Effects of Dietary Protein on the Induction of DNA Synthesis and Expression of Growth-Related Genes in Liver and Kidney of Growing Rats

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Summary To investigate molecular mechanisms of growth control by protein nutrition, we examined whether nutritive quality of protein affects the induction of DNA synthesis in liver and kidney of growing rats in relation to expression of growth-related genes such as c-myc, c-fos, c-Ha-ras, and ornithine decarboxylase (ODC). Rats were adapted to 2-h meal feeding schedule at first with laboratory chow for 10 days and then with a protein-free diet for 3 days prior to experiments. When protein-free diet was fed to the rats, the levels of c-myc, ODC and c-Ha-ras mRNAs increased in the liver within 2 days. However, substantial changes in the levels of those mRNAs were not observed in the kidney. The level of c-fos mRNA in these tissues was too low to detect by our method. Feeding of casein diet to rats that had been maintained on protein-free diet for 3 days caused a rapid decrease in the level of c-myc mRNA and induced DNA synthesis in the liver. On the other hand, zein diet, which lacks tryptophan and lysine, did not lower the c-myc mRNA level nor induced DNA synthesis in the liver. However, if zein diet was supplemented with tryptophan and lysine, a decrease in c-myc mRNA level and an induction of DNA synthesis were observed. The levels of ODC and c-Ha-ras mRNAs were not changed by feeding of casein or zein diet. Neither casein nor zein induced DNA synthesis and changed the levels of the mRNA in the kidney. The amount of food intake during the 2-h feeding period was not different among the diets. These results suggest that the liver cells are arrested in G1 phase during the feeding of protein-free diet and good quality of protein is required to progress the cell cycle to enter S phase.

Key Words proteins quality, c-myc, growth, liver, kidney, dietary proteins, protein-free diet, DNA synthesis, rats

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It is well-known that the quality of protein nutrition greatly affects the growth of young animals (1-3). The nutritive quality of protein is usually determined by measuring the weight gain or the nitrogen balance of animals after feeding of test proteins. On the other hand, recent knowledge of molecular biology indicates that cell growth and proliferation require growth signals and their transduction. The aim of our study is to determine whether protein nutrition affects signal transduction for cell growth in the tissues of growing animals depending on its nutritive quality. For this purpose, we examined changes in the expression of several growth related genes, such as c-myc, c-fos, c-Ha-ras and ornithine decarboxylase (ODC) in the liver, in comparison with those in the kidney after feeding of casein or zein (corn protein lacking tryptophan and lysine) diet, because the DNA synthesis in the liver can be induced by dietary manipulation (4-7) and the products of those genes are thought to be involved in the cascade of signal transduction (8-10). Furthermore, c-myc, c-fos and ODC are known to be expressed transiently in regenerating rat liver after partial hepatectomy (4,11-15) or in renal hypertrophy after unilateral nephrectomy (16,17). In the present study, we demonstrated that the expression of c-myc mRNA and DNA synthesis in the liver can be modulated by the nutritive quality of dietary protein and discussed the possibility that protein nutrition not only supplies material for body components but also plays a role as a signal for the progression of the liver cell cycle in young growing rats.

EXPERIMENTAL

Materials. [α-32P] Deoxycytidine-5' triphosphate (3,000 Ci/mmol) and [methyl-3H] thymidine (2.0 Ci/mmol) were purchased from ICN Biomedicals Inc. (U.S.A.). The Multiprime DNA-labeling system was purchased from Amersham Corp. Casein and dextrin were obtained from Ishizu Pure Chemicals (Osaka). Laboratory chow, mineral mixture, vitamin mixture and oil (cod oil: soybean oil, 1:4) were from Oriental Yeast Co. (Tokyo), and zein was from Nacalai Tesque (Kyoto). Oligo (dT)-cellulose column (Type 3) was the product of Collaborative Research (Waltham, MA). Human c-myc (0.467 kb), human c-fos (0.484 kb) and Harvey murine sarcoma virus v-Ha-ras (0.56 kb) cDNAs were purchased from Takara Shuzo. A 0.7-kb PstI fragment of rat liver ornithine decarboxylase cDNA (18), a 2.0-kb HindIII fragment of β-actin cDNA (19) and a 1.2-kb HindIII fragment of rat albumin cDNA (20) were prepared. 3,5-Diaminobenzoic acid dihydrochloride and other chemicals were purchased from Nacalai Tesque.

