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A circannual rhythm in trypsin-like protease activity of the mouse submaxillary gland and the influence of the Oriental medicines on it

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Abstract

A circannual rhythm in trypsin-like protease activity of the submaxillary gland was observed in male mice of B10A and B10 strains. The seasonal variation in trypsin-like protease activity of the submaxillary gland was observed only in male mice, with high values in winter and low values in summer. The seasonal variation in trypsin-like protease activity in males was abolished following castration and was absent in female mice, which indicates that the biorhythmicity is androgen-dependent. The effect of Rehmanniae Radix and Corni Fructus on trypsin-like protease activity was affected by the circannual rhythmicity. In summer, the trypsin-like protease activity, which was originally low in value, was more increased after *in vivo* administration of drug extract, whereas in winter, the trypsin-like protease activity, which was relatively high in value, was less affected after drug treatment.

Key words trypsin-like protease, circannual rhythm, Rehmanniae Radix, Corni Fructus, testosterone, mouse.

Abbreviations BAPNA, *Nα*-benzoyl-D, L-arginine-*p*-nitroanilide ; Hachimi-jio-gan (Ba-Wei-Di-Huang-Wan), 八味地黄丸.

Introduction

It is generally recognized that biorhythms are fundamental features of biological systems.[1,2] Circadian is one of the well studied biorhythms. Circannual rhythms, on the other hand, are also widely observed in many biological aspects of organisms, especially in the clinical features of human diseases. Circannual rhythmicity in the incidence of infectious diseases, for example, is quite prominent phenomena and is conventionally accepted as clinical background for diagnosis and prevention.[3-5] Circannual rhythmicity in morbidity and mortality of cardiovascular diseases, and in the incidence of allergic diseases, asthma, some genetic diseases and sterilitas temporalis, has been frequently described.[6-12] The circannual rhythmicity in clinic features are not quite understood at the present stage, but the seasonal variation of some physiological and biochemical functions, which subsequently changes susceptibility of the host to pathological factors, may play a key role in those diseases.

In the recent past two decades, studies searching for circannual rhythms of physiological parameters have gained much progress. Circannual rhythmicity has been detected in circulating hormones, such as growth hormone (GH), follicular stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH), thyroxin, cortisol, testosterone and renin, circulating minerals, such as sodium (Na), potassium (K), chloride (Cl), calcium (Ca), phosphorus (P) and magnesium (Mg), and circulating proteins, choline and cholesterol.[13-15]

Trypsin-like protease in the mouse submaxillary gland represent a family of androgen-de-

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pendent enzymes.[16] We have studied the androgen-like effect of Hachimi-jio-gan (Ba-Wei-Di-Huang-Wan) and it's components using trypsin-like protease as marker enzyme.[17,18] We have found that Hachimi-jio-gan or Aconiti Tuber and Rehmanniae Radix, the major component of Hachimi-jio-gan, had an androgen-like effect on both B10A and B10 strains.[19,20] Our study presents an interesting circannual biological rhythm in the trypsin-like protease activity of the mouse submaxillary gland in both B10A and B10 strains. The annual variation of trypsin-like protease activity of the submaxillary gland is androgen-dependent, *i.e.*, it exists only in male mice, but not in female mice, and the circannual rhythm in male mice is abolished by castration. Furthermore, the circannual rhythm in trypsin-like protease activity of the mouse submaxillary gland influences the drug effect in an inversely proportional pattern, *i.e.*, the higher the trypsin-like protease activity, the less the drug effect.

Materials and Methods

Animals : Eight-week-old B10A and B10 mice, male and female, were used in this experiment. The animals were kept in rooms with controlled temperature (23±2℃) and humidity (55±10%) and with a 12-hr dark-light cycle. The animals were supplied with standard mice chow and water *ad libitum*. Castration of the male mice was carried out under nembutal anesthesia (0.01 mg/g B.W.) with bilateral excision of testis and associated epididymides at an age of 4 weeks and kept for another 4 weeks before use, or at an age of 6 weeks and kept for another 2 weeks.

