 10 weeks. When PH was per-

Table 1. Composition of diets

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Ad libitum</th>
<th>40% CCI</th>
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<tr>
<td></td>
<td>g</td>
<td>kcal</td>
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<tr>
<td>Sucrose</td>
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<td>Methionine</td>
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<td>Saltflower oil</td>
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<td>18.0</td>
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<tr>
<td>AIN vitamin mix</td>
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<td>3.95</td>
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<td>AIN vitamin mix</td>
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<td>Inositol</td>
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</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
<td>397.6</td>
</tr>
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Energy, kcal/g 3.98 3.96
Protein/tot., kcal 0.302 0.302
Carbohydrate/tot., kcal 0.636 0.595
Fat/tot., kcal 0.045 0.075
formed at 15 weeks of age, the mean body weight of group A rats (272 g) was 27% greater than that of age-matched CCI group B rats (199 g). Liver to body weight ratios for each dietary group were equal, with mean whole liver weights (group A, 10.4 g; group B, 7.5 g) comprising 3.8% of total body weight. Within 48 hr postoperatively, 50% by weight of hepatic parenchymal regeneration had occurred in both dietary groups, and full regeneration of hepatic weight was accomplished by the second postoperative week.

Expression of Oncogene and Growth Factor mRNA. Patterns and levels of hepatic mRNA expression of c-fos, c-myc, c-Ha-ras, c-Ki-ras, c-sis, c-erbB, c-erbR, c-jun, TGF-α, and TGF-β as influenced by dietary calorie level during regenerative proliferation were determined by densitometric scanning of Northern blots. The hepatic mRNA expression of c-fos, c-myc, c-Ha-ras, and c-Ki-ras was activated by PH and their patterns and levels of expression are shown in Figs. 1 and 2. Hepatic expression of TGF-α, TGF-β, c-jun, c-erbB, c-erbA, and c-sis was weak to undetectable and was not substantially different in either dietary group. As shown in Figs. 1 and 2, c-myc and c-Ha-ras mRNAs were not substantially influenced by dietary energy level and were expressed in similar pattern and magnitude in both dietary groups.

In contrast, hepatic expression of c-Ki-ras mRNA activated following PH was influenced by dietary energy level. Hepatic expression of c-Ki-ras mRNA after PH among group A ad libitum fed rats remained elevated >3-fold greater than basal prehepatectomy levels through the 168th postoperative hour (Fig. 1). However, hepatic expression of c-Ki-ras mRNA following PH among CCI group B rats, though detected earlier, remained elevated above basal levels only transiently (48 hr) (Fig. 2).

Fig. 3 shows Northern blots of hepatic RNA extracted from paired samples of surgically removed liver and regenerating liver of individual group A or B rats, probed for c-fos expression. Basal c-fos mRNA expression in liver surgically removed at the time of two-thirds hepatectomy is compared to the c-fos mRNA expression level in the regenerating liver of the same animal 15, 30, 45, and 60 min after PH. As depicted in Figs. 1–3, hepatic expression of c-fos mRNA, though activated subsequent to PH, is lowered by controlling calorie intake. Peak hepatic expression of c-fos among group A animals occurred approximately 45 min postoperatively and was 6.5 times greater than basal prehepatectomy c-fos expression. However, this peak of hepatic c-fos mRNA expression was stringently suppressed by controlling calorie intake and was not detected at any interval of assessment.

Regenerative DNA Synthesis. The patterns and levels of DNA synthesis during hepatic regeneration were determined morphologically by counting the percentage of labeled nuclei on autoradiographs and biochemically by measuring the levels of [3H]thymidine incorporated in 100-μg samples of hepatic DNA of group A or B rats. The levels of DNA synthesis during hepatic regeneration, as determined by biochemical analysis, are shown in Fig. 4. During the principal wave of regenerative hepatic proliferation, 18–36 hr postoperatively, mean levels of hepatic [3H]thymidine incorporation were greater among CCI group B rats at every interval of assessment. The morphologic pattern of active DNA synthesis induced during regenerative proliferation followed the metabolic zones of the hepatic acinus. Earliest hepatocellular DNA synthesis occurred in perportal hepatocytes (zone 1 of the hepatic acinus) 12–18 hr after PH. Only at 12 hr post-PH was the DNA LI of group A rats (0.5% ± 0.8%) similar to that of group B rats (0.6% ± 1.1%). The mean morphologic DNA LI of CCI group B rats was greater than that of ad libitum group A rats at every subsequent interval of assessment. DNA LIs at 18 hr post-PH were 2.8% ± 1.4% versus 4.2% ± 1.9%, at 24 hr post-PH were 49.6% ± 10.9%

Fig. 1. Mean hepatic c-fos, c-myc, c-Ha-ras, and c-Ki-ras transcription of ad libitum fed group A rats following PH. Ordinate, arbitrary units of oncogene mRNA expression determined by quantifying bands of oncogene transcripts with a laser densitometer. Note high peak of c-fos expression and prolonged elevation of c-Ki-ras expression.