Animals. Male Sprague-Dawley rats (4 weeks old, 94 ± 1 g, n = 101) were obtained from CLEA Japan Inc. (Tokyo) and treated following the “Guideline for Studies with Laboratory Animals, The Jikei University School of Medicine.” Each group of three or four rats was housed in a cage and maintained in an air-conditioned windowless room lighted from 06:00 to 18:00 h. The temperature was kept at 24 ± 1°C and the relative humidity at 60 ± 8%. They were given laboratory chow for 2 h each day (18:00-20:00). Water was available ad libitum. The body
weight of each rat and food consumption in each cage were recorded daily over the course of experiments.

RNA extraction and hybridization. Rats were killed by decapitation at the indicated times. The livers and kidneys were excised, rinsed in cold phosphate-buffered saline, blotted on a paper towel and were frozen in liquid nitrogen. The frozen tissues were kept at −80°C until use. Total RNA was extracted from frozen liver or kidney pooled from 3 animals by the acid phenol guanidinium thiocyanate procedure (21), and poly (A) RNA was isolated by oligodeoxynthymidilate cellulose affinity chromatography (22). Samples (5 μg each) were separated on 1% agarose/formaldehyde gels and blotted onto nitrocellulose membranes. Hybridization analysis was conducted as described previously (23). As hybridization probes, the cDNAs were 32P-labeled by the multiprime DNA labeling system according to the protocol provided from Amersham Corp. Each band hybridizing with the probes was detected and quantitated using Fujix Imaging Analyzer Bas 2000 (Fuji Photo Film Co., Tokyo).

Determination of the rate of DNA synthesis. Each rat was injected with 30 μCi of [3H]thymidine intraperitoneally and killed 1 h later. DNA synthesis in the liver and kidney was measured by the incorporation of [3H]thymidine into nuclear DNA (24).

Test diets. Protein-free diet contained the following ingredients (g/kg): dextrin 910, mineral mixture 40, vitamin mixture 10, oil 20, and cellulose powder 20. A 60% casein and a 60% zein diet was prepared by reducing an equivalent amount of dextrin, respectively. Supplementary diet contained the following ingredient (g/kg): zein 250, tryptophan 4, lysine HCl 23 and other components were the same as protein-free diet (25). All diets were mixed with tap water (430 ml/kg diet) to form a paste.

Statistical analysis. For evaluation of experimental results, data on DNA synthesis were analyzed by one-way ANOVA followed by Scheffe’s test (26). Differences were considered significant at p < 0.05. Values in the text are mean±SE.

RESULTS

We employed a 2 h restricted feeding schedule to observe rapid changes in the expression of growth related genes after feeding of test proteins. Figure 1 shows typical results on changes in body weight and food intake during the adaptation to the regimen. They lost body weight during the first 3 or 4 days because of the restricted feeding time. They then adapted to the regimen and gained body weight at a rate of 2 g per day. On day 13, rats were given a protein-free diet in the same time schedule. Although the amount of food intake did not change, the increase in their body weight immediately ceased by feeding of the protein-free diet. By using this regimen, we first examined the effect of protein-free diet on the expression of growth related genes in the tissues of growing rats. Figure 2 shows that the levels
Fig. 1. Changes in body weight and diet intake during restricted feeding. Sixteen male rats (4 weeks old) were housed in 5 cages (3–4 rats per cage) in an air-conditioned windowless room lighted from 06:00 to 18:00 h and were fed laboratory chow for 2 h each day (from 18:00 to 20:00 h). After 13 days, diet was changed to a protein-free diet and they were fed on the same time schedule for 3 days as indicated by the horizontal bar. Body weight was recorded daily except on days 4 and 5, and the results are expressed as mean ± SE (n=16). Food consumption in each of 5 cages was recorded and average food intake of the rats in each cage was calculated. The results are expressed as mean ± SE (n=5). Error bar not shown was in the symbol.

of c-myc, ODC and c-Ha-ras mRNAs in the liver changed upon feeding of the protein-free diet. A quantitative analysis by using an imaging analyzer indicated that the c-myc mRNA level increased 4–5 fold in 2 days after the dietary change and kept the same level during feeding of the diet. The levels of ODC and c-Ha-ras mRNAs increased 8- and 5-fold, respectively, on day 3 (Fig. 2, left panel). It has been reported that the level of albumin mRNA decreased gradually when rats were fed protein-deficient diet for 10 days (27,28). Our results also indicated a decrease in the level of albumin mRNA in the liver of rats fed protein-free diet, though at least 80% of the albumin mRNA remained after 4 days (Fig. 2, left panel). Although the level of c-myc mRNA in the kidney increased slightly, the other mRNA levels examined did not change during feeding of the protein-free diet (Fig. 2, right panel). The levels of c-fos mRNA in both tissues were too low to detect by our method (data not shown). We also examined the levels of c-myc mRNA in muscle and brain and observed that the level of the mRNA did not change in these tissues by feeding of the protein-free diet (data not shown). Therefore, the expression of the growth-related genes in the liver was specifically affected by
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Fig. 2. Changes in the expression of growth-related genes in liver and kidney of rats after feeding of protein-free diet. Fifteen rats were divided into 5 groups (3 rats per cage) and maintained as described in EXPERIMENTAL. One group of 3 rats were fed laboratory chow for 10 days. The other 4 groups were first given laboratory chow, and then the diet was changed to the protein-free diet on days 7, 8, 9 and 10, respectively. All rats were killed in the evening (16:00-18:00 h) of day 11. Poly(A) RNA was prepared from pooled livers and kidneys from 3 rats of each group, and Northern blot analysis was performed with 32P-labeled albumin (liver), β-actin (kidney), c-myc, ODC and c-Ha-ras cDNAs, respectively. Bands of each mRNAs were visualized with an imaging analyzer.