Extraction of Rehmanniae Radix and Corni Fructus : Rehmanniae Radix (Jukujio, 熟地黃), *Rehmannia glutinosa* (GAERTN) LIBOSCH. var *hueichingensis* CHAO et SCHIH, produced in Henan (河南) province of China, and Corni Fructus (Sanshuyu, 山茱萸), *Cornus officinalis* SIEB. et ZUCC, produced in Korea, were obtained from Ohminedo Co., Ltd. (Nara, Japan). Ten percent (w/v) of Rehmanniae Radix in 99% ethyl alcohol and 10% (w/v) of Corni Fructus in 99% ethyl alcohol were heated for 3 hr at the temperature of 55℃. The extracts, filtered by passage through cotton, were evaporated by mild heating in a vacuum and continuous stirring until dense syrups were obtained. The Rehmanniae Radix syrup (0.8 g B.W./day) and the Corni Fructus syrup (4.6 g/kg B.W./day) were suspended in water respectively and was administered orally to each animal by means of a metal gastric tube at 2 : 00 to 3 : 00 p.m. daily for 14 days. The control mice were administered with distilled water.

Tissue preparation : The mice were sacrificed by cervical dislocation. The submaxillary glands were removed, and the blood was washed away in ice cold saline and mice were dissected free of the adipose and lymphatic tissues. The tissue was homogenized in 9 volumes of deionized water at 4℃ in glass homogenizers and centrifuged twice at 16,000×g for 30 min. The supernatants were used immediately for the measurement of enzyme activity, protein content assay and electrophoretic analysis.

Spectrophotometric assay for trypsin-like protease activity : Trypsin-like protease activity in submaxillary glands was measured by the method of Taie and Ogita.[21] The tissue extract (0.5 ml) was incubated at 37℃ for 60 min with 1.0 ml of substrate solution containing 1 mM $N\alpha$-benzoyl-D, L-arginine-p-nitroanilide (BAPNA), 2% of dimethylsulfoxide (DMSO) and 50 mM phosphate buffer (pH 7.6). The reaction was stopped by the addition of 0.5 ml of 20% perchloric acid. The mixture was then centrifuged at 3,000×g for 30 min at room temperature, and 1.0 ml of the supernatant was mixed with 1.0 ml of pre-cooled 0.2% sodium nitrite solution and kept in an ice bath for 10 min. One milliliter of 0.5% ammonium sulfamate solution was added to the solution to destroy the excess sodium nitrite. Two milliliters of 0.05% N-1-naphthylethylenediamine dihydrochloride solution was added and the solution was incubated at room temperature for 30 min. The absorbance at 546 nm density was measured in a Hitachi model 200/20 spectrophotometer. Protein content in the supernatant was measured by the method of Lowry *et al.*[22] Highly purified bovine serum albumin (Sigma

Chemical Co.) was used as a standard. One unit was defined as the amount of enzyme required to hydrolyze 1 μmol of BAPNA in 1 min at 37℃. Specific activity was indicated as one unit of enzyme in 1 mg of total protein.

Electrophoresis : Three microliters of double diluted tissue extract was placed on a vertical 8% polyacrylamide gel slab and electrophoresis was carried out at a constant current of 1 mA/cm of gel width for 2 hr by the method of Ogita and Markert[23] and isozyme activities of trypsin-like protease were visualized by use of specific staining technique of Isobe and Ogita[24] employing BAPNA as substrate.

Statistical analysis : The difference between the control group and the experimental groups were statistically evaluated using the Student's t-test.

Results

The trypsin-like protease activity of the submaxillary gland in mice was measured at two month intervals for two years (from October, 1986 to September, 1988). The mean values at each point of the observations showed a typical circannual rhythm in male mice with peak values in December－January, and February－March and a nadir in June－July (Fig. 1). In contrast, no circannual rhythm was observed in female and castrated male mice in trypsin-like protease activity of the submaxillary gland (Fig. 1). The values of trypsin - like protease activity of male mice in winter months (December－February) were significantly higher than those in summer months (June－August) with $p<0.001$ and the results were highly reproducible in two-year period observations (Fig. 2). Although the values of trypsin-like protease activity in male mice changed dramatically from summer to winter, the bands of protease isozymes, named A to F as revealed by zymogramatic analysis, were still consistent, which was qualitatively different from those of castrated male mice and female mice (Fig. 3).

