Effect of Maternal Passive Autoimmunization against Myostatin on Growth Performance in Chickens*

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ABSTRACT : Myostatin is a negative regulator of skeletal muscle growth and a loss of functional myostatin protein increases muscle hypertrophy and hyperplasia in cattle. The present study was conducted to investigate whether maternal passive immunization against myostatin would improve growth performance in chickens. A complete broiler myostatin cDNA was cloned and it was expressed into two transcripts as 1,128 bp and 985 bp by alternative splicing. A conjugated mature myostatin (350 bp) was used to induce autoimmunization and maternal passively immunized chickens was used for the experiment. It was confirmed that there was a maternal passive immunization against myostatin at zero weeks of age, but its effect was reduced by 6 weeks of age. The auto-immunized groups showed smaller body weights than those of control group during the growing period and the difference was getting bigger with time until 6 weeks of age. These results suggest that passive autoimmunization against myostatin used in this study is not potent enough to stimulate growth performance in chickens. *(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 7 : 1017-1021)*

Key Words : Myostatin, Autoimmunization, Muscle, Growth, Chicken

INTRODUCTION

A recently discovered member of the transforming growth factor (TGF)-β superfamily of growth and differentiation factors, myostatin, has been shown to act as a negative regulator of skeletal muscle growth (Grobet et al., 1997; Kambadur et al., 1997; McPherron et al., 1997; McPherron and Lee, 1997). Knockout of the myostatin results in considerable acceleration of body weight and muscle growth in mice (Chen, 2001). The increased muscle hypertrophy and hyperplasia or double muscling observed in Belgian Blue and Peidmontese cattle is due to a loss of functional myostatin protein (McPherron and Lee, 1997; Westhusin, 1997; Thomas et al., 2000). A deletion of myostatin 11-nucleotide coding sequence in Belgian Blue cattle and a missense mutation in the gene sequence in Peidmontese cattle have expressed a truncated or non-functional myostatin proteins (Kambadur et al., 1997; McPherron and Lee, 1997). It is a goal of researchers and animal producers to promote muscle growth with maintaining the quality of meat. Compared to callipyge lambs known as the muscle hypertrophy but poor meat quality (Koomaraie et al., 1995) double muscling in cattle has a good marbling score as well as muscle hypertrophy (Hendricks et al., 1973; Wegner et al., 2002). To improve the muscle growth, muscle-specific gene expression vector system was developed and injected into muscle (Xie et al., 2004) but it was not a feasible scheme to augment growth in livestock. Therefore, myostatin is one of major target gene to utilize in farm animal industry. Our hypothesis is that myostatin auto-immunized mother delivers the myostatin antibody to the next generation (maternal passive immunization) resulting in inhibition of endogenous myostatin in embryo which in turn stimulating muscle development in chicken. The objective of the present experiment was to isolate chicken myostatin and to induce growth improvement by myostatin auto-immunization in chicken.

MATERIALS AND METHODS

Cloning of myostatin in chicken

A muscle tissue was collected from white leghorn chicken at each of the days 10, 15 and 20 of embryo stage and days 1, 3, 5, 7 and 14 of post-hatch stage and adult. Muscle tissue was aseptically removed, immediately frozen in liquid nitrogen, and stored at -70°C until RNA isolation. Total RNA was isolated using guanidinium thiocyanate-phenol-chloroform (Chomczynski and Sacchi, 1987). Two primers (forward 5'-TTCATATGCAAAAGCTA GCAG TCTATG-3' and reverse 5'-TTCTCGAGTGAGCACCC GCAACGAT-3') were designed for RT-PCR to encompass

entire coding sequences of avian myostatin according to Genbank. The amplified PCR product was cloned into pGEM TA cloning vector (Promega). The cloned myostatin cDNA was sequenced by double-stranded dideoxy chain termination method (USB Sequence version 2.0) and was confirmed myostatin in Genbank database. The confirmed myostatin gene was cloned into pET vector (Novagen) or pMAL vector (New England Biolabs. Inc.) to induce myostatin or fused myostatin with maltose binding protein. After treatment of enzyme factor Xa, myostatin protein was purified using affinity chromatography. The purified myostatin protein was separated on SDS-PAGE and the correct size of myostatin was confirmed.

