Monitoring of Blood Cytokines by PIT-1 Genotypes in Day 150 Male Pigs

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ABSTRACT : Several studies have been done regarding carcass traits and growth in pigs. Recently, these have progressed to examine increases in economic traits, including meat quality and meat quantity, by using candidate genes. One of them is the pituitary-specific protein PIT-1, a member of the POU (Pit-Oct-Unc) family of transcription factors playing an important regulatory role in developmental processes. In addition, muscle development is known to be regulated in part by growth factors and cytokines locally produced. Therefore, studies were performed to analyze PIT-1 genotypes and serum cytokines (IGF-I, IGF-II, TGF-β1, EGF, cortisol, DHEA-S, IL-2, and IL-6) in castrated male pigs for their possible involvement in the development of carcass traits. The genotypes of PIT-1 gene were analyzed by PCR-RFLP with *MspI* restriction enzyme. But, only CD and DD genotypes, not CC genotype, have been detected. Based on PIT-1 genotyping, a significant difference in EGF expression beween CD type (78.8 ng/ml) and DD type (46.0 ng/ml) was detected ($p<0.05$), whereas other cytokines did not show any statistical significance depending on PIT-1 genotypes. Collectively, these results suggest the possibility that EGF could affect the formation of carcass traits. ***(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 12 : 1659-1664)***

Key Words : PIT-1, Growth Factor, Cytokine, Hormone, Pig

INTRODUCTION

Meat quality is a primary factor that is directly related to taste, and is affected by age, sex, feeding condition, processing method of slaughter, and storing condition (Lahucky et al., 1997). In the past, pigs have been bred toward high lean content by selection for decreased backfat thickness and high growth capacity, resulting in pigs becoming more sensitive to stress and thus increasing abnormal pork formation (Schworer et al., 1980). However, studies have recently progressed to overcome these problems for better meat quality. Especially, several studies have reported that specific genes can be used as the candidate gene or genetic marker for meat quality or quantity (Maclennan et al., 1990; Clamp et al., 1992; Lebret et al., 1999). One candidate gene is the pituitary-specific protein PIT-1 that is a member of the POU-domain family of transcription factors playing important regulatory roles in developmental processes (Gonzalez-Parra et al., 1996).

PIT-1 participates in growth hormone (GH), prolactin (PRL), and thyroid-stimulating hormone (TSH) gene expression by the binding to the promoter regions and in the proliferation of the cells that produce these hormones (Ingraham et al., 1988; Nelson et al., 1988). The expression of these genes (GH, PRL, and TSH) can be modulated by hormonal changes, possibly via PIT-1 in part.

A number of studies have shown that the PIT-1 gene is associated with variation in birth weight and weaning weight, average daily gain and backfat in pigs (Yu et al., 1995; Stancekova et al., 1999). In cattle, the PIT-1 gene is associated with body weight, milk, protein and fat yield (Renaville et al., 1997). These reports suggested that the PIT-1 gene could be used as a candidate gene for selecting animals for growth and carcass traits.

Also, there are many reports that cytokines including growth factors affect muscle development. Florini et al. (1996) found that insulin-like growth factor-I (IGF-I) directly affects muscle growth and development as a GH mediator. Peng et al. (1997) reported that epidermal growth factor (EGF) plays a role in skeletal muscle growth and maintenance in growing pigs during the later stage of development. In addition, proliferation of porcine myogenic cells is suppressed by both transforming growth factor (TGF)-β1 and TGF-β2 in a dose-dependent manner (Pampusch et al., 1990).

Other cytokines are also known to be involved in the regulation of muscle development. Dehydroepiandrosterone-sulfate (DHEA-S) may have an anti-obesity effect and relate to the subcutaneous fat depth (Clore, 1995; Wise et al., 1995), suggesting its possible relation to carcass traits. The report that cortisol levels decreased in hypophysectomized pigs (Latimer et al., 1993), suggested a possibility that cortisol may be related to PIT-1 expression due to its regulation by the pituitary. Unlike other cytokines, interleukins (IL-2 and IL-6) regarding their direct roles in muscle development and carcass traits have not been reported. But, there are reports that IL-2 was related to the immune system and IL-6 to muscle proteolysis (Goodman, 1994; Ebisu et al., 1995; Sterin-Borda et al., 1996), indicating their indirect roles in muscle development.

To extend the existing knowledge, studies are required to identify serum cytokines involved in muscle development. And, the expression of resultant cytokine is

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expected to be different depending on certain genotypes, thus it may be used as a marker for either muscle development or carcass traits. Therefore, this study was performed to monitor several cytokine concentrations in pigs at 30 days before slaughter in order to establish the standard criteria which can predict carcass traits in pigs. Also, the relationship of those cytokines with PIT-1 genotypes was investigated.