The effect of Rehmanniae Radix on trypsin-like protease activity of the mouse submaxillary gland in males was affected by circannual rhythms. The trypsin-like protease activity in male mice was increased to a larger extent in summer ($p<0.001$), in which the trypsin-like

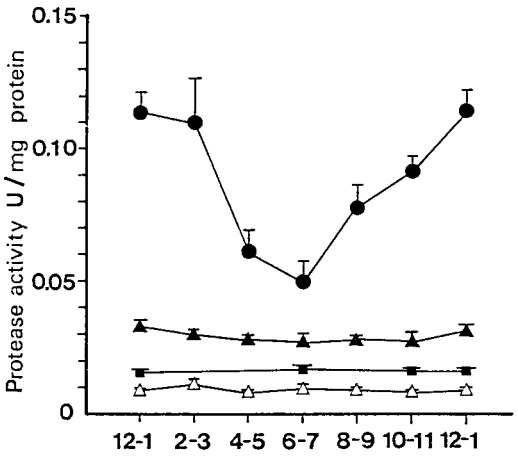

Fig. 1 Trypsin-like protease activity of the submaxillary gland of ● : male (n=15－16), ▲ : castrated male for 2 weeks (n=7－8), ■ : castrated male for 4 weeks (n=6), and △ : female (n=6－7) mice in different months of the year (1986－1988). n : number of animals.

Each point represents mean value and bar indicates S.E. Horizontal axis line shows month.

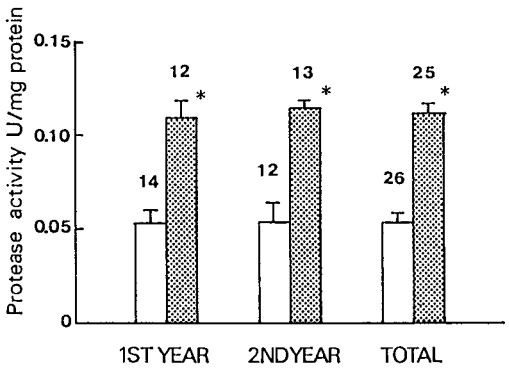

Fig. 2 Comparison of trypsin-like protease activity of the submaxillary gland of male mice in summer and in winter in two year periods.

First year observation was from October, 1986 to September, 1987, and second year was from October, 1987 to September, 1988. Each column represents the mean value and bar indicates S.E. Numbers in parentheses above the bar are animals tested. Open column indicates the mean value in summer (between June and August) and dotted column represents the mean value in winter (between December and February). * : Significantly different with $p<0.001$.

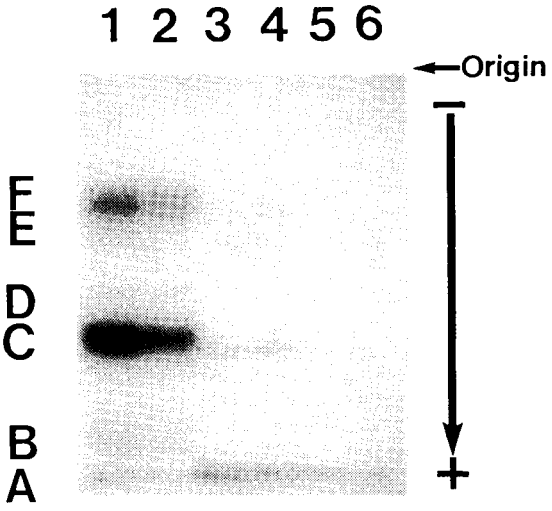

Fig. 3 Zymograms of trypsin-like protease in submaxillary gland. A to F show the bands of protease isozymes. Lane 1 : male mouse in December ; Lane 2 : male mouse in July ; Lane 3 : castrated mouse for 2 weeks in December ; Lane 4 : castrated mouse for 2 weeks in July ; Lane 5 : female mouse in December ; Lane 6 : female mouse in July.