protein nutrition.

It has been reported that mitotic activity (29) and DNA synthesis (5–7) could be induced by feeding a casein diet if animals had been maintained on low-protein or protein-free diets prior to the feeding of protein. Then, we examined whether quality of protein affected the induction of DNA synthesis and expression of growth related genes in liver and kidney of growing rats that had been adapted to a 2-h feeding schedule and then fed protein-free diet for 3 days in the same manner. When 60% casein diet was fed to these animals on day 3, the hepatic level of c-myc mRNA, which had increased 4–5 fold during the feeding period of protein-free diet (Fig. 2), decreased rapidly and returned to the original level by 8 h after the feeding (Fig. 3a and b, left panel). A significant increase in the rate of DNA synthesis was observed at 21 h after feeding. In contrast, zein diet neither decreased the level of c-myc mRNA nor induced DNA synthesis. The levels of other mRNAs in the liver did not change upon feeding of either diet (Fig. 3a, left panel). Food intake during the 2-h feeding period was not different between the rats fed either casein or zein. These results clearly indicated that the quality of protein consumed affected the expression of c-myc genes and DNA synthesis in the liver of growing rats. On the other hand, the expression of growth related genes did not differ in the kidney of the
Fig. 3
rats fed either casein or zein and DNA synthesis could not be induced by either diet (Fig. 3a and b, right panel).

In is known that the nutritive quality of dietary protein improves if deficient amino acids are supplemented to the diet. Therefore, we next examined whether zein diet supplemented with tryptophan and lysine could affect the expression of c-myc mRNA and induction of DNA synthesis in the liver. Figure 4 shows changes in the levels of albumin, c-myc, c-Ha-ras and ODC mRNAs, and the rate of DNA synthesis in the liver of growing rat after feeding of zein diet supplemented with tryptophan and lysine. The results demonstrated that the diet could decrease the level of c-myc mRNA and induce DNA synthesis in the liver (Fig. 4a and b), while the levels of albumin, c-Ha-ras and ODC mRNAs were not changed (Fig. 4a).

**DISCUSSION**

In the present study, we employed a 2-h restricted feeding schedule. Although the growth of rats was slower than that of rats maintained on ad libitum feeding, their body weight increased linearly at a rate of 2 g per day after adaptation (Fig. 3).
Fig. 4. Changes in the expression of growth-related genes and the induction of DNA synthesis in liver after feeding zein diet supplemented with tryptophan and lysine. Twenty-nine rats were fed laboratory chow for 10 days as described. Rats were given protein-free diet for 3 days, and then fed 24% zein diet supplemented with tryptophan and lysine. (a) Groups of 3 rats were killed at the indicated time points. As a 0 time control, 3 rats were killed before feeding test diet. Poly(A) RNA was prepared from pooled livers of 3 rats and Northern blot analysis was performed as described. (b) The rate of DNA synthesis at each time point was determined as described under Fig. 3(b). Results were expressed as mean±SE (n=3) except for the result at time point 27h, which was the mean of 2 rats. Statistical analysis of the DNA synthesis data among the time points was performed as described in EXPERIMENTAL. a, b: mean values of each symbol not sharing a common letter are significantly different (p<0.05). Error bar not shown was in the symbol. The relative change in the expression of c-myc mRNA was determined as described under Fig. 3(b).

1). Food intake during the 2-h feeding period was relatively large and was not different among the given diets (12±1g, n=16). By using this regimen, we demonstrated in the present paper that the expression of c-myc mRNA and DNA synthesis...
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synthesis in the liver of growing rats changed depending on the nutritive quality of dietary protein.