MATERIALS AND METHODS

Animals

Blood from jugular veins was collected in day 150 castrated male pigs (three-way cross breed ; Duroc× Yorkshire×Landrace). Sera were made by allowing blood samples to stand at room temperature for 1-2 h followed by centrifugation at 1,000×g for 30 min (Daughaday et al., 1980) and aliquots were stored at -70°C until used.

DNA extraction

Porcine genomic DNA was extracted from the clotted blood (Seo et al., 1999). In brief, 250 ul of lysis solution (360 μg/ml proteinase K, 150 mM sodium chloride, 50 mM EDTA, 2% SDS) was mixed with clotted blood and the mixture was incubated at 55°C for 12 h. Then 5.5 M NaCl and 600 ul of phenol:chloroform (25 : 24) were added, it was centrifuged for 10 min at 5,000×g. The supernatant was mixed with absolute ethanol and the mixture was centrifuged again under the same conditions. The pellets were dried, resuspended with TE solution (10 mM Tris-Cl, 1 mM EDTA), and stored at -20°C.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

PIT-1 genotypes were analyzed by PCR-RFLP using primers as reported (Stancekova et al., 1999). Primers were: forward 5'-AAAATCAGAGAACTTGAAAAGTTTGCC-3' and reverse 5'-GGCTTCCCCAACATTTGTTTGGG-3'. The reaction was performed with AccuPower^{TM}Premix-Top (Bioneer Co., Korea) containing 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase, 1 mM dNTP under the following conditions with a GeneAmp PCR System 2400 (Perkin Elmer Co., USA): 1 cycle at 95°C for 3 min, 63°C for 40 sec, and 72°C for 2 min; 30 cycles of 95°C for 40 sec, 63°C for 40 sec, and 72°C for 2 min; 1 cycle at 72°C for 7 min, 63°C for 40 sec, and 72°C for 2 min; hold at 4°C. RFLP was performed by adding *MspI, RsaI*, and *BamHI* restriction enzyme to each sample followed by incubation at 37°C overnight. DNA samples were analyzed using 2% agarose gel electrophoresis.

IGFs radioimmunoassay (RIA)

IGF-I was analyzed by radioimmunoassay and IGFs iodination using a chloramine T method (Lee and Henricks, 1990). One microgram of recombinant human IGF-I (GroPep Pty Ltd., Australia) was iodinated to a specific activity of 300 μCi/μg protein using 1 mCi $Na^{125}I$ (Amersham Pharmacia Biotech Ltd, Sweden). Iodinated IGF-I was purified on a Sephadex G-50 column and aliquots were stored at -20°C until used.

Serum IGF binding proteins were removed using an acid-ethanol method (Daughaday et al., 1980). Briefly, each samples were acidified with acid-ethanol (87.5% ethanol, 12.5% HCl) and stood for 30 min at room temperature. Then samples were centrifuged at 1,800×g for 30 min, they were neutralized with 0.2 ml of 0.855 M Tris-base. IGFBPs-removed supernatant was mixed with 0.1 ml of RIA buffer (30 mM sodium phosphate, 0.02% protamine sulfate, 10 mM EDTA, 0.05% Tween-20, 0.02% sodium azide; pH 7.5), and incubated with rabbit anti-human IGF-I polyclonal antiserum (GroPep Pty Ltd.; final dilution of 1:10,000) and 20,000 cpm [^{125}I] IGF-I in RIA buffer for 18 h at 4°C. Then, 0.1 ml of goat anti-rabbit IgG antibody (GroPep Pty Ltd.) was added and the mixture was incubated for 1 h followed by an additional 1 h incubation with 0.1 ml of normal rabbit serum at 4°C. After addition of 1 ml RIA buffer, the tubes were centrifuged for 30 min at 3,000 × g at 4°C. The supernatant was aspirated and the pellet was counted in a gamma-counter.

IGF-II was analyzed by the same method, except for rabbit anti-human IGF-II polyclonal antiserum as a primary antibody (GroPep Pty Ltd.).

Concentrations of serum TGF-β1 and EGF

Serum TGF-β1 was determined by TGF-β1 Emax ImmunoAssay System (Promega Co., USA). To measure the active form of TGF-β1, serum was diluted with DPBS. Then, 1 μl of HCl was added to 50 μl of diluted sample and the mixture was incubated for 15 min at room temperature. After incubation, 1 μl of NaOH was added and the neutralized mixture was used for the assay. Flat-bottom 96 well plates were coated with TGF-β1 coat mAb and the captured TGF-β1 was bound by a second specific polyclonal antibody (pAb). The amount of specifically bound pAb was detected using a species-specific antibody conjugated to horseradish peroxidase.

