발생과 생식 제 6권 제 1호, 31~35 (2002)
Dev. Reprod. Vol. 6, No. 1, 31~35 (2002)

Urine Analysis in Transgenic Mice Expressing the Growth Hormone-releasing Factor

Byung-Nam Cho†, Hoi-Kyung Jung, Yong-Dal Yoon[1] and Kelly E Mayo[2]

†School of Life Science, The Catholic University of Korea, Puchon, Gyeonggi-do 420-743, Korea
[1]Department of Biology, College of Natural Sciences, Hanyang University, Seoul 133-791, Korea
[2]Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208, USA

성장호르몬 방출인자를 발현하는 형질전환 생쥐에서 소변분석

조병남† · 정회경 · 윤용달[1] · Kelly E Mayo[2]

가톨릭대학교 생명과학부, [1]한양대학교 생명과학과, [2]노스웨스턴 BMBCB

ABSTRACT : The major urinary proteins(MUPs) of mice that bind hydrophobic molecules known as pheromones are regulated in part by the actions of growth hormone. The expression of the MUPs was therefore investigated in transgenic mice that express a human growth hormone-releasing factor gene from a metallothionein gene promoter(MT-GRF) and as a result have elevated growth hormone levels. MUPs were severely down-regulated in the urine of these animals compared to normal mice or to control transgenic mice expressing another gene(the inhibin a subunit) from the same metallothionein promoter(MT-Inh) and more MUPs disappeared in male mice than female ones. MUPs were also down-regulated in the urine of the MT-GRF-injected mice. In addition, it was observed that the urine of the MT-GRF mice included a high molecular weight protein that co-migrates with the major serum protein albumin, indicating an impairment in glomerular filtration within the kidney. The urinary loss of serum proteins was more severe in male MT-GRF mice than female ones. Thus the overexpression of human GRF mimics changes observed in MUP protein expression and glomerular function in other models of growth hormone hypersecretion with sex-dependent differential effects.

Key words : Growth hormone-releasing factor, Transgenic mice.

요 약 : 생쥐에서 페르몬으로 알려진 소수성의 분자와 결합하는 major urinary proteins(MUPs)는 부분적으로 성장 호르몬(GH)의 조절을 받는다. 본 연구에서는 MT-GRF로 형질전환되어 성장호르몬이 증가된 생쥐에서 MUP의 발현을 조사하였다. 이 MT-GRF로 형질전환된 생쥐의 소변에서 MUP이 대조군보다 심하게 저하되어 나타났고, 암컷보다 수컷에서 더 저하되어 나타났다. 또한 MT-GRF를 근육에 주사한 생쥐에서도 MUP는 저하되어 나타났다. 부가하여, MT-GRF로 형질전환시킨 생쥐의 소변에서 전기영동상에서 albumin과 동일하게 이동하는 고분자의 단백질이 다량으로 관찰되었는데 신장의 사구체 여과가 손상되었음을 암시하고 있다. 이 혈장 단백질의 손실도 암컷보다 수컷에서 심하게 나타났다. 결과적으로 GRF의 과다생성은 성별에 차별화된 영향을 미치면서 GH로 형질전환된 동물모델에서 관찰되는 MUP의 발현과 사구체 기능의 변화를 동일하게 유발하고 있었다.

INTRODUCTION

The mouse major urinary proteins(MUPs) are members of the lipocalin superfamily of proteins that characteristically bind small hydrophobic molecules known as pheromones(Bacchini et al., 1992; Bocskei et al., 1992; Robertson et al., 1996; Lehman-McKeeman et al., 1998). The MUPs have been suggested to function in territorial marking, kin recognition, and orientation by slowly releasing the pheromones while drying after urination (Cavaggioni et al., 1987; Robertson et al., 1996). Large quantities of MUP are predominantly produced in the liver and rapidly excreted in the urine(Finlayson et al., 1965). Normal male mice excrete 5- to 20-fold more MUPs than do females (Norstedt and Palmiter, 1984). The mouse genome contains approximately 35 MUP genes(Bennett et al., 1982; Bishop et al.,

†Correspondence: School of Life Science, The Catholic University of Korea, 43-1 Yokkok2-dong, Wonmi-gu, Puchon city, Kyonggi-do, 420-743, Korea. (Tel) 82-32-340-3358, (Fax) 82-32-340-3765, E-mail: ocean@ catholic .ac.kr