A 350 bp mature myostatin was amplified by PCR using 5' primer (TTC ATA TGG ATT TTG GCC TTG ACT GTG) and 3' primer (TTC TCG AGT GAG CAC CCG CAA CGA T), and the product was cloned into Nde I and Xho I sites of pET expression system (pET-BME350) (Novagen) for histidine-tagged myostatin fusion protein. Myostatin was induced using IPTG (1 mM) at pET-BME350 transformed BL21 (DE3) E. coli for 3 h. The mature myostatin (15 kDa) was confirmed at SDS-PAGE and purified for myostatin conjugation with KLH (Keyhole Limper Hemocyanin), a carrier protein.

Production of myostatin auto-immunized chicken

For myostatin auto-immunization, conjugated mature myostatin was produced by MBS (m-maleimidobenzoyl-N-hydroxysuccinimide) coupling method (Dendren et al., 1989) with carrier protein KLH (Keyhole limpert hemocyanin) (Elliot et al., 1995). Briefly, 5 mg of KLH were dissolved in 0.5 ml of 0.01 M-phosphate buffer (pH 6.0), and then activated with 70 µl of MBS solution for 30 min at room temperature. The mixture was eluted by PD-10 column, and measured at 280 nm. 5 mg of myostatin protein dissolved in 1 m of 0.01 M PBS and added the conjugated MBS/KLH. The mixture was dialyzed against phosphate buffer at saline (PBS), pH 7.5. Conjugated myostatin (25-125 µg/chicken) was injected to the legs of female broiler chicken (n = 20, 18 weeks of age) and determined the level of antibodies against myostatin by ELISA after 1, 2, 3, and 4 weeks injection, respectively. The second and third conjugated myostatin was injected to the chicken when the level of antibody titer was dropped up to 1/3 of maximal titer. The fertilized eggs were produced from the auto-immunized chicken and the blood was sampled for antibody titer from hatched chick and 6 weeks old chicken, respectively. ELISA plates were coated with uniform amount (2.0 µg/well) of recombinant chicken myostatin in PBS buffer and incubated for 18 h ant 37°C. The coating mixture was removed and wells were filled with blocking buffer (PBS containing 0.1% BSA and 0.02% sodium azid). After a 1 h incubation 4°C, the blocking buffer was removed, and the plates were washed three times

with PBS containing 0.05% Tween 20 and 0.02% sodium azid. Serum form an animal immunized with conjugated myostatin were thawed, diluted (1:20 or 1:400) in PBS with 0.1% BSA, 0.05% Tween 20, and 0.02% sodium azide, plated in triplicate in 50 µl volumes, and incubated at 37°C. Alkaline phosphates conjugated goat anti-chicken IgG (1:5,000 in Tween-PBS; BETHYL Lab., INC, TX) was added to the appropriate plates and incubated for 18 h ant 37°C. For the colorimetric reaction, p-nitrophenyl phosphate (1.0 mg/ml in carbonate buffer) was added to each well, allowed to develop for 1 h at room temperature, and then measured at 405 nm using a ELISA reader (Genelab. Diagnostic, Germany). The body weight was measured every week for growth curve.

Statical analysis

Data were analyzed by the Duncan's multiple range tests using General Linear Model (GLM) procedure of the SAS program package (SAS, 1988).

