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Proliferative activity of the parathyroid cells in rats with secondary hyperparathyroidism

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Abstract Proliferative kinetics of parathyroid cells in secondary hyperparathyroidism (HPT) are still unknown. We examined the histopathological changes and proliferative activity of parathyroid cells in spontaneously hypercholesterolemic (SHC) rats that exhibit secondary HPT and in normal Sprague Dawley (SD) rats from 3 weeks to 32 weeks of age. Proliferative activity [proliferating cell nuclear antigen(PCNA) labeling index], evaluated by means of immunohistochemical examination of PCNA, declined in SD rats with age from 10.8% at 3 weeks of age to 0.15% at 32 weeks of age. In SHC rats, a PCNA labeling index of 11.6% declined to 3.12% at 14 weeks of age and rebounded to 6.15% at 26 weeks of age. Parathyroid glands increased in size as determined by the maximum cross-sectional area, but in SHC rats, the increase was significantly greater, paralleling the progression of renal dysfunction, and at 32 weeks they were almost three times larger than in SD rats. Parathyroid hormone (PTH) levels in SHC rats also rose sharply after 20 weeks and reached 611 pg/ml at 32 weeks, while PTH in SD rats remained unchanged at approximately 110 pg/ml. This study showed that in the course of developing HPT in SHC rats, there is a large increase in the size of the parathyroid gland, a concomitant increase in PTH levels, and a PCNA labeling index that is higher than in normal SD rats.

Key words Secondary hyperparathyroidism · Parathyroid cell · Spontaneously hypercholesterolemic rat · Proliferative activity · Proliferating cell nuclear antigen

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Introduction

Hyperparathyroidism (HPT) is an endocrinological disorder of abnormal calcium metabolism owing to excessive secretion of parathyroid hormone (PTH) [5]. The cause, in some HPT cases, has been considered to be related to an abnormal gene, and the details have already been described [2]. However, it is not clear how HPT develops and advances clinically. It is important to study the cellular proliferative kinetics of the parathyroid glands in order to elucidate the mechanism involved in the progression of HPT.

Proliferating cell nuclear antigen (PCNA) is known as a cell proliferation marker [6, 20]. PCNA is mainly synthesized during the G1 phase of the cell cycle, and its amount is maximal during the S phase [7]. Therefore, proliferative activity was determined by observing PCNA expression in the cell nuclei and counting both the PCNA-positive and the PCNA-negative cells and expressed as the PCNA-labeling index (%). Previously, we evaluated the proliferative activity of parathyroid cells in patients with HPT by examining the expression of PCNA using an immunohistochemical procedure [19]. Subsequently, it has been shown that there is high cellular proliferative activity in adenoma, and in secondary hyperplasia and also in parathyroid cell proliferation during the development of secondary HPT [19].

Details of physiological proliferation of human parathyroid cells have been described [13]. However, the relationship between cell proliferation and enlargement of the parathyroid gland in the natural course of events leading to secondary HPT is still unknown. In human studies, proliferative activity of parathyroid cells can be determined after the surgical removal of the parathyroid gland, but it is difficult to evaluate the results even under impartial conditions because of large individual differences. In the conventional experimental rat model for secondary HPT, chronic renal failure was induced in a very short period of time by imposing surgical stress on normal rats [14]. However, this model is not appropriate for evaluating physiological and long-term changes in

parathyroid cellular proliferation with secondary HPT. For this reason, we focused our attention on the spontaneously hypercholesterolemic (SHC) rats, in which chronic renal failure progresses gradually, and secondary HPT is definitely exhibited without any kind of treatment [18]. The proliferative activity of parathyroid cells estimated by the PCNA labeling index, in the course of developing secondary HPT, was studied from the onset to the final stage in SHC rats and compared with that in normal rats.

Materials and methods

The SHC/Ta rat was established by Imai et al. [8] from Jcl:Sprague Dawley (SD) rat strain. The genetically altered SHC rats develop an age-related increase in plasma cholesterol, urea nitrogen, creatinine, and urinary protein on normal rat food diet. These changes are more severe in males than in females. The mean life span of the male rats is approximately 32 weeks, owing to chronic renal failure. The enlarged parathyroid glands associated with secondary hyperparathyroidism were found in the male SHC rat after 24 weeks of age [18]. In this study, we utilized these characteristics of the SHC rats.