1982; Clark et al., 1982), which can be classified into six gene groups(Derman, 1981; Shahan et al., 1987). The MUP genes are under multihormonal control by factors including testosterone, thyroxine and growth hormone(GH), and different MUP genes display different patterns of hormonal regulation(Knopf et al., 1983; Al-Shawi et al., 1992). Levels of the MUPs are low in normal female mice as well as in male transgenic mice that express the human growth hormone gene from a heterologous metallothionein promoter(MT-GH mice, Norstedt and Palmiter, 1984). Expression of human growth hormone-releasing factor (hGRF), a major neuroendocrine regulator of GH secretion, in transgenic mice(MT-GRF mice, Hammer et al., 1985) results in many of the same changes observed with direct GH overexpression, including increased serum GH levels and increased somatic growth. In addition, glomerular enlargement has been reported in the kidney of both MT-GH and MT-GRF transgenic mice(Doi et al., 1988). However, there are additional phenotypic features that differ between MT-GH and MT-GRF transgenic mice, including reduced female fertility(unique to the MT-GH mice) and pituitary somatotroph hyperplasia(unique to the MT-GRF mice)(Hammer et al., 1985; Mayo et al., 1988). This study was therefore undertaken to investigate whether the changes in MUP expression observed in MT-GH mice also occur and whether the additional physiological changes occur in response to growth hormone -releasing factor overexpression in the MT-GRF transgenic mice.

MATERIALS AND METHODS

1. Transgenic Animals

The generation of the MT-GRF transgenic mice has been described(Hammer et al., 1985). Briefly, the MT-GRF transgene was constructed in such way that the mouse metallothionein-I gene promoter was fused to a human GRF minigene so that a single intron of the hGRF gene was retained. In order to exclude the effect of the mouse metallothionein-I gene promoter itself, the control MT-Inh line of mice was generated using the same metallothionein promoter and a rat inhibin α subunit cDNA clone(Cho et al., 2001). These mouse lines were maintained by breeding to control C57BL/6xSJL F1 or CD-1 mice for the MT-GRF and MT-Inh strains, respectively. Offspring from the already well established lines(line 765-2 and line 801-5) of the MT-GRF transgenic mice and mostly line 8 among three lines of the MT-Inh control transgenic mice were analyzed for the presence of the respective transgenes by DNA dot-blotting of tail samples removed at the time of weaning. Hybridization was performed as described elsewhere(Mayo et al., 1988; Cho et al., 1993) and cDNA clones specific for human GRF or rat inhibin α subunit were used as hybridization probes. All animals were maintained on water and laboratory chow provided *ad libitum*.

2. DNA Injection

Plasmid DNA for injection was purified using a slightly modified alkaline lysis method(Sambrook et al., 1989). Injection of 200 μg of MT-GRF in 50 μl of 10% sucrose in saline was performed twice, with 7 days between injections, into the quadriceps of two months of mice as described(Danko et al., 1997). The urine was collected at day 1, 2, and 3 after the MT-GRF injection.

3. Protein Analysis

The urine from the mice at 3 to 4 months of age was collected by bladder massage as described(Norstedt and Palmiter, 1984). Briefly, urine was centrifuged for 3 min at 12,000× g to remove particulates. The supernatant was put into SDS sample buffer(2%SDS; 60 mM Tris-HCl, pH 6.8; 10% glycerol; 5% β -mercaptoethanol) and heated at 100℃ for 90 sec. Blood was isolated by bleeding from the tail vein and serum was obtained after the blood was allowed to clot for 1 hour. Urine or serum samples were analyzed by polyacrylamide gel electrophoresis using 12%(w/v) acrylamide slab gels in a discontinuous buffer system as described by Laemmli(Laemmli, 1970). The gels were electrophoresed at 25 mA until the dye front had reached the bottom, and stained with Coomassie Blue R-520(Sigma Chemical Company). Molecular weights were established using SDS-PAGE protein standards(BioRad). All experiments were repeated using multiple animals and multiple gels.

RESULTS AND DISCUSSION

To investigate MUP expression in normal, MT-Inh control and MT-GRF transgenic mice, urine samples were analyzed using SDS/polyacrylamide gel electrophoresis. These data are presented in Fig. 1. As expected, the most abundant proteins in the urine of normal male mice were clustered near 20,000

