Androgen-dependent effect of Rehmanniae Radix on trypsin-like protease of the mouse submaxillary gland

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Abstract

Oral administration of Rehmanniae Radix (R.R.) increases trypsin-like protease activity of the submaxillary gland in both B10A and B10 strain mice. This effect is not shown in females and castrated male mice of both strains, which indicates that the effect of R.R. is androgen-dependent, but R.R. itself has no androgen-like effect. However, the R.R. effect on trypsin-like protease is induced in castrated male mice by administration of R.R. (0.8 g/kg B.W./day) and testosterone propionate (TP) (0.5 mg/30 g B.W./2 days, i.p.) two weeks after castration for two weeks. The effect of R.R. is absent in both female mice and Tfm mice, an androgen receptor defect mutant strain, with administration of both R.R. and TP for two weeks. The results suggest that testosterone and its receptors are crucial for the effect of R.R. on trypsin-like protease of the mouse submaxillary gland. The responses of mouse submaxillary trypsin-like protease to R.R. in male as well as castrated male of B10A and B10 strain mice are slightly different between strains. The two strains of mice are derived from congenic ancestors and are different from each other only at the H-2 locus region of chromosome, and therefore it is conceivable that the regulatory mechanisms of R.R. effect on trypsin-like protease may involve the H-2 region of chromosome 17.

Key words trypsin-like protease, Rehmanniae Radix, testosterone, strain-related difference, sexual dimorphism.

Abbreviations R.R., Rehmanniae Radix (Jukujio ; 熟地黄) ; TP, testosterone propionate ; GCT, granular convoluted tubule ; BAPNA, *N*-benzoyl-D, L-arginine-*p*-nitroanilide ; Tfm, testicular feminization mouse ; Hatimi-ziô-gan (Ba-Wei-Di-Huang-Wan), 八味地黄丸 ; Rokumi-ziô-gan (Liu-Wei-Di-Huang-Wan), 六味地黄丸 ; syndrome of kidney deficiency (Shen-Xu-Zheng), 腎虚証.

Introduction

Hatimi-ziô-gan (Ba-Wei-Di-Huang-Wan) and Rokumi-ziô-gan (Liu-Wei-Di-Huang-Wan) are the main recipes for the syndrome of kidney deficiency (Shen-Xu-Zheng) in oriental medicine. The syndrome of kidney deficiency is characterized by reproductive system symptoms, such as genetopathy, impotence, prospermia, spermatorrhea, habitual abortion and infertility, and other symptoms, such as lumbago, lassitude, giddiness, tinnitus, asthma, forgetfulness. It has been shown that the hypothalamo-hypophysio-gonad, hypothalamo-hypophysio-adrenal gland and hypothalamo-hypophysio-thyroid disfunctions are involved in this syndrome.[1)]

Since the discovery of kallikreins in the submaxillary (or submandibular) gland in 1936,[2)] a great number of biologically active polypeptides has been purified from, or claimed to be present in the submaxillary gland of the mouse and other species, some of which are androgen-dependent, including nerve growth factor,[3-5)] epidermal

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growth factor[6,7] renin[6,8] and several esteroproteolytic enzymes.[9-11] These observations suggest that those androgen-dependent substances are synthesized and stored in granular convoluted tubule (GCT) cells of the submaxillary gland. The androgen-mediated differentiation of GCT cells in the gland is fundamentally different from that of androgen target tissues, such as prostate and the external genitalia, in that the sexual dimorphism of GCT cells is not apparent until the onset of puberty.[12-14] The character of androgen-mediated differentiation of GCT cells and androgen-dependent biologically active substances provides us an opportunity for monitoring the various changes of androgen response, which may be involved in the syndrome of kidney deficiency. Taie *et al.*[15,16] showed that both Hatimi-ziô-gan had an *in vivo* androgen-like effect on trypsin-like protease (arginine aminopeptidase) in the mouse submaxillary gland, which activity was significantly reduced or completely abolished in female or castrated male mice. Since testosterone is the major androgen hormone in vertebrates, the question that whether this sex hormone is involved in androgen-like effect of Hatimi-ziô-gan and Rokumi-zio-gan, or R.R., the major element in both recipes, on trypsin-like protease in the mouse submaxillary gland becomes one of the very important issues of our study.