We used 90 male Jcl:SD rats (purchased from Clea Japan Inc., Tokyo, Japan) and 90 male SHC/Ta rats (purchased from Kiwa experimantal laboratory animals Co., Ltd., Wakayama, Japan) and sacrificed at 3, 8, 14, 20, 26, and 32 weeks of age, 15 in each group. They were housed in plastic cages in the Animal Laboratories for Medical Research of Asahikawa Medical College. The animals were given commercial food pellets (CE-2, Clea Japan Co., Tokyo, Japan) and water ad libitum. They were kept under constant environmental conditions: temperature of 22°C, relative humidity of 45%, and light/dark cycle of 14 h/10 h with lights on between 0500 hours and 1900 hours. The rats were transferred to individual metabolic cages 24 h before sacrifice and 24-h urine samples were collected.

Rats were sacrificed by deep ether anesthesia and laparotomized. We drew blood samples from the inferior caval vein and removed the kidneys. Normal rats have two parathyroid glands, one each on the left and right side, which are usually buried in the thyroid gland. A neck incision was made, and the two parathyroid glands were removed with thyroidal tissues. The blood samples were centrifuged, and their serums were submitted to SRL Inc., Tokyo, Japan to test for calcium, phosphorus, urea nitrogen, creatinine, triglyceride, and total cholesterol. Urine samples were also examined for creatinine, calcium, and phosphorus. Rat PTH was measured using double-antibody immunoradiometric assay using a Rat Parathyroid Hormone Kit (Immutopics Inc., San Clemente, Calif.).

Pathological evaluation

The kidneys and parathyroid glands with thyroid were fixed in 10% buffered formalin solution for 24 h. The tissues were then dehydrated and embedded in paraffin. The kidney sections were stained with hematoxylin and eosin (H&E). The parathyroids, two from each rat, were carefully cut to expose a maximum area, and ten serial sections of the parathyroid glands, 4-μm thick, were mounted on aminoalkylsilane-treated glass slides (Dako, Glostrup, Denmark) and air-dried for more than 24 h at room temperature. Eight of the ten sections were stained with H&E to evaluate the size of the parathyroid gland, cell components, and structure of the enlarged glands. After taking microphotographs of eight sections, the long (α mm) and short (β mm) axes of each parathyroid gland were measured, and the cross-sectional area (A) was calculated as follows: $A(mm^2)=(\alpha/2)\times(\beta/2)\times\pi$. We chose the section with the largest area and defined it as the maximum area for that gland. This procedure was repeated in the two parathyroids from each rat and obtained a total of 30 maximum area data for each rat group.

The two remaining sections of the ten sections were employed in the following immunohistochemical studies. The sections were deparaffinized and rehydrated through a graded alcohol series and treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. After blocking nonspecific background staining by treating with 10% normal rabbit serum for 15 min, the first antibody, monoclonal mouse anti-PCNA antibody PC-10 (Dako, Glostrup, Denmark), was applied at a dilution of 1:100 at 37°C for 60 min. The second antibody, biotinylated anti-mouse immunoglobulin (Ig)G+A+M, was then applied, followed by the streptavidin-peroxidase conjugate immunoperoxidase (Nichirei, Tokyo, Japan) reaction. The reaction was visualized with 3,3' diaminobenzidine hydrochoride (DAB; Nichirei, Tokyo, Japan) containing 0.06% hydrogen peroxide. Each step, except between the blocking of nonspecific staining and application of the first antibody, was followed by washing with phosphate-buffered saline. The sections were counterstained with methyl green and overlayed with coverslips. As a positive control, we used formalin-fixed, paraffin-embedded sections of the human tonsil. In the negative control, the primary antibody was omitted. The immuno-positive reactions were noted within the cell nuclei, and the cells whose nuclei were clearly brown from the DAB, instead of green from methyl green, were interpreted as positive. We took microphotographs of two sections and counted a total of 2000 parathyroid cell nuclei in each section in areas where they were distributed normally. Because we had two PCNA sections from one parathyroid, we averaged the two counts, and the PCNA labeling index (%) was calculated as follows: the number of positive cells/2000×100. This procedure was repeated for the two parathyroids from each rat, and thus a total of 30 PCNA labeling indexes for each rat group was obtained.