RESULTS AND DISCUSSION

Cloning of myostatin cDNA in chicken

A complete broiler myostatin cDNA was cloned and it was revealed that myostatin cDNA was expressed into two transcripts as 1,128 bp and 985 bp by alternative splicing (Figure 1A). The mature myostatin was consisted of 339 bp (787-1,125 bp) sequences that encoded 113 amino acids. The intact myostatin transcript has 375 deduced-amino acids, identified by nucleotide sequencing. The intact form of chicken myostatin was identical to AF019621 in Genbank previously reported by McPherron and Lee (1997). The smaller transcript (truncated isoform) has 143 bp deletion at 374-516 bp from the initation codon, ATG, and contains 129 amino acid open reading frames by reading frame shift (Figure 1B). To authors' knowledge, the truncated isoform of myostatin has not been reported to date. The 143 nucelotides deletion causes a frame-shift, which is predicted to result in a truncated protein that terminates 5 codons downstream of the alternatively spliced site. Thus, the truncated transcript is expected to produce small polypeptide because it has 138 amino acids less than 266 amino acids of normal pro-peptide region. In embryonic stage, the steady-state level of intact myostatin mRNA was gradually increased in the developing period, but decreased at day 1 post-hatching, while it increased again at day 3 post-hatching and then maintained constant until adult chicken (data not shown). The steady-state level of truncated myostatin mRNA was low in embryonic stages and increased at day 3 post-hatching and then maintained constant until adult chicken (data not shown). The physiological role of truncated isoform of myostatin remains to be answered in the future.
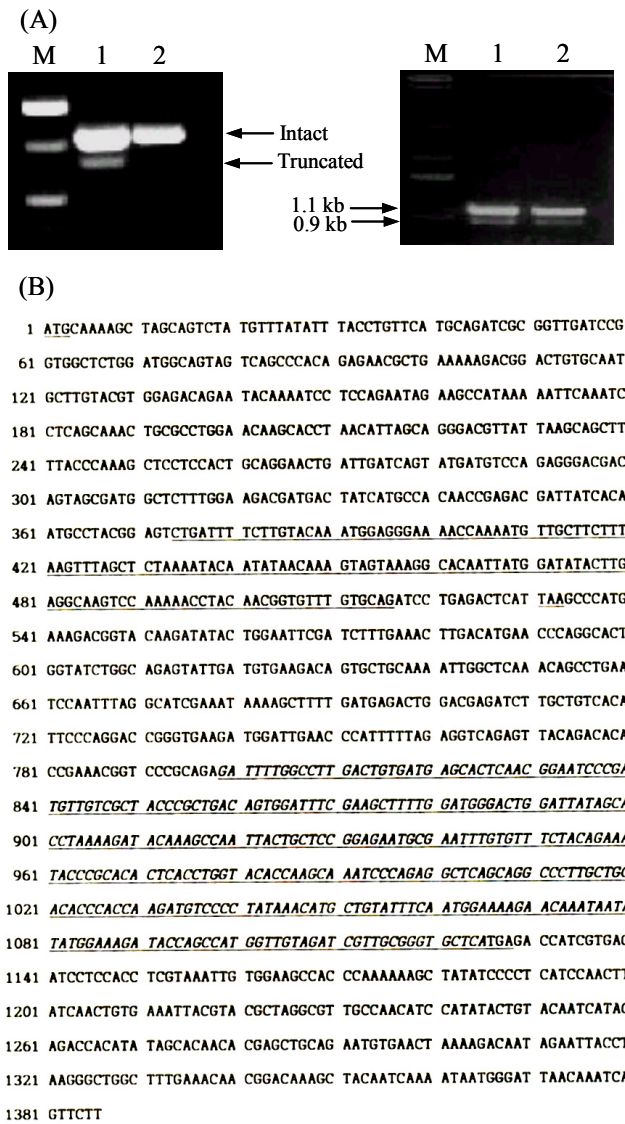
(A)

(B)