Materials and Methods

Animals : Eight-week-old B10A and B10 mice, male and female, and Tfm mice were used in this experiment. The animals were kept in rooms with controlled temperature (23±2℃) and humidity (55±10%) and 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.) at an age of 4 weeks and mice were kept for another 4 weeks before use, or castration was carried out at an age of 6 weeks, mice were kept for another 2 weeks. Some of the castrated male mice were injected with TP (0.5 mg/30 g B.W.) intraperitoneally every other day during the Rehmanniae Radix feeding.

Extraction of Rehmanniae Radix : Rehmanniae Radix (R.R.), *Rehmannia glutinosa* (GAERTN) LIBOSCH. var. *hueichingensis* CHAO et SCHIH, was obtained from Ohminedo Co., Ltd. (Nara, Japan) (produced in Henan (河南) province of China.). Ten percent (w/v) of R.R. in 99% ethyl alcohol was heated for 3 hr at the temperature of 55℃. The extract, filtered by passage through cotton, was evaporated by mild heating in a vacuum and continuous stirring until a dense syrup was obtained. The syrup 0.8 g, 3.2 g, 6.4 g/kg B.W./day, respectively, was suspended in water 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, the blood washed away in ice cold saline and mice were dissected free of the adipose and lymphatic tissue. 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 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.[17] The tissue extract (0.5 ml) was incubated at 37℃ for 60 min with 1.0 ml of substrate solution containing 1 mM BAPNA, 2% of dimethylsulfoxide and 50 mM phosphate buffer (pH 7.6). The reaction was stopped by 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*.[18] Highly purified bovine serum (Sigma Chemical Co.) was used as a standard. The results are expressed as a unit of protease activity per milligram protein. One trypsin-like protease activity was defined as the amount of enzymes that hydrolyzed 1.0 μmol of *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) per minute.

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[19] and isozyme activities of trypsin-like protease were visualized by use of the specific staining technique of Isobe and Ogita[20] employing BAPNA as substrate.

Free serum testosterone measurement : Free testosterone in the blood circulation was tested by the radioimmunoassay method using free [^{125}I]-testosterone and anti-testosterone antibody precoated on the test tubes (Diagnostic Products Corporation, Los Angeles, CA, U.S.A.). Blood was collected by intraperitoneally bleeding under ether anesthesia and serum was separated. Fifty microliters of serum from each animal was incubated with 1.0 ml of free [^{125}I]testosterone in testosterone antibody-coated tube at 37℃ for 4 hr. Both free testosterone in the sample and free [^{125}I]testosterone were competitively bound to the antibody. The bound [^{125}I]testosterone was then counted in a gamma couuter. The counts were inversely related to the concentration of free testosterone in the sample. A calibration was made with commercially available testosterone from the same company.

Results

The trypsin-like protease activity of B10A and B10 strain used in this study showed androgen-dependent, however B10 strain showed high response compared to B10A strain (Fig. 1). The androgen-dependent effect was also revealed by

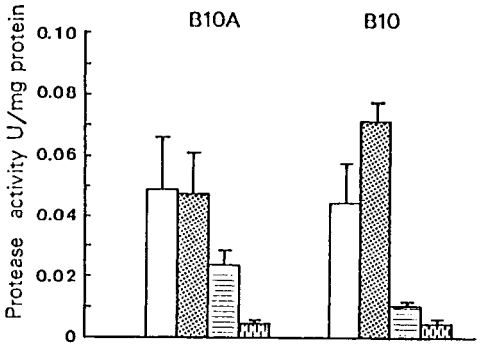

Fig. 1 Trypsin-like protease activity of the submaxillary gland in B10A and B10 strain mice. Each column represents the mean value for 5 samples and the bar indicates 1 S.E. Open column : male mice ; dotted column : castrated male mice given TP (0.5 mg/30 g B.W./2day, i.p.) for 2 weeks ; horizontal hatched column : female mice ; vertical hatched column : castrated mice for 4 weeks.