Statistical analysis

Statistical significance was calculated using a nonparametric test (Wilcoxon-Mann-Whitney test) in the case of a simple comparison. For multiple comparisons, Scheffe's post-hoc test was used only if one-way factorial analysis of variance (ANOVA) showed a significant difference. The correlation coefficient and its significance were calculated using Spearman's rank correlation. Statistical significance was assumed when the *P* value was less than 0.05. All results were expressed as the mean±SEM of the mean.

Results

Growth curves of normal SD rats and SHC rats are shown in Fig. 1. Body weights in both groups increased from approximately 39 g at 3 weeks of age to an average weight of 550 g at 19 weeks of age. After 21 weeks, significant differences in the body weights between the two groups were evident. SHC rats began to lose weight and, by 32 weeks of age, were on average 225 g lighter than the SD rats, which had continued to gain in weight.

Total cholesterol (Fig. 2) and triglyceride were unchanged in both groups at a level of approximately 80 mg/dl until 8 weeks of age, when these levels increased sharply in SHC rats and continued to rise 403±30.9 mg/dl by 32 weeks of age. In SD rats, these levels remained unchanged at approximately 82 mg/dl. Total renal function, serum urea nitrogen, creatinine, and creatinine clearance were also gradually aggravated. Serum urea nitrogen (Fig. 3) stayed constant at below 18.7 mg/dl in both groups until 20 weeks of age when an increase was observed only in the SHC rats. By 26 weeks and 32 weeks of age, the levels had increased significantly to 65.4±5.87 mg/dl and 114±21.2 mg/dl,

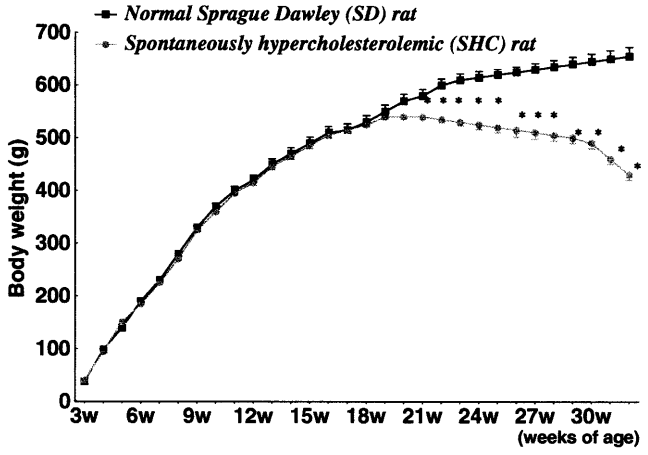

Fig. 1 Growth curves in body weight of normal Sprague Dawley (SD) rats (n=15) and spontaneously hypercholesterolemic (SHC) rats (n=15). The weights were almost equal until 19 weeks of age. After 21 weeks of age, SHC rats gradually decreased in body weight (*P<0.001). *Error bars* represent SEM

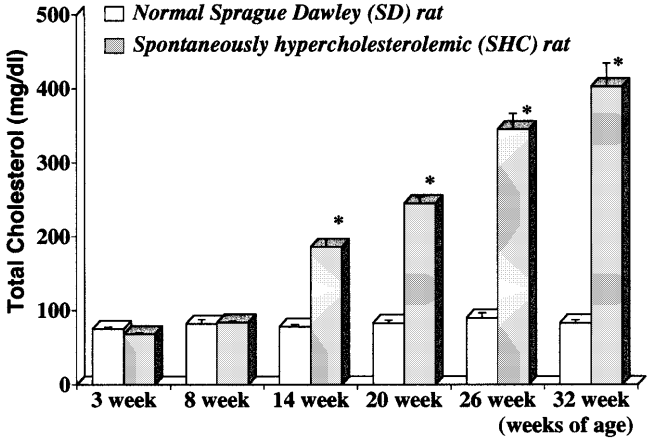

Fig. 2 Lipid metabolism was represented by serum concentration of total cholesterol. Triglyceride change in each group was similar to total cholesterol. Serum total cholesterol gradually increased with age in spontaneously hypercholesterolemic (SHC) rats after 14 weeks of age (*P<0.001). *Error bars* represent SEM. n=15, each group