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   1 ATGCAAAAGC TAGCAGTCTA TGTTTATATT TACCTGTTCA TGCAGATCGC GGTTGATCCG
  61 GTGGCTCTGG ATGGCAGTAG TCAGCCCACA GAGAACGCTG AAAAAGACGG ACTGTGCAAT
 121 GCTTGTACGT GGAGACAGAA TACAAAATCC TCCAGAATAG AAGCCATAAA AATTCAAATC
 181 CTCAGCAAAC TGCGCCTGGA ACAAGCACCT AACATTAGCA GGGACGTTAT TAAGCAGCTT
 241 TTACCCAAAG CTCCTCCACT GCAGGAACTG ATTGATCAGT ATGATGTCCA GAGGGACGAC
 301 AGTAGCGATG GCTCTTTGGA AGACGATGAC TATCATGCCA CAACCGAGAC GATTATCACA
 361 ATGCCTACGG AGTCTGATTT TCTTGTACAA ATGGAGGGAA AACCAAAATG TTGCTTCTTT
 421 AAGTTTAGCT CTAAAATACA ATATAACAAA GTAGTAAAGG CACAATTATG GATATACTTG
 481 AGGCAAGTCC AAAAACCTAC AACGGTGTTT GTGCAGATCC TGAGACTCAT TAAGCCCATG
 541 AAAGACGGTA CAAGATATAC TGGAATTCGA TCTTTGAAAC TTGACATGAA CCCAGGCACT
 601 GGTATCTGGC AGAGTATTGA TGTGAAGACA GTGCTGCAAA ATTGGCTCAA ACAGCCTGAA
 661 TCCAATTTAG GCATCGAAAT AAAAGCTTTT GATGAGACTG GACGAGATCT TGCTGTCACA
 721 TTCCCAGGAC CGGGTGAAGA TGGATTGAAC CCATTTTTAG AGGTCAGAGT TACAGACACA
 781 CCGAAACGGT CCCGCAGAGA TTTTGGCCTT GACTGTGATG AGCACTCAAC GGAATCCCGA
 841 TGTTGTCGCT ACCCGCTGAC AGTGGATTTC GAAGCTTTTG GATGGGACTG GATTATAGCA
 901 CCTAAAAGAT ACAAAGCCAA TTACTGCTCC GGAGAATGCG AATTTGTGTT TCTACAGAAA
 961 TACCCGCACA CTCACCTGGT ACACCAAGCA AATCCCAGAG GCTCAGCAGG CCCTTGCTGC
1021 ACACCCACCA AGATGTCCCC TATAAACATG CTGTATTTCA ATGGAAAAGA ACAAATAATA
1081 TATGGAAAGA TACCAGCCAT GGTTGTAGAT CGTTGCGGGT GCTCATGAGA CCATCGTGAG
1141 ATCCTCCACC TCGTAAATTG TGGAAGCCAC CCAAAAAAGC TATATCCCCT CATCCAACTT
1201 ATCAACTGTG AAATTACGTA CGCTAGGCGT TGCCAACATC CATATACTGT ACAATCATAC
1261 AGACCACATA TAGCACAACA CGAGCTGCAG AATGTGAACT AAAAGACAAT AGAATTACCT
1321 AAGGGCTGGC TTTGAAACAA CGGACAAAGC TACAATCAAA ATAATGGGAT TAACAAATCA
1381 GTTCTT
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Figure 1. The intact and truncated myostatin cDNAs in chicken by RT-PCR and nucleotide sequences of chicken myostatin. (A) Myostatin cDNA was expressed into two transcripts as 1,128 bp (intact isoform) and 985 bp (truncated isoform) by alternative splicing. (B) The nucleotide sequences belonging to the 143 bp region deleted in the alternative-spliced, truncated myostatin cDNA are underlined. The nucleotide sequences encoding mature myostatin are indicated by italic type and underline.

Induction of auto-immunization against myostatin in chicken

The myostatin autoimmunization was induced by the injection of conjugated myostatin to adult chicken every 4 weeks intervals after second injection (Figure 2). The titer of myostatin antibody was maintained at a high level after second injection as compared to controls. The myostatin autoimmunization group was divided into low (1.033±0.087, n = 8) and high titer groups (1.523±0.050, n = 8) based on the results of titering of antibody from hens. The titer of antibody from control group was 0.323±0.032, n = 11. The fertilized eggs were obtained from myostatin
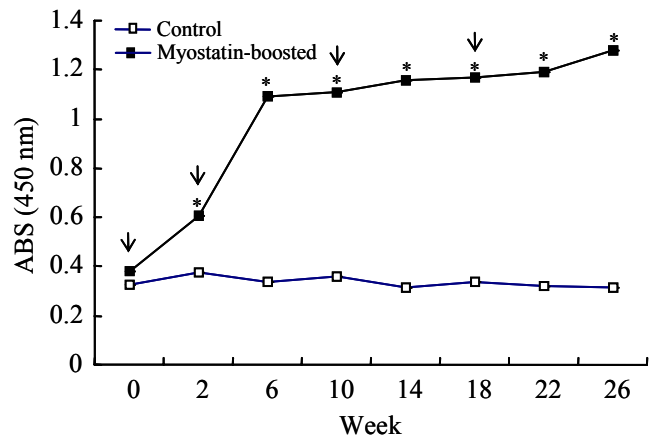