zymogramatic analysis. Six bands of protease, named A to F, were clearly shown in male B10A and B10 strain mice. In the male B10A strain, five of the six bands, from band B to F, gradually disappeared 2 weeks and 4 weeks after castration, and only band A which is characterized in female mice was not affected by castration. The pattern of protease isozymes was completely converted to that of male pattern administration of testosterone to the castrated mice for 2 weeks (Fig. 2). Male B10 strain mouse also showed the same zymogram pattern (data not shown). The trypsin-like protease activity of the male mouse submaxillary gland was significantly increased by oral administration of R.R. extract in both B10A and B10 strains, but the responses in both strains were slightly different. In B10A male mice, the protease activity was significantly increased after treatment with a small dose (0.8 g/kg B.W./day) and a large dose (6.4 g/kg B.W./day) of R.R., but was not changed with a medium dose (3.2 g/kg B.W./day) of R.R. In B10 male mice, the protease activity was not changed after treatment with both small and medium doses of R.R. and was significantly increased with a large dose of R.R. (Fig. 3A). The trypsin-like protease activity of castrated male mice was significantly reduced by

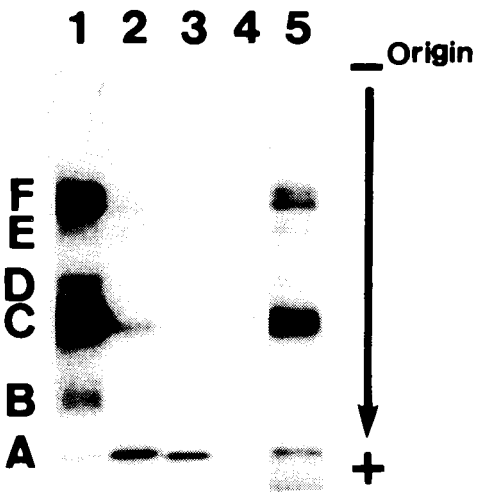

Fig. 2 Zymograms of trypsin-like protease in submaxillary gland in B10A mice.
A to F show the bands of protease isozymes. Panel 1 : male mice ; panel 2 : castrated mice for 2 weeks ; panel 3 : castrated mice for 4 weeks ; panel 4 : female mice ; panel 5 : castrated mice given TP (0.5 mg/30 g B.W./2 days, i.p.) for 2 weeks.

small, medium and large doses of R.R. in B10A mice, but not in B10 strain mice (Fig. 3B). The protease activity of female mice was not significantly changed by any doses of R.R. in both B10A and B10 mice (Fig. 3C). Similarly, six isozyme bands of protease, bands A to F, which show androgen-dependence, were all increased in male but not female and castrated male mice following R.R. feeding. The response in B10A and B10 strains was also slightly different (Fig. 4). The protease activity of the submaxillary gland was not affected following administration of R.R. and TP for 2 weeks in female mice. A similar result was obtained in castrated male mice when both R.R. and TP were given 4 weeks after operation (Fig. 5A). However, a significant increase ($p<$ 0.01) of trypsin-like protease activity was shown in those castrated B10A mice when both R.R. and TP were given 2 weeks after surgery. Administration of TP did not show a significant increase of trypsin-like protease activity compared to administration of R.R. and TP. No significant