respectively. Serum calcium and urine calcium and phosphorus did not change, but the serum phosphorus rose significantly only in SHC rats at 32 weeks of age (data not shown).

PTH levels remained unchanged at approximately 110 pg/ml in SD rats, but in SHC rats, a gradual increase was observed beginning at 14 weeks of age and attained levels of 257±31.3 pg/ml, 430±89.9 pg/ml, and 611± 124 pg/ml at 20, 26, and 32 weeks of age, respectively. Changes in PTH levels indicated the gradual onset of secondary HPT and concomitant progression of renal dysfunction in SHC rats (Fig. 4).

The kidneys of SHC rats tended to enlarge before renal dysfunction appeared. In histopathological findings of the 26-week-old SHC rat kidneys, focal and segmental glomerular hyalinosis and sclerosis with severe tubular dilatation were evident in more than half of the glo-

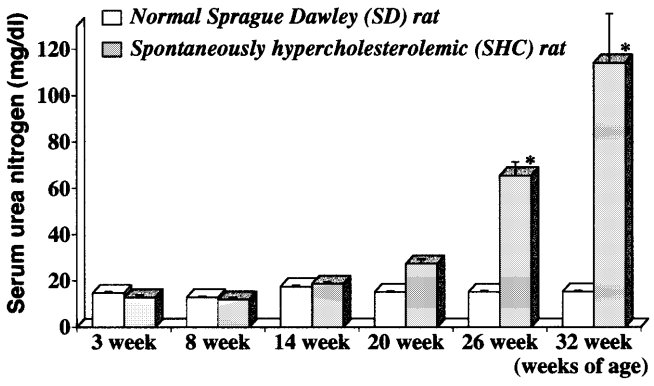

Fig. 3 Total renal function was represented by serum concentration of urea nitrogen. Serum creatinine and creatinine clearance of each group were similar to urea nitrogen. Serum urea nitrogen gradually increased in spontaneously hypercholesterolemic (SHC) rats after 20 weeks of age. Statistical significance was confirmed after 26 weeks of age (*P<0.001). *Error bars* represent SEM. n=15, each group

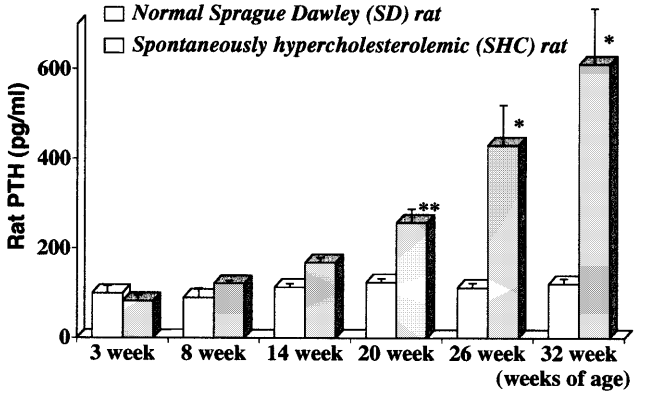

Fig. 4 Serum concentration of parathyroid hormone (PTH) level in spontaneously hypercholesterolemic (SHC) rats started to rise from 14 weeks of age and became significantly higher than that of Sprague Dawley (SD) rats after 20 weeks of age (*P<0.001, **P<0.01). *Error bars* represent SEM. n=15, each group