Fig. 2. Mean hepatic c-fos, c-myc, c-Ha-ras, and c-Ki-ras transcription of CCI group B rats following PH. Ordinate, same as in Fig. 1. Note absence of major c-fos expression peak and abbreviated c-Ki-ras expression.

Fig. 3. Northern blot analysis of hepatic RNA from individual rats, hybridized to 32P-labeled probe for c-fos expression. The arrow indicates the band of c-fos transcripts at 2.3 kilobases. The basal c-fos mRNA expression prior (P) to regenerative proliferation of liver surgically removed at PH is compared to levels in the liver of the same rat during hepatic regeneration (R). 15, 30, 45, and 60 min after PH. (A) Ad libitum fed rats. (B) CCI rats.
versus 67.1% ± 9.1%, at 28 hr post-PH were 29.5% ± 6.3% versus 39.5% ± 8.1%, and at 36 hr post-PH were 14.3% ± 3.9% versus 17.4% ± 4.9% for group A and group B rats, respectively. The photomicrographs in Fig. 5 show the greater extent and distribution of hepatic DNA synthesis that occurred among group B compared to group A rats 24 hr post-PH.

Autoradiographs of liver, spleen, urinary bladder, thymus, and small and large intestine from each dietary group were evaluated 12, 18, 24, 28, and 36 hr post-PH. Mean DNA LI for spleen (12.1%), urinary bladder (0.1%), thymus (1.8%), small intestine (16.5%), and large intestine (13.7%) following PH were not significantly influenced by dietary energy level.

**DISCUSSION**

CCI may impart its healthful, beneficial effects by influencing cellular proliferation. Reduced developmental (11) or maintenance (12, 13) rates of cell replication might reduce the risk of early genetic lesion fixation, delay deteriorative aging, and perhaps increase adaptability to environmental stimuli by maintaining adequate or even greater inducible cellular responses (5). This premise is supported by the findings that, although cell replication during growth, and perhaps even maintenance proliferative rates, are somewhat reduced by controlling calorie intake (11–13), induced immunologic functions of CCI animals are equal to or even greater than those of ad libitum fed controls (5, 15, 16).

The regulated regenerative proliferation of hepatocytes in response to PH represents a coordinated, inducible increase in cell replication. To test whether the inducible responses of animals fed a CCI level are equal to or perhaps even greater than full-fed controls, we determined the influence of dietary energy level on the hepatocellular proliferative burst and on the expression of oncogenes and growth factors following PH.

We have previously described the induced patterns and levels of oncogene expression in response to PH and have shown that of the genes assessed, primarily c-myc, c-fos, and c-Ha-ras expression is elevated during regenerative proliferation (20). In the current study, expression of c-myc, c-fos, c-Ha-ras, and c-Ki-ras was elevated during hepatic regeneration following PH. The patterns and levels of c-fos and c-Ki-ras expression were further influenced by the level of
dietary energy. The 4- to 6-fold increase in c-fos expression that occurred as a peak within the first postoperative hour in ad libitum fed group A rats was not detected among CCI group B rats during any interval of assessment up to 672 hr after PH. The protracted expression of c-Ki-ras among ad libitum fed group A rats from the 8th through the 16th postoperative hour was substantially abbreviated among CCI group B rats, with elevated c-Ki-ras expression occurring only from the 3rd through the 48th postoperative hour.

The mechanism by which CCI delays aging, prevents disease, and extends longevity is not fully understood. Animals maintained on a CCI level can have enhanced induced immunologic capabilities, increased enzyme-mediated repair of DNA, increased free-radical scavenging, and lowered rates of cellular replication (5, 11–13, 33, 34). Whereas fasting delays and reduces DNA synthesis during PH-induced hepatocellular regeneration (21), animals fed a CCI level respond with an equivalent or greater hepatocellular proliferative burst than ad libitum fed controls. Although a comparable profile of oncogenes is activated during hepatic regenerative proliferation regardless of dietary energy level, mRNA expression levels of c-fos and c-Ki-ras are reduced by controlling calories.

Taken together, the reduced cellular replication during growth and maintenance that accompanies CCI shown previously (11–13), and the protected or perhaps even augmented induced cellular proliferation and suppressed levels of oncogene mRNA expression during hepatocellular regeneration that accompanies CCI shown here, suggest that influences on cellular proliferation may, in part, explain the healthful contributions of controlling dietary calories.

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