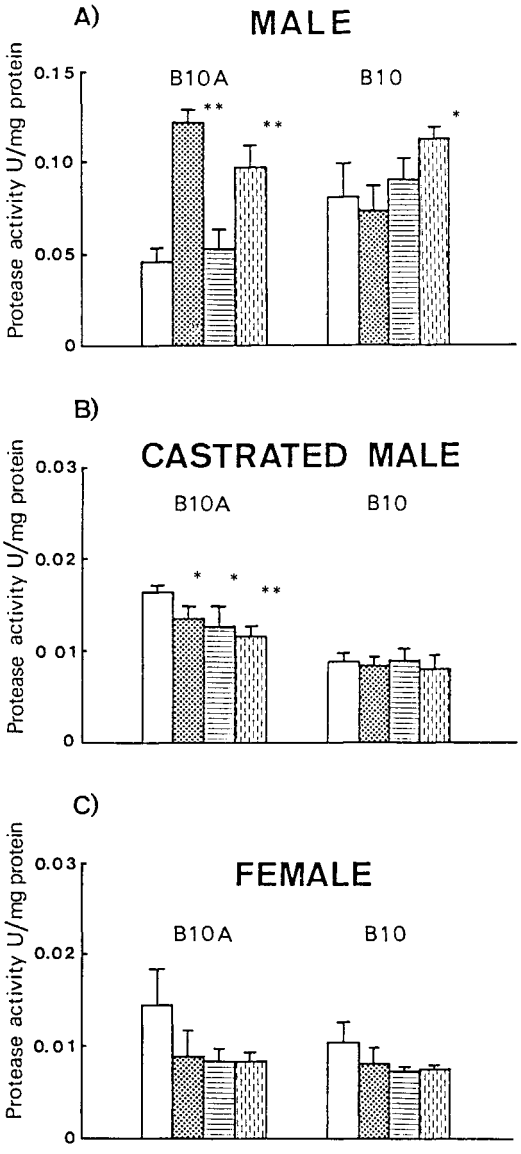

Fig. 3 The effect of R.R. on trypsin-like protease of submaxillary gland in male (A), castrated male (B) and female (C) of B10A and B10 mice.
Columns represent the mean value for 14 (male), 10 (castrated male) and 5 (female) samples, respectively. A bar indicates 1 S.E. Open column : mice given distilled water as control ; dotted column : mice given 0.8 g of R.R. extract per kg B.W. per day ; horizontal hatched column : mice given 3.2 g of R.R. extract per kg B.W. per day ; vertical hatched column : mice given 6.4 g of R.R. extract per kg. B.W. per day. * and ** : significantly different from control with $p<0.05$ and $p<0.01$, respectively.

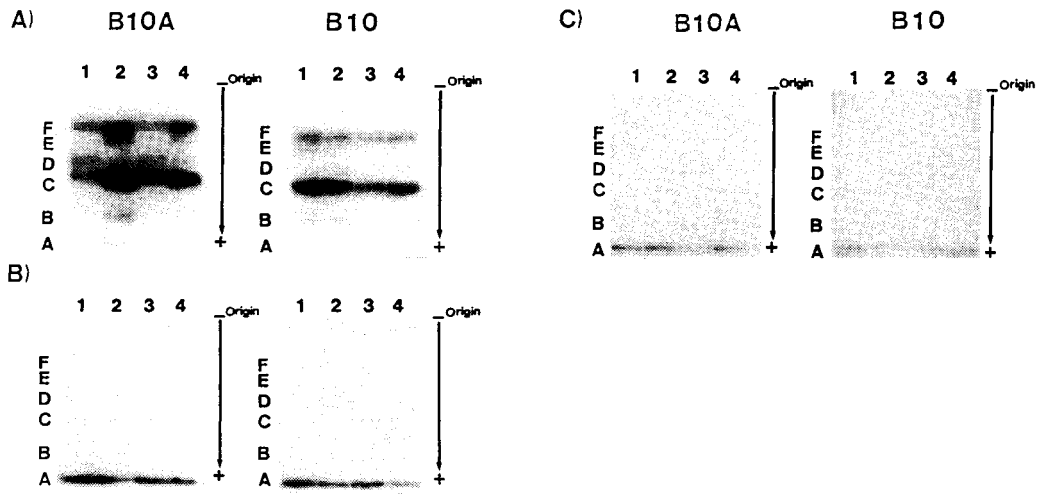

Fig. 4 The effect of R.R. on trypsin-like protease of the submaxillary gland in male (A), castrated male (B) and female (C) of B10A and B10 strain mice.