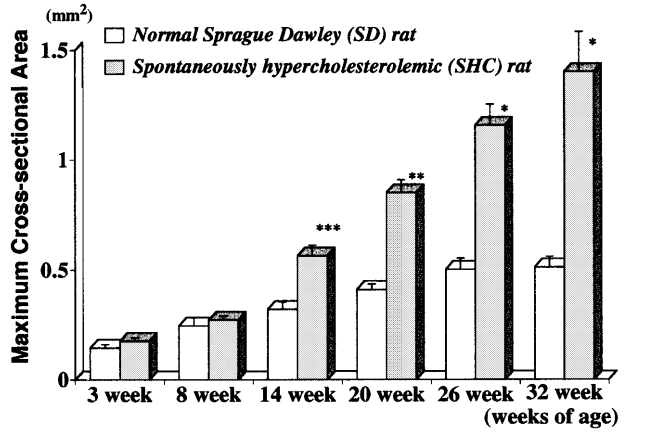

Fig. 5 The size of the parathyroid gland estimated by the maximum cross-sectional area in the histological sections. The parathyroid size of spontaneously hypercholesterolemic rats is significantly increased after 20 weeks of age (*P<0.001, **P<0.01). *Error bars* represent SEM. n=30, each group

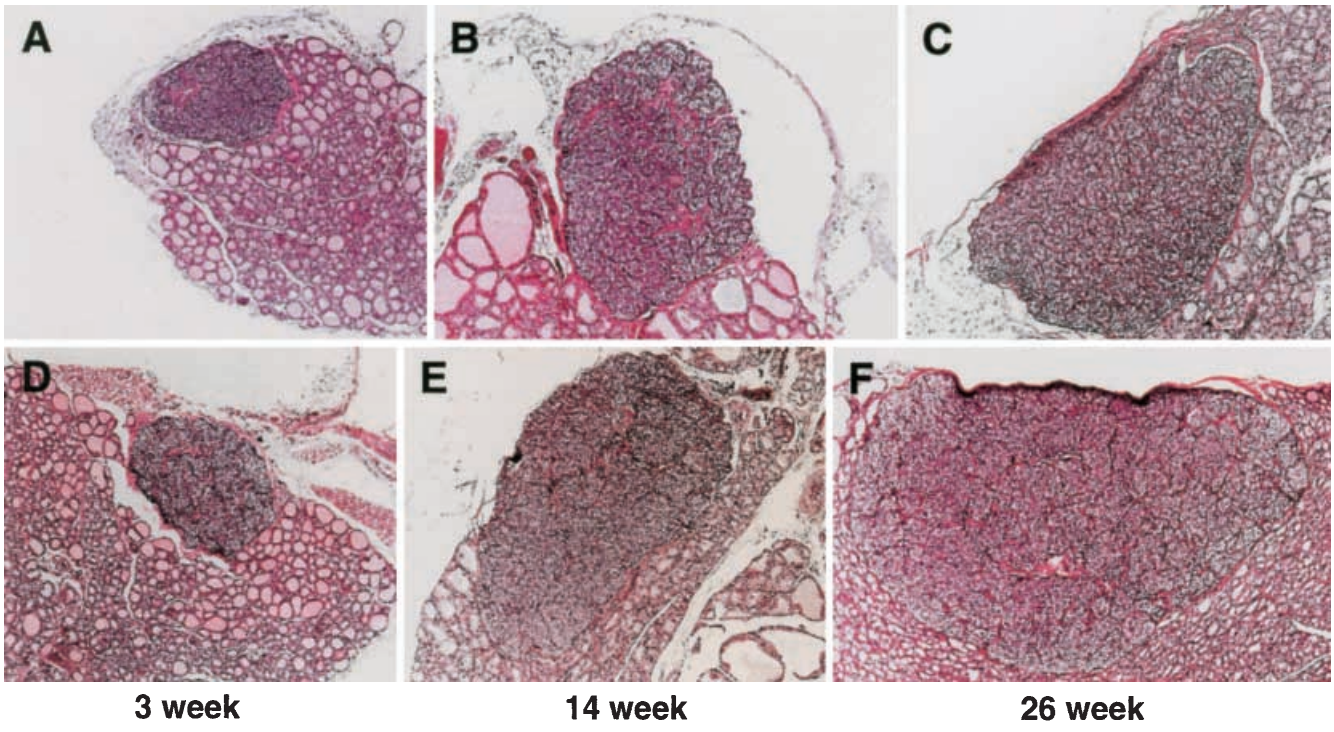

Fig. 6 Microscopic findings of parathyroid glands in Sprague Dawley (SD) rats and spontaneously hypercholesterolemic (SHC) rats. The parathyroid glands of SHC rats enlarged a little at 14 weeks of age and obviously enlarged at 26 weeks of age. **A** SD rat (3 weeks of age); **B** SD rat (14 weeks of age); **C** SD rat (26 weeks of age); **D** SHC rat (3 weeks of age); **E** SHC rat (14 weeks of age); and **F** SHC rat (26 weeks of age). Hematoxylin and eosin stain, magnification ×33