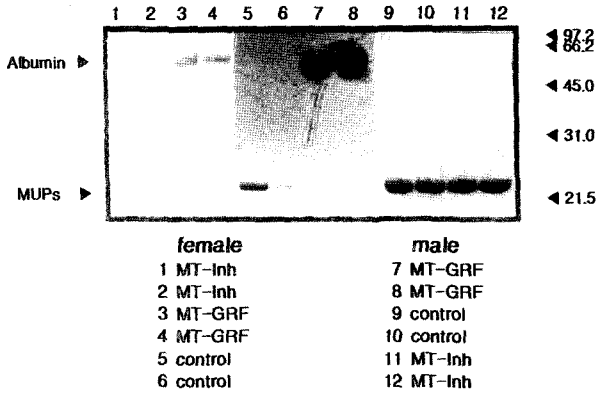

Fig. 1. Analysis of urinary proteins in control and MT-GRF transgenic mice. Urinary proteins were analyzed by polyacrylamide gel electrophoresis. Male and female mice that represent normal non-transgenic(control), a control transgenic(MT-Inh) which was adopted to exclude the effect of the MT-promoter itself or the test transgenic (MT-GRF) lines are as indicated. Molecular weight standards are shown, along with arrows indicating the migration of the major urinary proteins, (MUPS), and the major serum protein, (Albumin). Equivalent amount of urine(1 μl) from individual mouse was loaded in each lane and stained with Coomassie Blue R-520.

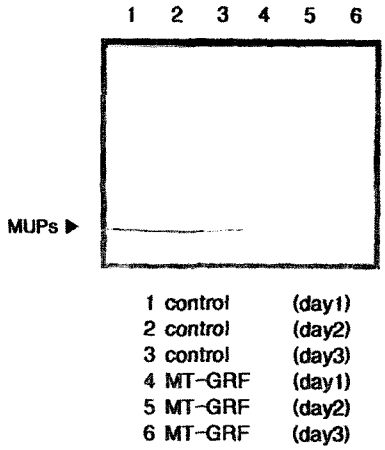

Fig. 2. Analysis of urinary proteins in MT-GRF-injected mice. Urinary proteins were analyzed by polyacrylamide gel electrophoresis. Female mice that represent normal(control), MT-GRF-injected(MT-GRF) are as indicated. Equivalent amount of urine(1 μl) from individual mouse was loaded in each lane. Day 1~3 represent the days after MT-GRF injection into muscle.

daltons, the known sizes of the MUPs. Also, there was significantly less MUP expression in normal female mice(Fig. 1, lanes 5 and 6) compared to males(Fig. 1, lanes 9 and 10). A similar pattern of MUP expression was observed when the control MT-Inh transgenic mice were examined, indicating that expression of a transgene from the MT promoter does not alone result in any change in MUP expression(Fig. 1, lanes 1, 2, 11, and 12). In contrast, urine samples from the MT-GRF transgenic mice showed a profound change in the pattern of MUP expression. As shown in Fig. 1, there are essentially no detectable MUPs observed in the urine of either female(lanes 3 and 4) or male MT-GRF mice(lanes 7 and 8). The down-regulated MUP was also observed at day 1 through day 3 in the urine of mice after injection of the MT-GRF(Fig. 2, lanes 3, 4, and 5). Direct DNA injection into muscle for transient expression of gene was used as reported(Danko et al., 1997). In our study, DNA injection under the control of MT promoter was adopted since MT promoter had been used for gene therapy (Trojan et al., 1994) and MT promoter had been known to be active *in vivo*(Palmiter et al., 1983; Cho et al., 2001).

It was previously reported in MT-GH transgenic mice that MUP levels are reduced to 3~12% of normal(Norstedt and Palmiter, 1984), and our findings suggest that a similar or more severe reduction is observed in response to overexpression of GRF in transgenic mice. This is consistent with a direct role for GH in the regulation of MUP gene expression(Al-Shawi et al., 1992; Keeney et al., 1993; Johnson et al., 1995). It is interesting that this regulation of MUP expression is observed even though the hGRF transgene likely causes chronically elevated GH levels, rather than the normal pulsatile pattern of GH secretion. The secretory pattern of GH differs between the sexes, being much more pulsatile in male rodents than female ones, and this is likely to be important for the sex-differences in MUP expression observed in normal mice. These findings also indicate that increased expression of the endogenous mouse GH gene in the MT-GRF transgenic mice leads to a MUP phenotype much like that observed in response to the exogenous human GH transgene in the MT-GH transgenic mice, likely through actions on a subset of about 12 MUP genes that are known to be GH responsive(Al-Shawi et al., 1989; 1992).