A to F show the bands of protease isozymes. Panel 1 : mice given distilled water as control ; panel 2 : mice given 0.8 g of R.R. extract per kg B.W. per day ; panel 3 : mice given 3.2 g of R.R. extract per B.W. per day ; panel 4 : mice given 6.4 g of R.R. exract per kg B.W. per day.

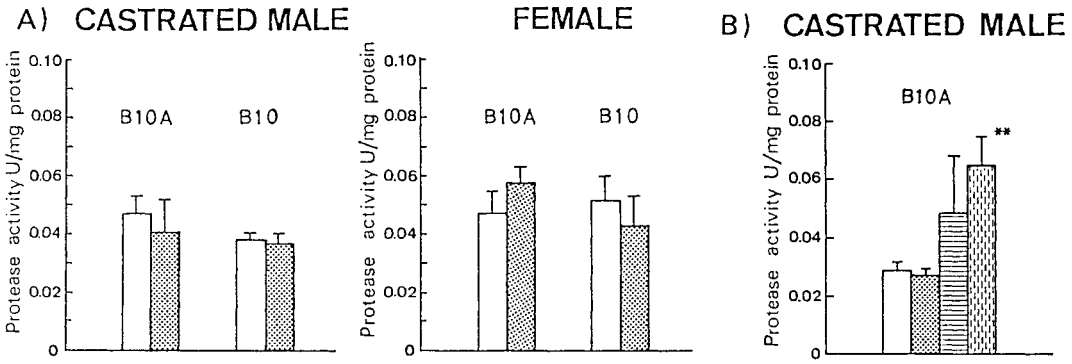

Fig. 5A The effect of R.R. and TP trypsin-like protease of the submaxillary gland in castrated (A) and female (B) mice.

Castration was carried out at an age of 4 weeks and both R.R. and TP were given 4 weeks later for 2 weeks. Each column represents the mean value for 6 samples and the bar indicates 1 S.E. Open column : mice given distilled water daily and 0.5 mg of TP per 30 g B.W. per 2 days, i.p. : dotted column : mice given 0.8 g (B10A), 6.4 g (B10) of R.R. extract per kg B.W. per day and 0.5 mg of TP per 30 g B.W. per 2 days, i.p.

Fig. 5B The effect of R.R. and TP on trypsin-like protease of the submaxillary gland in castrated B10A mice.

Castration was carried out at 6-weeks and both R.R. and TP were given 2 weeks later for 2 weeks. Each column represents the mean value for 5 samples and the bar indicates 1 S.E. Open column : mice given distilled water saline (0.25 ml/30g B.W./2 days, i.p.) as control ; dotted column : mice given R.R. extract (0.8 g/kg B.W./day) and saline (0.25 ml/30g B.W./2 days, i.p.) ; horizontal hatched column : mice given distilled water and TP (0.5 mg/30 g B.W./2 days, i.p.) ; vertical hatched column : mice given R.R. extract (0.8 g/kg B.W./day) and TP (0.5 mg/30 g B.W./2 days, i.p.). ** : significantly different from control with $p < 0.01$.

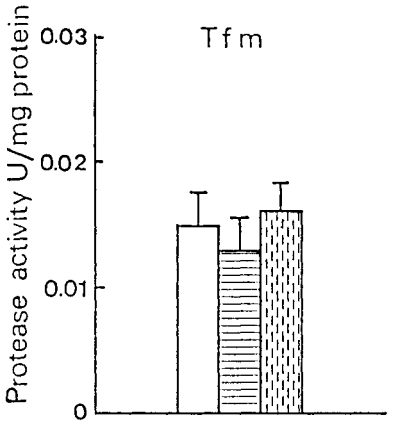

Fig. 6 The effect of R.R. on trypsin-like protease of the submaxillary gland of Tfm mice. Each column represents the mean value for 4 samples and the bar indicates 1 S.E. Open column : mice given distilled water and saline (0.25 ml/30 g B.W./2 days, i.p.) as control ; horizontal hatched column : mice given distilled water and TP (0.5 mg/30 g B.W./2 days, i.p.) ; vertical hatched column : mice given R.R. extract (0.8 g/kg B.W./day) and TP (0.5 mg/30 g B.W./2 days, i.p.).