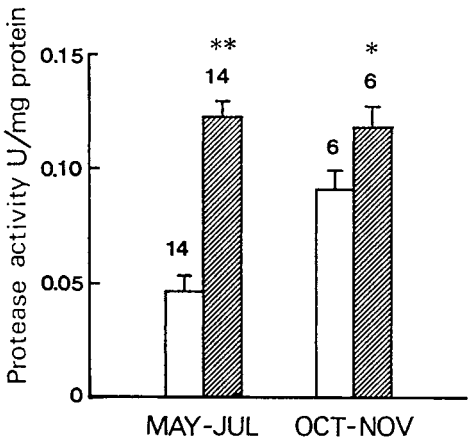

Fig. 4 Comparison of the effect of Rehmanniae Radix on trypsin-like protease of the submaxillary gland in summer (May − July) and in winter (October − November) in male mice.

Each column represents the mean value and bar indicates S.E. Numbers in parentheses are animals tested. Open column : mice given distilled water as control ; hatched column : mice given 0.8 g of Rehmanniae Radix extract/kg B.W./day for 2 weeks. * : $p<0.05$; ** : $p<0.001$.

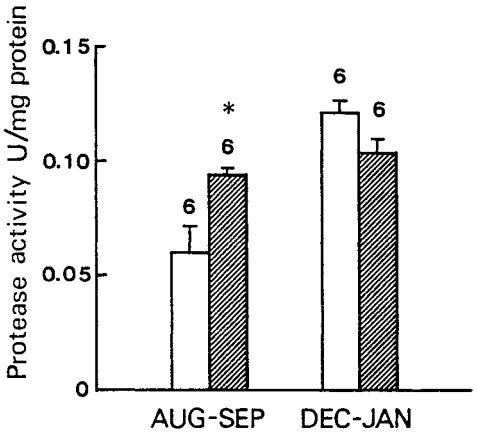

Fig. 5 Comparison of the effect of Corni Fructus on trypsin-like protease of submaxillary gland in summer (August − September) and winter (December − January) in male mice.

Each column represents the mean value and bar indicates S.E. Numbers in parentheses are animals tested. Open column : mice given distilled water as control ; hatched column : mice given 4.6 g of Corni Fructus extract/kg B.W./day for 2 weeks. * : Significantly different with $p<0.01$.

protease activity before treatment was relatively lower, whereas the trypsin-like protease activity was slightly increased in winter months ($p<0.05$), in which the trypsin-like protease activity before drug administration was relatively higher (Fig. 4). Similar results were observed in the effect of Corni Fructus, in which the trypsin-like protease activity was greatly increased in summer months by administration of Corni Fructus for 2 weeks ($p<0.01$), whereas the trypsin-like protease activity was even slightly decreased ($p<0.05$) after Corni Fructus feeding (Fig. 5). No significant effect was observed in castrated male and female mice upon administration of Rehmanniae Radix (Fig. 6).

Discussion

Circadian and circannual biological rhythms, having been widely documented for a multitude of physiological variables of both plant and animals species, including man, are quite familiar to scientists. Obviously, with respect to evolution, cir-