Figure 2. Induction of myostatin autoimmunization in adult female broiler. The myostatin autoimmunization was induced by the injection of the conjugated myostatin every 2 or 4 weeks intervals and titered for the myostatin antibody using ELISA. Data represent the mean±SE for hens (control n = 11; myostatin boosted group n = 16). ↓: Appears the boost time of conjugated myostatin. * p<0.05, compared between the control and the boosted group at the same week.

autoimmunized broiler chicken and the average egg weights of control, low and high titer groups were 60.8±0.4 g (n = 87), 59.5±0.8 g (n = 43), and 60.8±0.4 g (n = 45), respectively. We expected that the passive immunization against myostatin in chicken could affect the weight of eggs produced from active immunized hens but the egg weight was not affected from myostatin auto-immunization. The average egg weight in control, low and high titer groups were 60.1 g, 60.3 g and 60.5 g, respectively. The chick survival rates in control, low and high titer groups were 80%, 80% and 94.8%, respectively.

To confirm the passive autoimmunization against myostatin, the titer of myostatin antibody was measured from chickens at the time of hatching (0 week) and at 6 weeks of age that produced from control and myostatin autoimmunized adult chickens (Figure 3). As expected, the autoimmunized groups have shown higher titer of myostatin antibody than that of control group at hatching. The low and high titer groups have shown a 2.3- and 3.4-fold increase, respectively as compared to the control group. This result demonstrated that myostatin auto-immunized mother delivered the myostatin antibody to the next generation named maternal passive immunization. However, the titer of antibody from treatment group was diluted and reached to almost the same level as the control group at 6 weeks of age. Without additional booster immunization on the second generation, the auto-immunization against myostatin was affected on the early stage of growth in chicken.

Growth performance of chicken derived from maternal passive autoimmunization

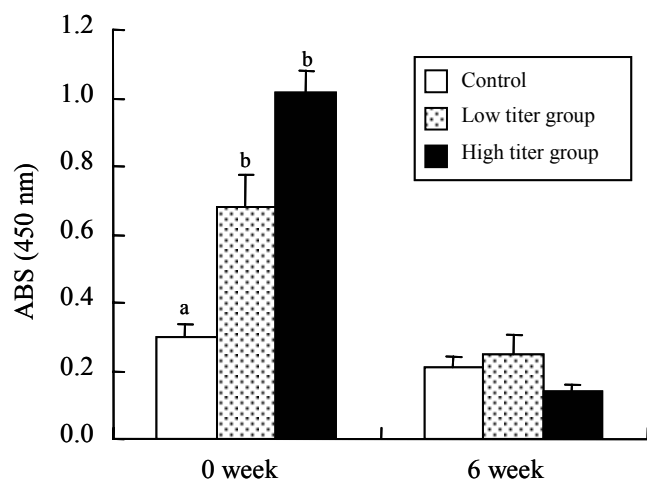The positive relationship between birth weight and

Figure 3. Titer of myostatin antibody at 0 and 6 weeks of age in chickens induced myostatin-autoimmunization from the sixth antibody boosting. Data represent the mean±SE for hens (week 0: control n = 4; low titer group n = 8; high titer group n = 4; week 6: control n = 26, low titer group n = 12, high titer group n = 9). a, b: p<0.05, compared between the control and the low, high titer groups on the same week.