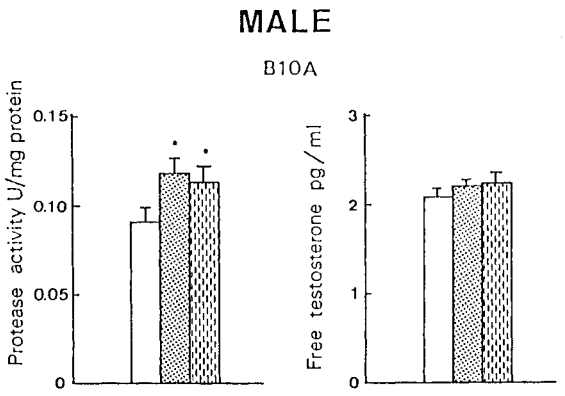

Fig. 7 Free circulating testosterone level in relation to the effect of R.R. on trypsin-like protease of the mouse submaxillary gland. Each column represents the mean value for 5 samples and the bar indicates 1 S.E. Open column : mice given distilled water as control ; dotted column : mice given R.R. extract (0.8 g/kg B.W./day) ; vertical hatched column : mice given R.R. extract (6.4 g/kg B.W./day). * : significantly different from control with $p<0.05$.

difference was shown when the mice were given R.R. plus saline at the same time (Fig. 5B). The effect of R.R. on trypsin-like protease was not shown in Tfm mice (Fig. 6). The level of free testosterone in blood circulation of B10A male mice was not affected by giving different doses of R.R. extract in which a significant effect on protease was observed (Fig. 7).

Discussion

Consistent with the observations of other investigators,[9,10] the present data show that the trypsin-like protease activity of the mouse submaxillary gland is androgen-dependent, indicated by its low activity in female and castrated male mice, and a complete recovery by administration of TP to the castrated male mice. The protease activity of the submaxillary gland is also significantly increased following administration of R.R. for 2 weeks in male mice, but this effect is not shown in both female and castrated male mice. This indicates that the effect of R.R. on trypsin-like protease is also an androgen-dependence. Unlike testosterone, administration of R.R. to the castrated mice does not convert the trypsin-like protease activity from female pattern to the that of male activity, which suggests that R.R. does not have androgen-like effect by itself, but its effect on target cells relies on male sex hormones. Since testosterone, one of the major androgens, is synthesized by the interstitial (Leydig) cells of the testes and its concentration in blood circulation is apparently diminished by castration,[21] we then supposed that the R.R. effect could be induced in female and castrated male mice via a supply of the proper amount of testosterone. In fact, this did not occur in female mice (Fig. 5A), as well as in castrated male mice when both R.R. and TP were given 4 weeks after castration. However, this did happen in those castrated mice when both R.R. and TP were given 2 weeks after surgery. Mainwaring *et al.*[22] showed that after castration, the receptor binding affinity for androgen in target cells was gradually decreased. Baulieu *et al.*[23] reported

that less cytoplasmic androgen receptors were present in the rat prostate gland after long-term castration. Roy and co-workers[24] showed that the level of hepatic cytosol receptor activity in adult male rats was gradually decreased after castration and was hardly detected at 18 days after castration. These suggest that in castrated male mice, long-term castration gradually reduces the number of androgen receptors and receptor binding affinity on target cells, and hence more difficulty is expected for recovery of the response of target cells to androgen hormones. So, the R.R. effect was shown only in those mice who were given both R.R. and TP 2 weeks after castration, but not those who were given both drugs 4 weeks after castration. Although the target cells of female mice have the ability to respond to male sex hormones, R.R. effect was still not shown in female mice when both R.R. and TP were administered. This could be, at least partially, explained by the fact that estrogen, whith is a dominant sex hormone in female animals, acts as a potent inhibitor of androgen binding receptors. Roy *et al.*[24,25] showed that hepatic androgen receptor activity was well induced by administration of dihydrotestosterone in ovariectomized female rats rather than in normal female rats, and treatment of the adult male rats with estradiol for 8 days completely inhibited cytosol androgen receptor activity.

