DIETARY RESTRICTION MAINTAINS THE BASAL RATE OF SOMATOTROPE RENEWAL IN LATER LIFE IN MALE RATS

Isao Shimokawa, Masato Tomita, Yoshikazu Higami,
Tomoaki Okimoto, Toshio Kawahara and
Takayoshi Ikeda

Department of Pathology
Nagasaki University School of Medicine
1-12-4 Sakamoto
Nagasaki City 852, Japan

ABSTRACT

We investigated the impact of dietary restriction on the basal rate of somatotrope renewal in the pituitary gland. Bromodeoxyuridine (BrdU), a thymidine analog, was administered continuously for 1 week in male F344 rats at 3, 8 and 20 months of age (mo), fed ad libitum (AL) or diet restricted from 1.5 mo (DR). Combined immunostainings for BrdU and GH visualized newly formed somatotropes as well as pituitary cells in tissue sections. The rate of incorporation of BrdU by anterior pituitary cells (BrdU-labeled nuclei/100 nuclei) was not influenced by the dietary regimen or age. The fraction of BrdU-labeled somatotropes relative to all labeled cells precipitously decreased to the same level in both dietary groups between 3 and 8 mo, although the fraction was greater in DR rats at 3 mo. In AL rats, the fraction decreased further between 8 and 20 mo, while it stabilized in DR rats. Our results suggested that dietary restriction maintains the basal rate of somatotrope renewal in later life in male rats. Although one must also estimate the effects of dietary restriction on apoptotic cell death in pituitary cells, the present study provides evidence that dietary restriction modulates somatotropes cell turnover and preserves the cell population for GH secretion during aging.

KEY WORDS

Aging, dietary restriction, somatotrope, cell renewal, growth hormone

INTRODUCTION

Impaired growth hormone (GH) secretion in response to secretagogues in aged animals is considered as one of the causative factors underlying the age-related reduction in plasma GH levels (1-3). Although age-related

To whom all correspondence should be addressed:
Isao Shimokawa, MD
Department of Pathology,
Nagasaki University School of Medicine
1-12-4 Sakamoto,
Nagasaki City 852, Japan
Phone: +81 (958) 49-7050
Fax: (958) 49-7052
e-mail: shimo@net.nagasaki-u.ac.jp

changes at the hypothalamic level remain upstream for the reduction in spontaneous secretion of GH, studies examining the pituitary response to secretagogues are necessary since GH releasing hormone (GHRH), or its analogues, has been used to improve physical activity in the elderly (4).

In a series of recent studies from our laboratory, we have suggested that a reduction in the number of somatotropes during aging may account, at least in part, for the impaired response of the pituitary gland to GHRH (5-7). Furthermore, lifelong dietary restriction, a powerful antiaging intervention in laboratory rodents (8-10), slows the age-related reduction in the number of somatotropes (6, 7). An age-related reduction in cell renewal rate can be linked to functional impairment in most organs in aged animals (11). In a recent review, Warner et al. (12) emphasize the importance of modulation of cell turnover, renewal and death, to our understanding of the antiaging effects of dietary restriction.

In the present study, we investigated the modulatory effect of dietary restriction on somatotropes cell renewal during aging. The continuous labeling method of bromodeoxyuridine (BrdU) with a mini-osmotic pump was used to measure the cumulative rate of somatotrope cell renewal over a period of one week, since the basal rate of renewal is expected to be very low (13) and diurnal variations of pituitary cell proliferation has been reported (14, 15). The present study showed that dietary restriction results in preservation of the basal rate of cell renewal in aged animals, rather than exerting a suppressive effect on cell proliferation.

MATERIALS AND METHODS

Experimental Animals

Specific-pathogen-free male F344 weanling rats at 4 weeks of age were purchased from Charles River Laboratory Japan (Yokohama, Japan). They were housed singly and maintained in a barrier facility (temperature: $21 \pm 1^\circ\text{C}$, 12:12 hours light/dark cycle with light on at 5:00 A.M.) in the Laboratory Animal Center at Nagasaki University School of Medicine (NUSM). The animals were cared for and used in accordance with the guidelines of NUSM.