In addition to the observed change in MUP expression, urine from both male and female MT-GRF transgenic mice included a predominant high molecular weight protein(Fig. 1, lanes 3, 4, 7 and 8) that was not observed in normal mice or in the MT-Inh transgenic control mice(Fig. 1). Because of an earlier report that glomerular function might be impaired in transgenic mice that overexpress GH(Doi, et al., 1988), we compared the mobility of

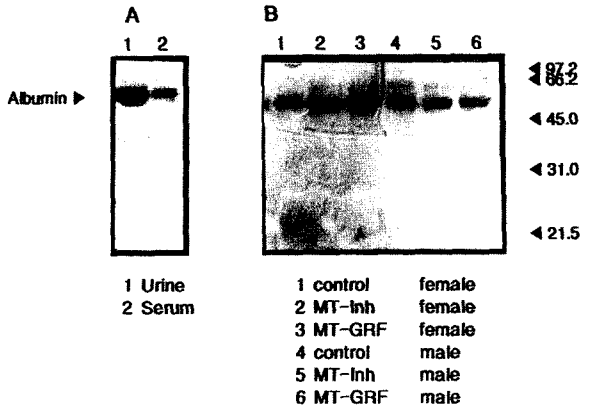

Fig. 3. Analysis of serum proteins in control and MT-GRF transgenic mice. A: Serum and urinary proteins in MT-GRF transgenic mice were analyzed by polyacrylamide gel electrophoresis. One microliter of urine and 0.1 μl of serum were loaded and stained with Coomassie Blue R-520. Note that the protein observed in the MT-GRF mouse co-migrates with the mouse serum albumin. B: Serum proteins were analyzed by the same method. Male and female mice that represent nontransgenic(control), a control transgenic(MT-Inh) or the test transgenic(MT-GRF) lines are as indicated. Equivalent amount of serum(0.1 μl) from individual mouse was loaded in each lane.

this protein with the mobility of the major serum protein albumin by analyzing serum samples from control, MT-Inh control transgenic and MT-GRF transgenic mice of both sexes. These data are presented in Fig. 3. Indeed, the predominant protein observed in the MT-GRF mouse urine co-migrates on SDS/polyacrylamide gels with the mouse serum albumin, consistent with an impairment of kidney function in these mice(Fig. 3, A). The urinary loss of serum proteins is more severe in male MT-GRF transgenic mice(Fig. 1, lanes 7 and 8, arrowhead) than female ones(Fig. 1, lanes 3 and 4, arrowhead). In spite of the large loss, the overall albumin levels in MT-GRF transgenic mice are similar to those of control and MT-Inh control transgenic mice(Fig. 3, B). Recent reports revealed that different portions of the GH molecule mediate different actions such as glomerular enlargement and somatic growth(Yang et al., 1993; 1997). Whether the MUPs reduction and kidney malfunction caused by GH are mediated by different portions of GH molecule remains to be unknown.

Overall, these results demonstrate that in another animal model of GH hypersecretion, the MT-GRF transgenic mouse, striking reductions in MUP expression in the urine are observed, confirming an important role for growth hormone in this regulation. In addition, changes in kidney glomerular function lead to a large loss of serum proteins into the urine of these mice with sex-differences, indicating a profound disregulation of urinary proteins metabolism in this animal model, which may mimic some diseases of growth hormone hypersecretion in man.

ACKNOWLEDGMENTS

The author thanks Dr. Richard Palmiter (University of Washington, Seattle) for providing the mouse metallothionein-I promoter.

REFERENCES

Al-Shawi R, Ghazal P, Clark AJ, Bishop JO (1989) Intraspecific evolution of a gene family coding for urinary proteins. J Mol Evol 29:302-313.

Al-Shawi R, Wallace H, Harrison S, Jones C, Johnson D, Bishop JO (1992) Sexual dimorphism and growth hormone regulation of a hybrid gene in transgenic mice. Mol Endocrinol 6:181-190.

Bacchini A, Gaetani E, Cavaggioni A (1992) Pheromone binding proteins of the mouse, *Mus musculus*. Experientia 48:419-421.

Bennett K, Lalley P, Barth R, Hastie N (1982) Mapping and structural genes coding for the major urinary proteins in the mouse: combined use of recombination inbred strains and somatic cell hybrids. Proc Natl Acad Sci USA 79:1220-1224.

Bishop JO, Clark A, Clissold P, Hainey S, Franke U (1982) Two main groups of mouse urinary protein genes, both largely located on chromosome 4. EMBO J 1:615-620.

Bocskei Z, Groom CR, Flower DR, Wright CE, Phillips SEV, Cavaggioni A, Findlay JBC, North ACT (1992) Pheromone binding to two rodent urinary proteins revealed by x-ray crystallography. Nature 360:186-188.

Cavaggioni A, Sorbi RT, Keen JN, Pappin DJ, Findlay JB (1987) Homology between pyrazine-binding protein from nasal mucosa and urinary proteins. FEBS Letters 212:225-228.

Clark AJ, Clissold PM, Bishop JO (1982) Variation between mouse major urinary protein genes isolated from a single inbred line. Gene 18:221-230.