The oral administration of R.R. had no effect on the trypsin-like protease of Tfm mice. Tfm mice were proved to be a specific kind of mutant animal, who have normal testosterone-secreting testes but defective testosterone receptors in target tissues, therefore, they cannot respond to the testosterone.[26,27] Since the other aspects of the cells are normal in Tfm mice and only the gene coding for testosterone receptors is abnormal, the lack of the effect of R.R. on protease activity further suggests that testosterone and its receptors are crucial for R.R. to be effective.

The free testosterone in serum of B10A strain mice upon administration of R.R. was also tested. No significant difference was shown. The result is not surprising, since more than 90% of testosterone circulating in the plasma is bound to testosterone-binding globulin,[28] the concentration of free testosterone does not reflect the actual level of circulating androgen hormones. An increase in total circulating testosterone level in response to R.R. is still not excluded in this study. Further investigation needs to be done in order to fully understand the results.

Administration of TP, trypsin-like protease activity was increased on both strains ; the B10 strain showed higher response to TP than B10A strain. In addition, the R.R. effect on trypsin-like protease of the mouse submaxillary gland is different in B10A and B10 strain mice, as revealed in Figs. 3 and 4, in that the trypsin-like protease activity in B10A males is significantly increased by small doses and large doses of R.R. but not by medium doses of the drug, whereas the trypsin-like protease activity in B10 males is increased only by administration of large doses of R.R. The protease activity in castrated male mice is significantly reduced in B10A mice by administration of R.R., but is not affected in B10 mice. These results suggest that the effects of R.R. to trypsin-like protease in the mouse submaxillary gland are different from that of TP. The two strains of mice are derived from congenic ancestors and are different from each other only at the H-2 locus region of chromosome 17.[29] The different responses of two strains of mice to R.R. imply that the regulatory mechanisms of the R.R. effect on trypsin-like protease of the mouse submaxillary gland may involve H-2 region on chromosome. It has been long realized that strain-related difference is one of the important elements in Oriental medicine, *i.e.*, it may affect the manifestation of the syndromes and the treatment. Our finding in the present paper provides evidence of genetic basis for the strain-related difference in response to the effect of R.R. Further study on this aspect will certainly help us to understand the syndromes of Oriental medicine and will improve the efficiency of treatment.

和文抄録

熟地黄成分はマウス顎下腺トリプシン様プロテアーゼ活性を上昇させる効果のあることがB10Aと

B10系統マウスの雄に認められたが，去勢した雄と雌には認められなかった。これは熟地黄の効果が精巣依存性であり，熟地黄エキス成分には直接的な男性ホルモン様作用のないことを示唆している。去勢2週間後のB10A雄マウスに熟地黄エキスと低濃度のテストステロンプロピオネートを同時に投与した場合，トリプシン様プロテアーゼ活性上昇について協力効果が認められた。テストステロンレセプターを遺伝的に欠損したTfmマウスに熟地黄エキス並びにテストステロンを同時に投与してもトリプシン様プロテアーゼ活性値の上昇が認められなかった。以上の結果は熟地黄がトリプシン様プロテアーゼ活性を上昇させる機構にテストステロンレセプターの関与が考えられる。また，熟地黄成分に対する応答性の差異をマウス顎下腺トリプシン様プロテアーゼ活性値を指標として雄，去勢した雄，雌それぞれについて比較した。B10A及びB10マウスにおいて，いずれの場合も熟地黄成分に対する応答性に系統差のあることが認められた。B10AとB10のマウスは相互にH-2遺伝子座のみが異なるコンジェニックな系統であることから，熟地黄成分に対する応答性の系統差はマウスの第17染色体のH-2遺伝子座付近の差に基づいている可能性がある。

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