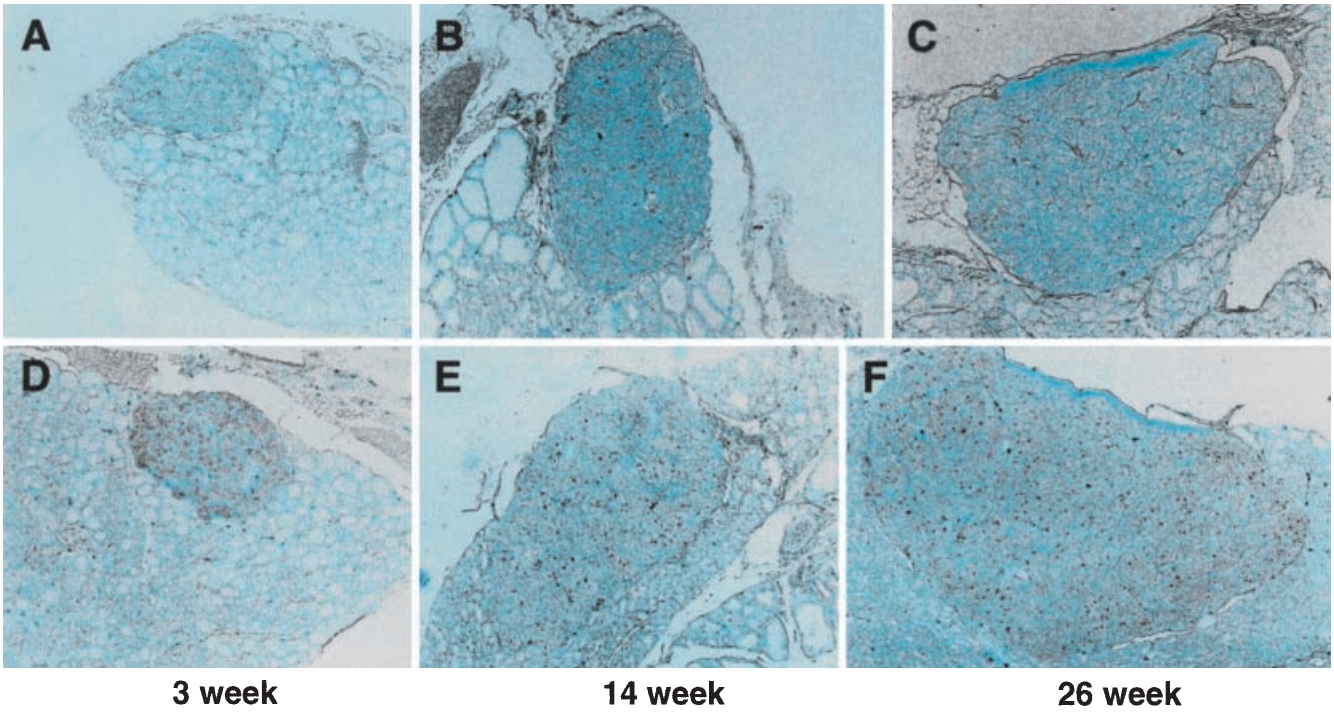

Fig. 7 Microscopic findings of proliferating cell nuclear antigen (PCNA) expression in the parathyroid glands in Sprague Dawley (SD) rats and spontaneously hypercholesterolemic (SHC) rats. Positive reactions are noted in the nuclei, which stained *dark brown*. The PCNA expression was reduced with age in SD rats and little reaction was observed at 14 weeks and 26 weeks of age. In SHC rats, the PCNA expression remained at high levels compared with SD rats of the same age. **A** SD rat (3 weeks of age); **B** SD rat (14 weeks of age); **C** SD rat (26 weeks of age); **D** SHC rat (3 weeks of age); **E** SHC rat (14 weeks of age); and **F** SHC rat (26 weeks of age). Magnification ×33