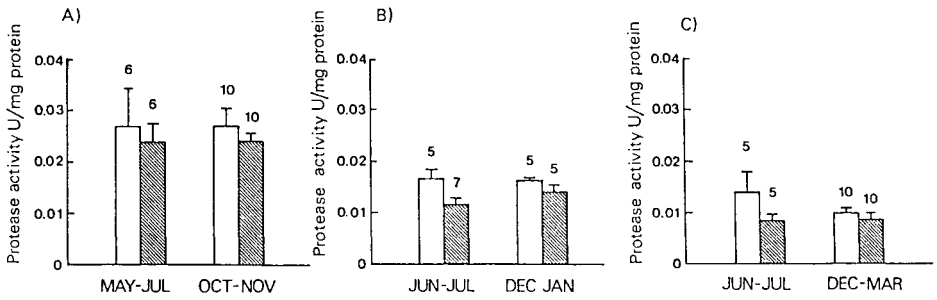

Fig. 6 Comparison of the effect of Rehmanniae Radix on trypsin-like protease of submaxillary gland in summer (May－July or June－July) and in winter (October－November or December－January or December－March) in castrated male mice for 2 weeks (panel A) and in castrated male mice for 4 weeks (panel B) and in female mice (panel C).
Each column represents the mean value and bar indicates S.E. Numbers in parentheses are animals tested. Open column : mice given distilled water as control ; hatched column : mice given 0.8 g of Rehmanniae Radix extract/kg B.W. per day for 2 weeks.

cadian biorhythms appear to be related to the rotation of the earth around its axis, and circannual biorhythms appear to be related to the rotation of the earth around the sun. The cyclic changes in environmental factors, such as the alternation of light with darkness and/or of heat with cold, may play an important role in these biorhythms. During the process of long term evolution, many biorhythms are not merely passive responses to the cyclic changes in the environment ; they become endogenous, *i.e.*, they exist even if the environmental conditions are kept constant. The circannual rhythm of trypsin - like protease activity of mouse submaxillary gland in males, as reported in the present paper, is one example. The animals have been kept in controlled temperature and humidity without any seasonal change for generations in the laboratory, but the trypsin-like protease activity in male mice is still shown in a typical circannual rhythm with high values in winter months and low values in summer months. These results are quite reproducible in the period of two-year observations.

This circannual biorhythm in trypsin-like protease activity of the submaxillary gland exists only in male mice, but not in female mice, and the biorhythm is completely abolished in male mice following castration. Testosterone propionate treatment to castrated male mice of B10A strain (8 weeks old after 2 weeks of castration) in summer (July) or winter (December), showed no difference of the trypsin-like protease activity between the treatment in summer and in winter (data not shown). These results suggested that the circannual rhythm in the trypsin-like protease mainly depended on plasma testosterone propionate level. It has been reported that seasonal rhythm in plasma testosterone, with high values in winter and low values in summer, was found in rams, rats, and monkeys.[25-27]

In vivo administration of Rehmanniae Radix and Corni Fructus increases trypsin-like protease activity of the submaxillary gland in male mice.[20] This drug effect is greatly affected by circannual rhythm in trypsin-like protease activity. The effect of Rehmanniae Radix on trypsin-like protease activity in males is relatively higher in summer months, when the original trypsin-like protease activity is low, and the effect of Rehmanniae Radix is significantly lower in winter months when the original value of trypsin-like protease activity is high. Similarly, the effect of Corni Fructus on trypsin-like protease activity is relatively high in summer when the trypsin-like protease value is low before administration of its herb extract, whereas the effect of Corni Fructus is not observed in winter when the original trypsin-like protease value is high. More interestingly, as

we have shown in male mice that the lower the trypsin-like protease activity, the higher the drug effect on trypsin-like protease, whereas in female and castrated male mice, the trypsin-like protease activity is also quite low, but no effect is induced by *in vivo* administration of its extract.

The increasing effect of Hachimi-jio-gan [17] and its component herbs [18-20] on trypsin-like protease in mouse submaxillary gland show the upper limit activity. These results indicate that Oriental medicines have influence on the homeostatic mechanisms.

和文抄録

我々はB10A及びB10系統マウスの顎下腺トリプシン様プロテアーゼ活性の年間リズムについて観察を行った。このリズムは雄マウスにおいてのみ観察され，冬期において活性が高く，夏期において活性が低かった。雄マウスのトリプシン様プロテアーゼ活性の年間リズムは，去勢により観察されなくなるため，男性ホルモン依存性のあることが示唆される。この年間リズムは熟地黄，山茱萸などの和漢薬によるトリプシン様プロテアーゼ活性の上昇効果に影響を及ぼした。すなわち，活性の低い夏期にこれらの生薬エキスを投与することにより，本酵素活性は明らかに上昇したが，活性の高い冬期においては生薬エキスの投与による酵素活性の上昇はほとんど認められなかった。

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