Figure 4. The growth curves of chickens produced by the maternal passive myostatin autoimmunization. Data represent the mean±SE for hens (control n = 28, low titer group n = 13, high titer group n = 11). * p<0.05, compared among the control, low and high titer group at the same week.

growth rate has been reported in some species (Dwyer et al., 1993; Rehfeldt et al., 1993; William, 1994). We expected that the maternal passive immunization onto eggs might affect on embryonic development that in turn muscle development during growing period. Because myostatin expression is detected in somites during embryonic myogenesis (McPherron et al., 1997; Oldham et al., 2001), and its expression is continued in postnatal muscle (Kambadur et al., 1997; McPherron et al., 1997), myostatin may play a role at all stages of myogenesis. The early stage of detection of higher titer of antibody in treatment group may be generated a useful information on growth performance. Therefore, the body weight of chickens was measured every week to monitor growth performance. At 0 week of age, the body weights of control, low and high groups were 41.3±0.5 g (n = 28), 40.2±1.3 g (n = 12), and 41.2±0.7 g (n = 11), respectively, with no differences among the treatments (Figure 4). After 1 week, however, the body weights of chicken have shown a significant difference between control and auto-immunized groups, but there was no difference within auto-immunized groups. The auto-immunized groups did not catch the control group and the difference was getting bigger along with time until 6 weeks of age we determined. The body weights of the low titer (747.7±45.5 g, n = 12) and high titer (691.9±45.8 g, n = 11) groups were a 15.2% and 21.6% smaller, respectively, than that of the control group (882.2±31.1 g, n = 28) at 6 weeks of age. Sillence et al. (1992) have reported that growth rate was increased by a passive immunization against ACTH in the rat, suggesting the possibility that animal growth may be enhanced by passive immunization against the peptide. The ACTH is catabolic hormone by
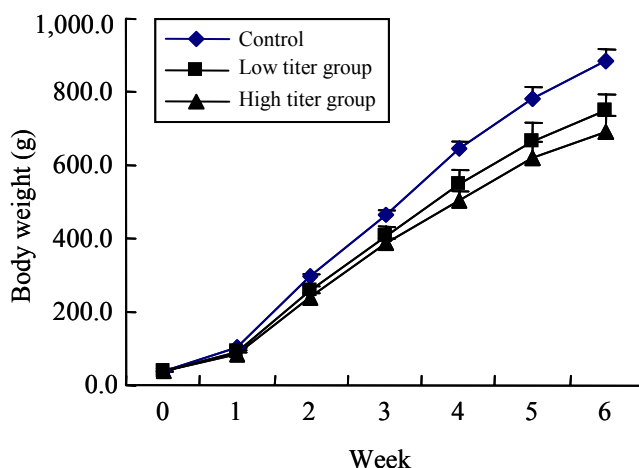
nature in the pig and rat, as evidenced by its negative effects on growth in these species (Chapple et al., 1989; Guo et al., 2000; Huang et al., 2000). As myostatin has a negative effect on growth, autoimmunization of myostatin might result in an increase in weight gain, possibly muscle mass in the chicken. Surprisingly, however, the higher titer groups of chicken have shown the smaller body weight during all weeks of age except at the time of hatching. We expected that the maternal passive myostatin autoimmunization could stimulate the muscle development during the embryogenesis and the early stage of growth of chicken. However, our result is opposite to what we expected. Our speculation is that the myostatin autoimmunization generates the physiological stress to the chicken resulting in the lower body weight. Another possibility is that the myostatin passive immunization was not enough to bring immunoneutralization of endogenous myostatin in chicken resulting in a negative growth performance.

In conclusion, the results of initial trial suggest that the passive autoimmunization against myostatin used in this study are not potent enough to stimulate growth performance, even that induces down regulation of growth rate in chicken. Therefore, the present autoimmunization and passive immunization against myostatin will not probably be effective for improving growth of chicken. It will be worthwhile, however, to further investigate the effect of passive immunization against myostatin with different dose of titer or different myostatin conjugates used in the present study.

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