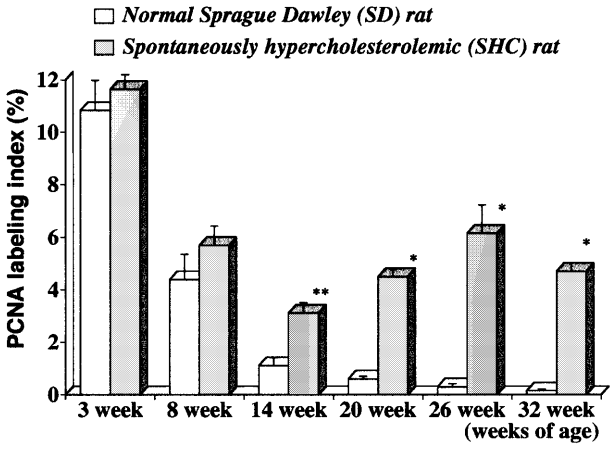

Fig. 8 The comparison of proliferating cell nuclear antigen (PCNA)-labeling index in Sprague Dawley (SD) and spontaneously hypercholesterolemic (SHC) rats. PCNA labeling index of SHC rats are statistically higher than that of SD rats after 14 weeks of age (*P<0.001, **P<0.01). *Error bars* represent SEM. n=30, each group

meruli. Lipid depositions in the glomeruli and tubular epithelial cells were also present.

The parathyroid glands in both groups grew in size, as determined by the maximum cross-sectional area (Fig. 5). In SHC rats, the glands increased in size from 0.18±0.01 mm^2 to 1.40±0.18 mm^2 by 32 weeks of age, while, in SD rats, the increase was smaller and grew from 0.15±0.02 mm^2 to 0.51±0.05 mm^2 by 32 weeks of age. Figure 6A–C shows microphotographs of the rat parathyroid glands in SD rats at 3, 14 and 26 weeks of age, respectively, and Fig. 6D–F shows microphotographs of the rat parathyroid glands in SHC rats at the same ages, stained with H&E. In SHC rats, chief cells are the only component in the markedly enlarged parathyroid gland, and oxyphil cells, which are commonly found in human secondary HPT, were not present. The glandular structure of SHC rats showed hyperplasia with diffuse growth pattern, but nodular growth, which can frequently be seen in human secondary HPT, was also not present.

Figure 7A–C shows immunohistochemical staining for PCNA in SD rats 3, 14, and 26 weeks of age, respectively, and Fig. 7D–F shows immunohistochemical staining for PCNA in SHC rats at the same ages. The PCNA labeling index of 10.8±1.12% in young SD rats decreased gradually with age to an index of 0.15±0.05% at 32 weeks of age (Fig. 8). This decrease appears to indicate a normal physiological change in the proliferative activity of the parathyroid cells with aging. The PCNA labeling index also decreased gradually in SHC rats until the age of 14 weeks, when the index increased and remained significantly higher than that in normal SD rats. At the age of 26 weeks, the index rose to 6.15±1.08%, a value similar to that at 8 weeks of age. At 32 weeks of age, the PCNA labeling index had decreased to 4.70±0.33%, but it was still significantly higher than in normal SD rats. No significant correlation was found between the PCNA labeling index and the size of the parathyroid gland, or between the PCNA labeling index and levels of PTH.

Discussion

This study demonstrated that the parathyroid glands of normal rats increased slightly, while their cellular PCNA labeling index gradually declined with aging. In SHC rats, the parathyroid glands enlarged rapidly with development of secondary HPT, and their cellular PCNA labeling index was significantly higher than in SD rats.