When rats were fed a protein-free diet, the levels of c-myc, ODC and c-Ha-ras mRNAs increased in the liver but not in the kidney, except for a slight increase in the level of c-myc mRNA (Fig. 2). The c-myc mRNA level in muscle and brain were low and not changed by feeding of the protein-free diet (data not shown). Thus, the liver is specifically affected by diets in the expression of these genes. Although hepatocytes of adult liver are normally quiescent, the hepatocytes of growing rats still proliferate in proportion to body growth (30). In fact, we observed that the mitotic activity of hepatocytes in growing rats maintained on casein diet. However, the activity decreased greatly when the casein diet was changed to the protein-free diet (unpublished observation). These observations indicated that nutrition altered the cell cycle of proliferating hepatocytes and might change the expression of growth related genes specifically in the liver of growing rats.

We observed that DNA synthesis in the liver of rats maintained on the protein-free diet was induced by feeding a casein diet but not by feeding a zein diet, while neither casein nor zein induced DNA synthesis in the kidney (Fig. 3b). Interestingly, c-myc mRNA decreased rapidly in the liver after feeding of the casein diet, whereas it remained at a high level in those fed the zein diet. In contrast, there was no significant difference between the levels of c-myc mRNA in the kidney of the rats fed either casein or zein diet. These results indicated that the expression of c-myc and DNA synthesis in the liver were regulated, depending on the nutritional quality of protein. This was further confirmed by feeding the rats a zein diet supplemented with deficient amino acids, tryptophan and lysine (Fig. 4). It is well known that c-myc expresses in G1 phase specifically when quiescent cells are stimulated to proliferate (31). Therefore, the accumulation of c-myc mRNA in the liver during feeding of protein-free diets may indicate that the cells are arrested at G1 phase and that good quality protein is required for the progression of cell cycle from G1 to S phase in the liver. The decrease in the level of c-myc mRNA in the liver may also reflect the progression of the cell cycle.

It has been reported that expression of ODC and c-Ha-ras mRNAs is induced in late G1 phase in response to growth stimuli (32,33). Although the levels of ODC and c-Ha-ras mRNAs in the liver increased during the feeding of protein-free diets, neither casein nor zein lowered the levels of the two mRNAs. We observed in cultured hepatocytes that the half-life of c-myc mRNA changes rapidly in response to medium amino acids (unpublished observation). Thus, c-myc mRNA accumulates in cultured hepatocytes with a half-life of about 100 min when amino acids are removed from the medium, and then the replenishment of amino acids to the medium causes a rapid decrease in the level of c-myc mRNA with a half-life of about 20 min. This observation suggests that the accumulation of c-myc mRNA in the liver during feeding of protein-free diet is caused by post-transcriptional regulation and that protein nutrition may affect the stability of c-myc mRNA. On
the other hand, the half-lives of ODC and c-Ha-ras mRNAs in cultured hepatocytes were not affected by medium amino acids, and the half-lives of the two mRNAs, 280 min and 630 min, respectively, are much longer than that of c-myc mRNA. Therefore, it can be speculated that transcriptional stimulation is involved in the increases in ODC and c-Ha-ras mRNA levels by feeding of protein-free diet, and that the increased levels of the two mRNAs will not decrease immediately, even if transcription of the two genes ceases by feeding a protein diet, because of their long half-lives.

The inability of zein diet to induce DNA synthesis may not be due to the lack of precursor amino acids required to synthesize factors necessary for the progression since we observed that ODC activity in the liver, which increased transiently at boundary of G1 to S phase of cell cycle (34), was induced rapidly after feeding of casein diet but not zein diet. However, tyrosine aminotransferase, one of the liver-specific enzymes, was induced by feeding either casein or zein diet (25). Interestingly, ODC mRNA did not increase after feeding of casein diet. In a previous paper from our laboratory, it was reported that casein but not zein increased the polysomal-associated ODC mRNA activity (35). Thus, the current results clearly indicate that the quality of protein nutrition regulates the induction of ODC, at least in part, at the level of translation (18), probably by controlling recruitment of ODC mRNA from the untranslatable pool (36).

Furthermore, it has been reported that amino acids were required for plasma to promote the entry of platelet-derived growth factor-treated competent Balb/c-3T3 cells into S phase, and that this requirement was abolished by the infection of the cells with simian virus 40 (37). Taken together with the above observations, we emphasize the possibility that the quality of protein nutrition may play a role as a signal for the progression of the liver cell cycle in young growing rat.

Although the level of c-myc mRNA in the kidney increased 2-fold during feeding of protein-free diet (Fig. 2), the level of c-myc mRNA and DNA synthesis did not change in response to dietary protein (Fig. 3). It has been reported that feeding a casein diet to young rats which had been maintained on a protein-free diet induced renal hypertrophy rather than hyperplasia (38). Thus, the difference in the expression of growth-related genes in response to the nutrition observed between liver and kidney suggests the involvement of different mechanisms in the control of tissue growth by nutrition.

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