During the first two weeks, the rats received water and food ad libitum (Charles River Formula, CRF-1, Oriental

Yeast Co., Tokyo). At 6 weeks of age, the animals were subdivided into two dietary groups, the ad libitum (AL) Group and dietary restricted (DR) Group. Group AL was allowed to have the same food ad libitum. The food provided to Group DR was restricted to approximately $70 \pm 5\%$ of the mean weekly intake of Group AL rats throughout life by providing $160 \pm 10\%$ of the mean daily intake of Group AL at 16:30 on three days of the week only; Monday, Wednesday, and Friday, i.e., the dietary regimen for dietary restriction in our laboratory was a modified alternate feeding. The pattern of food intake in both groups has been described in detail elsewhere (7). Briefly, the food intake altered a two-day cycle from Monday evening to Sunday morning, while little food was left from Sunday morning to Monday evening, as the meal provided on Friday evening was ingested until Sunday morning.

Incorporation of 5-Bromo-2'-Deoxyuridine (BrdU)

BrdU (Sigma Chemical Company, St. Louis, MO) was dissolved in drops of 1 N NaOH, and immediately diluted into 10 mg/ ml/ 100 g body weight with phosphate-buffered solution (PBS, pH 7.4), and sterilized by a sterile microfilter. BrdU was continuously administered in each rat for one week using the mini-osmotic pump (ALZET 2001, ALZA Corporation, Palo Alto, CA), according to the method described by De Bruine et al. (16). Under pentobarbital anesthesia, the pump was implanted in the subcutaneous tissue of the back. The operations was performed on Monday in each animal, between 0900 and 1100 in the morning. Seven days later, the rats were sacrificed under pentobarbital anesthesia between 0900 and 1100. The feasibility of this technique for measuring the cumulative rate of cell production by DNA synthesis *in vivo* has been established by several laboratories (16, 17).

Tissue Preparation

Dissected pituitary glands were immediately weighed and fixed in 4% paraformaldehyde overnight at 4°C. After that, the samples were routinely embedded in paraffin and cut into 6 μ m-thick sections. No tumors of the pituitary gland were evident macroscopically in all samples. Although hyperplastic cellular foci were found in the pituitary gland of two of five AL rats at 20 months, these foci were carefully excluded from the present analysis.

Immunohistochemistry

The avidin-biotin peroxidase complex (ABC) method for BrdU and the peroxidase antiperoxidase (PAP) method for GH were sequentially performed to visualize BrdU-incorporated somatotropes. Briefly, deparaffinized sections were first incubated in absolute methanol containing 0.3% H_2O_2 for 20 min to suppress the endogenous peroxidase activity. The sections were pretreated with 20 mg/ml of proteinase K (Wako Pure Chemical Industries, Japan) for 15 min at 37°C. In the next step, the

sections were immersed in 2N HCl for 30 min at 37°C to produce a partial denaturing of double-strand DNA and expose the antigenic sites of BrdU, then rinsed in borate buffer (pH 8.5). The sections were then incubated with 5% of normal horse serum (Vector Laboratories, Burlingame, CA) for 15 min at room temperature (R.T.) followed by incubation overnight with anti-BrdU antibody (DAKO A/S, Denmark) diluted in 1:80 at 4°C. The sections were incubated with the biotinylated antimouse IgG antibody (Vector Laboratories) diluted into 1:200 at R.T. for 30 min. The avidin-biotin peroxidase complex (Vector Laboratories) were applied at R.T. for 30 min. The substrate for peroxidase was 0.05% 3,3'-diaminobenzidine-tetrahydrochloride (DAB), 0.025% $CoCl_2$, 0.02% $NiSO_4(NH_4)_2SO_4$, and 0.001% H_2O_2 in PBS, which yielded dark gray reaction products. After rinsing the sections with running water, peroxidase activity was blocked again by 0.3% H_2O_2 -methanol. The primary antibody to rat GH (1:8000, Shikibo, Japan) was applied to sections overnight at 4°C after incubation with normal swine serum (DAKO A/S). The secondary antibody, swine anti-rabbit immunoglobulins (DAKO A/S) diluted into 1:100, and then PAP, were applied at R.T. for 30 min each. The substrate for peroxidase was 0.02% DAB and 0.005% H_2O_2 in PBS, resulting in brown reaction products. The sections were rinsed in PBS between steps of immunohistochemistry. Immunostained sections were washed, dehydrated and mounted. Counterstaining for nuclei was omitted. Negative control was achieved by replacing the primary antibodies by nonimmunized mouse and rabbit serum samples.