Cho BN, Suh YH, Yoon YD, Lee CC, Kim K (1993) Progesterone inhibits the estrogen-induced prolactin gene expression in the rat pituitary. Mol Cell Endocrinol 93:47-52.

Cho BN, McMullen M, Pei L, Yates CJ, Mayo KE (2001) Reproductive deficiency in transgenic mice expressing the rat inhibin α-subunit gene. Endocrinology 142:4994-5004.

Danko I, Williams P, Herweijer H, Zhang G, Latendresse JS, Bock I, Wolff JA (1997) High expression of naked plasmid DNA in muscles of young rodents. Hum Mol Genet 6:1435-1443.

Derman E (1981) Isolation of a cDNA clone for mouse urinary proteins:age- and sex-related expression of mouse major urinary protein genes is transcriptionally controlled. Proc Natl Acad Sci USA 78:5425-5429.

Doi T, Striker LJ, Quaife C, Conti FG, Palmiter RL, Behringer R, Brinster RL, Striker GE (1988) Progressive glomerulosclerosis develops in transgenic mice chronically expressing growth hormone and growth hormone releasing factor but not in those expressing insulinlike growth factor-1. Am J Pathol 131:398-403.

Finlayson JS, Asofsky R, Potter M, Runner CC (1965) Major urinary protein complex of normal mice:origin. Science 149: 981-982.

Hammer RE, Brinster RL, Rosenfeld MG, Evans RM, Mayo KE (1985) Expression of human growth hormone-releasing factor in transgenic mice results in increased somatic growth. Nature 315:413-416.

Johnson D, Al-shawi R, Bishop JO (1995) Sexual dimorphism and growth hormone induction of murine pheromone-binding proteins. J Mol Endocrinol 14:21-34.

Keeney DS, Murry BA, Bartke A, Wagner TE, Mason JI (1993) Growth hormone transgenicregulate the expression of sex-specific isoforms of 3β-hydroxysteroid dehydrogenase/5 4-isomerase in mouse liver and gonads. Endocrinology 133: 1131-1138.

Knopf JL, Gallagher JA, Held WA (1983) Differential, multihormonal regulation of the mouse major urinary protein gene family in the river. Mol Cell Biol 3:2232-2240

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the heads of bacteriophage T4. Nature 227:680-685.

Lehman-McKeeman LD, Caudill D, Rodriguez PA, Eddy C. (1998) 2-sec-butyl-4,5-Dihydrothiazole is a ligand for mouse urinary protein and rat a2u-globulin: physiological and toxical relevance. Toxicol Appl Phamacol 149:32-40.

Mayo KE, Hammer RE, Swanson LW, Brinster RL, Rosenfeld ME, Evans RM (1988) Dramatic pituitary hyperplasia in transgenic mice expressing a human growth hormone -releasing factor gene. Mol Endocrinol 2:606-612.

Norstedt G, Palmiter R (1984) Secretory rhythm of growth hormone regulates sexual differentiation of mouse liver. Cell 36:805-812.

Pamiter RD, Norstedt G, Gelinas RE, Hammer RE, Brinster RL (1983) Metallothionein-human GH fusion genes stimulate growth of mice. Science 222:809-814.

Robertson DHL, Cox KA, Gaskell SJ, Evershed RP, Beynon RJ (1996) Molecular heterogeneity in the major urinary proteins of the house mouse *Mus musculus*. Biochem J 316:265-272.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning- A laboratory manual, ed 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, pp1.38-1.39.

Shahan K, Denaro M, Gilmartin M, Shi Y, Derman E (1987) Expression of six mouse major urinary protein genes in the mammary, parotid, sublingual, submaxillary, and lachrymal glands and in the liver. Mol Cell Biol 7:1947-1954.

Trojan J, Johnson TR, Rudin SD, Blossey BK, Kelley KM, Shevelev A, Abdul-Karim FW, Anthony DD, Tykocinski ML, Ilan J, Ilan J (1994) Gene traphy of murine teratocarcinoma: Separate functions for insulin-like growth factors I and II in immunogenicity and differentiation. Proc Natl Acad Sci USA 91:6088-6092.

Yang CW, Striker LJ, Pesce C, Chen WY, Peten EP, Elliot S, Doi T, Kopchick JJ, Striker GE (1993) Glomerulosclerosis and body growth are mediated by different portions of bovine growth hormone. Studies in transgenic mice. Lab Invest 68:62-70.

Yang CW, Striker GE, Chen WY, Kopchick JJ, Striker LJ (1997) Differential expression of glomerular extracellular matrix and growth factor mRNA in rapid and slowly progressive glomerulosclerosis:studies in mice transgenic for native or mutated growth hormone. Lab Invest 76:467-476.