Serum EGF concentration was analyzed using an EGF Immunoassay kit (R&D Systems Inc., USA) based on the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for EGF was pre-coated onto a mocroplate. Standards and samples were pipetted into the wells and any EGF present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked pAb specific for EGF was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution that blended hydrogen peroxide with chromogen was added to the wells and color

developed in proportion to the amount of EGF bound in the initial step. The color development was stopped and the intensity of the colors was measured at 450 nm.

Concentrations of hormones and cytokines

Serum cortisol concentration was analyzed by solid-phase radioimmunoassay using a Coat-A-Count Cortisol kit (Diagnostic Products Co., USA), wherein ^{125}I-labeled cortisol competes for a fixed time with cortisol in the sample for antibody sites. Because the antibody was immobilized to the wall of a polypropylene tube, simply decanting the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radiolabelled cortisol. The tubes were counted for radioactivity.

Serum DHEA-S levels were measured by using an Enzyme Immunoassay Test kit (Diagnostic Automation Inc., USA) according to standard procedure based on the competition principle and the microplate separation. An unknown amount of DHEA-S present in the sample and a fixed amount of DHEA-S conjugated with horse-radish peroxidase were competed for the binding sites of a polyclonal DHEA-S antiserum coated onto the well. After one-hour incubation the microtiterplate was washed to stop the competition reaction. Having added the substrate solution, the concentration of DHEA-S was inversely proportional to the optical density measured.

Measurement of IL-2 and IL-6 in the serum was performed by ELISA kits (BioSource Europe S.A., Belgium), which were solid-phase enzyme amplified sensitivity immunoassays. The assay is based on an oligoclonal system in which a blend of monoclonal antibodies directed against distinct epitopes of IL-2 and IL-6 were used. Standards or samples were reacted with capture monoclonal antibodies coated on the microtiter well and with a monoclonal antibody labeled with horseradish peroxidase. After an incubation period, the microtiter plate was washed to remove unbound enzyme-labelled antibodies. Bound enzyme-labelled antibodies were measured through a chromogenic reaction.

Statistical analysis

Using the Student t-test procedure in a SAS package (1998), data were analyzed according to PIT-1 genotypes.

RESULTS

PCR-RFLP

In order to analyze PIT-1 genotypes, PCR products were digested with *MspI*, *Rsa*I, or *BamHI*, but the PIT-1 polymorphism was detected only by *MspI* (figure 1). The PIT-1 gene was reported to separate into three genotypes (CC genotype - 420 bp and 1,680 bp; CD genotype - 420 bp, 830 bp, 850 bp, and 1,680 bp; DD genotype - 420 bp, 830 bp, and 850 bp) (Yu et al., 1995). In this experiment, only CD (n=12) and DD (n=18) genotypes, not CC genotype, were detected.

Growth factor concentrations

Among the cytokines related to muscle development, several growth factors were investigated for their serum expression (table 1). Distribution of serum IGF-I concentrations appeared between 115 ng/ml and 350 ng/ml and the mean was 240 ng/ml. Serum IGF-II level ranged from 220 ng/ml to 420 ng/ml and showed 308 ng/ml on the average. Serum TGF-β1 existed in two forms: active form and latent form (Phillips et al., 1995), but this study analyzed the concentration of the active TGF-β1 form. As a result, the mean of TGF-β1 showed 119.75 ng/ml. Unlike other growth factors analyzed, EGF was expressed at very low concentrations. EGF existed 59.12 pg/ml on the average in castrated male pigs.

To find any relationship between cytokines and PIT-1 genotypes, the expressions of growth factors were analyzed on the basis of PIT-1 genotypes (table 2). Although distribution of serum IGF-I and IGF-II concentrations between CD type and DD type does not have a significant difference, the IGF-I level of DD type was larger than that of CD type, and the reverse pattern in IGF-II concentration was found. Like expressions of IGF-I and -II in both types, significant differences in TGF-β1 amounts between the two genotypes were not detected.

Especially, a significant difference in EGF amount between CD (78.8 ng/ml) and DD type (46.0 ng/ml) was detected ($p<0.05$, table 2), but the mean level of EGF in serum was lower than that of other respective growth factors.

Table 1. Antemortem growth factor concentrations in serum of pigs

Growth factors	No. of barrows	Mean±SD
IGF-I	30	240.0± 65.8
IGF-II	30	308.7± 53.0
TGF-β1	30	119.8± 45.4
EGF	30	59.1± 25.2

Table 2. Growth factor concentrations in serum-based PIT-1 genotypes in pigs (mean±SD)

	PIT-1 Genotypes	
	CD (n= 12)	DD (n= 18)
IGF-I	237.12 ± 50.51	241.97 ± 75.71
IGF-II	313.50 ± 58.35	305.56 ± 50.56
TGF-β1	116.90 ± 44.49	121.65 ± 47.13
EGF	78.79 ± 13.50[a]	46.00 ± 10.13[b]

[a,b] Mean±SD within a column with different superscripts differ ($p<0.05$).