Evaluation of BrdU-Incorporated Cells

Using a light microscope, the reaction products by Ni-Co-DAB (dark gray) for BrdU were differentiated easily from those by DAB (brown) for GH; Figure 1 shows a photomicrograph of these sections. The number of

Figure 1. Immunohistochemical demonstration of BrdU-labeled somatotropes in tissue sections (ad libitum fed rat at 8 mo, original magnification: $\times 400$). Growth hormone appears in the cytoplasm as a brown stain while BrdU is identified in the nucleus as a dark gray stain.

nuclei and immunostained cells were counted under the light microscope using an eye piece equipped a square test grid, without reference to age and dietary groups. The number of nuclei in the anterior pituitary (expressed as number per mm²) was calculated by counting the number of nuclei in eight fields of a section stained with hematoxylin and eosin under a magnification of 400 (objective lens: x40, eye piece: x10). The number of BrdU-labeled nuclei in the anterior pituitary was also counted in a similar manner in sections immunostained for BrdU. The rate of BrdU incorporation in anterior pituitary cells was expressed as the number of BrdU-labeled nuclei per 100 nuclei.

The number of cells positive for GH, referred to as somatotropes in this paper, was expressed as the number of cells per mm² by scanning 40 fields in immunostained sections under a magnification of 400. Anterior pituitary cells labeled for both BrdU and GH were scanned in 80 fields per pituitary gland under a magnification of 200; approximately 1×10^5 GH cells were analyzed for positive reaction with BrdU in each pituitary gland. The rate of somatotrope production or renewal was expressed as the number of BrdU-labeled somatotropes per 100 BrdU-labeled anterior pituitary cells.

Statistical Analysis

The two-factor analysis of variance (ANOVA) was used to test the data for the main effects of age (AGE) and diet (DIET) and the interaction between these two factors (AGE x DIET). Duncan's new multiple range test was used for multiple comparisons. Data were analyzed after logarithmic transformation, when appropriate, to satisfy assumptions required for linear models. The statistical significance was accepted at $p < 0.05$. Data represent the mean $\pm$ SEM of five rats except Group DR at 3 months; some data from one rat in this group were accidentally lost.

RESULTS

Background Data on the Pituitary Gland in Male F344 Rats Used in this Study

Table 1 summarizes the influence of aging and dietary restriction on the weight of the pituitary gland, and the number of nuclei and somatotropes in the anterior lobe. The pituitary weight increased with age in both dietary groups (AGE; $p < 0.0001$, AGE x DIET; $p = 0.0860$), although the weight was, as expected, less in DR rats (DIET; $p < 0.0001$). Similarly, the number of nuclei per mm² in the anterior lobe increased with age in both groups (AGE; $p < 0.0001$, AGE x DIET; $p = 0.4906$). There were no significant differences in the number of nuclei between the two dietary groups (DIET; $p = 0.0502$). The effect of age on the number of somatotropes per mm² was different in AL and DR rats (AGE; $p < 0.0001$, AGE x DIET; $p < 0.05$). The number of somatotropes significantly decreased in AL rats between 8 and 20 months, but not between 3 and 8

months. In DR rats, there was a significant decrease at 8 months relative to that at 3 months ($p < 0.05$), but the fall at 20 months was not significant. Accordingly, the number of somatotropes was significantly greater in DR than AL rats at 20 months ($p < 0.05$).

Table 1. Background data in male F344 rats used in the present study.