In the study of parathyroid growth in humans, it has been shown that the number of parathyroid cells and the weight of the parathyroid gland increase rapidly from the fetal stage to 4 years of age or so and reach a plateau around the age of 30 years [13]. According to mitotic activity, which is the classical index for cell proliferation, the rate of mitosis continued to increase, together with an increase in the number of parathyroid cells and the weight of the parathyroid gland, until the age of 30 years [13]. However, after 45 years of age, when the parathyroid weight gain stops, mitosis continued but only for the purpose of maintaining the number of cells in the whole parathyroid gland. That is, parathyroid cells are expected to divide only to replace dead cells [13]. In animal studies, the mitoses tend to fall with age in the guinea pig [3], and the mitotic response of the remaining parathyroid gland after hemiparathyroidectomy in the rat is lower than that in the thyroid gland after hemithyroidectomy [10]. Using PCNA expression instead of mitoses, we evaluated the proliferative activity of parathyroid cells in normal SD rats. Up to 32 weeks, cell proliferation of the parathyroid gradually decreased with age. Wang et al. [21] concluded that in normal rats between 8 weeks and 22 weeks of age, there were no significant changes in parathyroid volume, cell size, and cell number, and the parathyroid cell birth rate was very low during the 24-h expression of Ki-67. These results agreed well with the physiological proliferation of human parathyroid cells.

A stimulation to the parathyroid gland, which was mainly introduced by a hypocalcemic and hyperphosphatemic condition, induces an increase in the number of parathyroid cells, an increase in PTH mRNA, and an excessive secretion of PTH [9, 11]. Parathyroid cell proliferation is markedly involved in this condition. PCNA expression was examined by Naveh-Many et al. [12] using five-sixth nephrectomized rats, in which chronic renal failure had been induced. Subsequently, they demonstrated that parathyroid cell proliferation increased in secondary HPT, and the proliferation could be changed by dietary phosphate content. Administration of phosphorus (medium P) directly to parathyroid organ culture stimulated PTH secretion [16] and is an important factor for parathyroid growth not only in secondary HPT but also in normal rats [22]. Hypophosphatemia and $1,25(OH)_2D_3$ decrease the proliferative activity of parathyroid cells [15]. Total parathyroidectomy with autotransplantation was frequently performed as a surgical treatment for secondary HPT [17]. Unfortunately,

sometimes autografts grew larger, and secondary HPT developed postoperatively. In such cases, cell proliferative activity of a recurrent autograft was significantly greater than that of a previously resected parathyroid gland [19]. These results indicate that parathyroid cells can alter their proliferative potency, responding to various changes in the environment and have the unique property, unlike transplanted cells from other organs, of retaining their function.

The entire biological process of parathyroid cell proliferation in secondary HPT has not been studied. The genetically altered SHC rats develop spontaneously secondary HPT without any special treatment and are an ideal model for this study. In humans, even though parathyroid glands can be studied following surgery, the large differences among the patients, because they are in different stages of hyperparathyroidism, make it difficult to evaluate the results. In the present study, parathyroid cell proliferation in the early phase of secondary HPT was similar to that in normal rats. The PCNA labeling index increased with progression of secondary HPT and with concomitant increase in the size of parathyroid and PTH secretion.

Other biological indicators have been used recently to study parathyroid cell proliferation in secondary HPT. Alo et al. [1] compared fatty acid synthase with Ki-67, PCNA, and p53 expression in hyperplastic parathyroid. They concluded that fatty acid synthase might be a potent marker of highly proliferating parathyroid cells in secondary HPT. In a study of secondary HPT in the rat, the calcium-sensing receptor in parathyroid cells was shown to have decreased primarily in the area of PCNA positive cells, and this downregulation of the calcium-sensing receptor was associated with parathyroid cell proliferation [4].

It is interesting to note the great difference in the PCNA labeling index between normal and secondary HPT rats. In particular, parathyroid cells of SHC rats at the age of about 26 weeks were shown to have high proliferative activity and high secretion of PTH. We should utilize this highly proliferative property and establish parathyroid cell cultures for use as a model for experimental hyperparathyroidism in the future.

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