	Age (months)		
	3	8	20
Pituitary weight (mg) ^{a,b}			
AL	7.1 $\pm$ 0.5	10.5 $\pm$ 1.4#	15.6 $\pm$ 0.9#
DR	5.5 $\pm$ 0.3	6.1 $\pm$ 1.1#*	9.6 $\pm$ 0.1#*
Number of nuclei in the anterior pituitary (x10 ³ nuclei/mm ²) ^a			
AL	5.46 $\pm$ 0.21	6.42 $\pm$ 0.12#	7.54 $\pm$ 0.20#
DR	5.87 $\pm$ 0.16	6.79 $\pm$ 0.17#	7.59 $\pm$ 0.08#
Number of GH-immunostained cells (x10 ³ nuclei/mm ²) ^{a,c}			
AL	2.41 $\pm$ 0.11	2.28 $\pm$ 0.08	1.50 $\pm$ 0.13#
DR	2.60 $\pm$ 0.07	2.1 $\pm$ 0.15#	1.99 $\pm$ 0.05*

Data represent the mean $\pm$ SEM of 5 rats except for some data in Group DR at 3 months of age (n=4). * $p < 0.05$, Age effect by 2-factor ANOVA, $p < 0.05$, Diet effect by 2-factor ANOVA, $p < 0.05$, interaction between Age and Diet effects by 2-factor ANOVA, $p < 0.05$, compared with the younger age rats in the same dietary group, $p < 0.05$, compared with Group AL at the same age by Duncan's new multiple range test.

The Rate of BrdU Incorporation in Anterior Pituitary Cells

As demonstrated in Figure 2, the rate of BrdU incorporation in anterior pituitary cells (BrdU-labeled nuclei/100 nuclei) was not influenced by dietary regimen or age (AGE; $p = 0.5706$, DIET; $p = 0.6360$, AGE x DIET; $p = 0.0634$).

Figure 2. The rate of BrdU incorporation in the anterior pituitary cells. AL: ad libitum fed rats, DR: dietary restricted rats. Each bar represents the mean value $\pm$ SEM of five rats except Group DR at 3 months (n=4).

The Rate of Somatotrope Production

As demonstrated in Figure 3, the fraction of BrdU-labeled somatotropes among labeled anterior cells precipitously decreased during aging (AGE; $p < 0.0001$), but the pattern was different between AL and DR rats (AGE x DIET; $p < 0.0001$). In AL rats, the fraction decreased progressively between 3 and 20 mo. In DR rats, the fraction decreased to a level similar to that in AL

at 8 months, but it did not change between 8 and 20 months. The fraction was significantly higher in DR rats than in AL rats at 3 and 20 months ($p < 0.05$).

Figure 3. The fraction of BrdU-labeled somatotropes in BrdU-labeled pituitary cells. AL: ad libitum fed rats. DR: dietary restricted rats. # $p < 0.05$, versus younger rats in the same dietary group, * $p < 0.05$, versus Group AL at the same age by Duncan's new multiple range test. Each bar represents the mean value $\pm$ SEM of five rats except Group DR at 3 months ($n=4$).

DISCUSSION

Based on the background data on the pituitary gland, it is assumed that the cell population in the anterior pituitary expanded by a similar rate in AL and DR rats during the period of observation in the present study. The fraction of BrdU-labeled somatotropes among BrdU-labeled pituitary cells precipitously decreased from 3 and 8 months in both dietary groups. In mice, the fraction of BrdU-incorporated somatotropes among the entire population of incorporated cells gradually increases after birth and reaches a level of 25% at 22 days after birth (18). Therefore, one can conclude that the rate of production of somatotropes in the anterior pituitary reaches a maximum level during maturation; but the rate precipitously declines thereafter and stays at a basal level for cell renewal of somatotropes. On the contrary, the production rate of non-somatotropes could be increased after maturation. Mammatropes and chromophobes may be candidates for the cell type, although sometimes considered neoplastic growth in aged rats (19).

The most important finding in this study was that the basal rate of somatotrope renewal was maintained in DR rats at 20 months, while it diminished in AL rats. These findings are comparable with those reported by morphometric analysis and reverse hemolytic plaque assay, in which dietary restriction slowed the decrease in the number of somatotropes in later life of male rats (6, 7). To our knowledge, there is no report that has

previously examined the dietary effect on the pituitary response to GH secretagogues *in vivo*. The response should be better in DR rats than AL rats at old age, depending upon the cellularity of somatotropes.

Hypothalamic peptides as well as other hormonal factors regulate the division and differentiation of cells in the somatotrope lineage (20). Of these, GHRH plays a significant role in stimulating the production of somatotropes (21, 22). The synthesis and secretion of GHRH in the hypothalamus is thought to be reduced in aged rats fed ad libitum (23, 24). There is no direct evidence that GHRH is secreted in aged DR animals in a fashion similar to that observed in young counterparts fed ad libitum. However, a recent study reported that pulsatile secretion of GH similar to that in young AL rats is present in aged DR rats, but not in aged AL rats (25), suggesting that GHRH is secreted efficiently in DR rats in later life. Thus, preservation of the hypothalamic function may be attributed to maintaining the basal rate of somatotrope renewal in aging DR rats, leading to the protection of impaired pituitary response to GHRH during aging.

A suppressive effect of dietary restriction, either short-term or long-term, on cell proliferation in several organs has been emphasized in association with inhibition of tumorigenesis (12, 26-28). In a study by Keenan et al. (17), using a method similar to the one described in the present study, these workers showed that the cumulative rate of BrdU incorporation in pituitary cells was reduced in DR rats at 12 months of age, albeit statistically insignificant. In the present study, the rate of BrdU incorporation in anterior pituitary cells as well as in somatotropes did not decrease in DR rats at the ages examined. Since the pituitary weight, hence the total number of anterior pituitary cells in the gland, was apparently less in DR rats; it could be argued that dietary restriction suppresses cell proliferation or induces cell death or both in the anterior pituitary during the early phase of dietary restriction. However, it is questionable that dietary restriction simply suppresses cell proliferation in the pituitary gland as well as other tissues after the early phase of dietary restriction. Our results are comparable with those reported by Merry and Holeman (29); in male SD rats maintained on a restricted diet from weanling, a severe inhibition of DNA synthesis occurs during the first six months in the liver, kidney, heart, abdominal skin, while the dietary effect on DNA synthesis disappears after that period.

Recently, Warner et al. (12) emphasized the importance of apoptotic cell death on the removal of damaged cells and preneoplastic cells from the cell population during aging and dietary restriction. At present, we cannot estimate the basal rate of apoptotic cell death in somatotropes, since the rate is too low to detect in tissue sections by microscopy. We may say, however, that the rate of cell death in somatotropes may not change in DR rats between 8 and 20 months, since the basal rate of cell renewal and cell density of somatotropes did not differ significantly during that period. In other words,

intrinsic mechanisms implicated in cell death as well as cell renewal of somatotropes could be maintained in DR rats. In contrast, changes in the cell renewal rate and cell density in AL rats suggested that the regulatory mechanisms controlling cell turnover in somatotropes could be disrupted in AL rats.

The hypothesis of Warner et al. (12) relies on an experiment by James and Muskhelishvili (28). In that experiment using male C57BL/6 x C3H F1 mice, a hybrid prone to hepatoma, the rate of apoptosis was found to be greater in DR mice at 12 months of age. They also reported that a similar finding is observed in the liver of F344 rats. However, using the F344 rats, we could not confirm this phenomenon in our laboratory; the rate of apoptosis in the liver was increased with advancing age, while no acceleration by dietary restriction was observed (30).

In the pituitary gland, the cumulative rate of cell proliferation in the anterior pituitary was not altered by age or dietary restriction. By definition, the rate of apoptotic cell death should be balanced by a similar rate of cell renewal in order to maintain a steady cell population in an organ. Thus, the cumulative rate of apoptosis in the anterior pituitary should not be upregulated by dietary restriction, since the pituitary weight, thereby the cell population, increased in a similar manner during the period of observation. Further experiments are necessary to examine how standard dietary regimens for energy restriction, not fasting, modulates the basal rates of cell renewal and apoptotic cell death, thereby cell turnover in different tissues. We believe that dietary restriction does not simply modulate cell turnover by downregulating cell proliferation and upregulating cell death. Another important issue is how dietary restriction maintains the basal rate of cell turnover in later life, if the rate was decelerated or accelerated as compared to that in animals fed ad libitum.

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