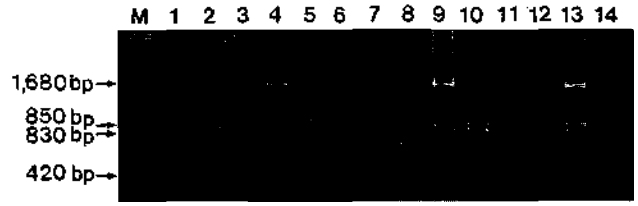

Figure 1. Representative polymorphism of the swine PIT-1 gene after *MspI* treatment. Samples were loaded onto a 2.0% agarose gel. Lanes 1, 3, 4, 6, 9, 13 - CD type, Lanes 2, 5, 7, 8, 10, 11, 12, 14 - DD type, M - marker

Hormone concentrations

To investigate the possibility of hormonal effect on carcass traits, serum cortisol and DHEA-S concentrations were measured (table 3). Mean concentrations of both hormones in male pig sera showed a high individual variation.

For further analyses of cortisol and DHEA-S, experimental groups were divided into two groups by PIT-1 genotypes and their serum concentrations were compared between CD and DD genotypes (table 4). However, these factors did not show any statistical significance based on PIT-1 genotypes.

To investigate the relationship between cortisol and DHEA-S, castrated pigs were divided into two groups of low and high levels by average cortisol concentration (45.5 ng/ml; table 5). The DHEA-S expression of the high cortisol group was lower than that of the low cortisol group, showing a negative relationship between serum cortisol and DHEA-S levels.

Cytokine concentrations by PIT-1 genotypes

The average concentration of IL-2 was 0.53 U/ml ranging from 0.30 U/ml to 0.80 U/ml (table 3) and individual variation was relatively low compared to other factors analyzed. Serum IL-6 was an average of 13.18 pg/ml, which ranged from 6 pg/ml to 38 pg/ml.

To find any relationship between cytokines and PIT-1 genotypes, the concentrations of IL-2 and IL-6 were analyzed on the basis of PIT-1 genotypes (table 4). Although significant differences in the concentrations of IL-2 and IL-6 were not detected between CD and DD genotypes, IL-2 and IL-6 levels in pigs with CD genotype tended to be higher than those with DD genotype.

Table 3. Antemortem hormone and cytokine concentrations in serum of pigs

Hormones	No. of barrows	Mean ± SD
Cortisol	30	45.4 ±24.7
DHEA-S	30	50.4 ±25.5
Interleukin-2	30	0.5 ± 0.1
Interleukin-6	30	13.2 ± 5.9

Table 4. Hormone and cytokine concentrations in serum-based PIT-1 genotypes in pigs (Mean±SD)

	PIT-1 Genotypes	
	CD (n= 12)	DD (n= 18)
Cortisol	41.36 ±27.94	48.09 ±22.74
DHEA-S	49.39 ±22.03	51.09 ±28.21
Interleukin-2	0.55 ± 0.10	0.52 ± 0.13
Interleukin-6	14.64 ± 7.75	12.20 ± 4.24

Table 5. Antemortem hormone concentrations in serum depending on average cortisol concentration in pigs (mean ±SD)

Group	No. of barrows	Cortisol (ng/ml)	DHEA-S (ng/ml)
High (>45.4 ng)	13	67.43±18.79	37.96±16.96
Low (<45.4 ng)	17	26.62± 9.51	52.31±20.00

DISCUSSION

The studies on PIT-1 expression have been progressed to find regulatory factors that are related to growth and carcass traits, such as average backfat thickness, longissimus muscle area, and weaning weight. But little is known about the relationship between PIT-1 genotypes, carcass traits, and muscle development. The PIT-1 binds to and transactivates the promoter sequences of the GH, PRL, and TSH-β subunit genes in mammals so that abnormalities of the PIT-1 gene cause a combined deficiency of GH, PRL, and thyrotropin (Irie et al., 1995; Tatsumi and Amino, 1999). Therefore, it is thought that understanding of the association between PIT-1 genotypes and muscle development may be important for efficient production of domestic animals, in particular, pigs. Recently, studies on PIT-1 gene polymorphism related to growth and carcass traits have been reported. Yu et al. (1995) reported that PIT-1 was a candidate gene in association with growth and carcass traits in pigs. And Stancekova et al. (1999) suggested that PIT-1 genotypes were related to backfat thickness and lean content.

In this study, for preliminary data on the correlation between PIT-1 genotypes and cytokines, day-150 castrated male pigs were divided into three groups depending on PIT-1 genotypes and their cytokine profiles were compared accordingly. When PIT-1 genotypes were analyzed by PCR-RFLP with *MspI*, CC genotype was not detected. This can be explained by the previous report by Yu et al. (1995) that C allele could be detected only in Chinese breeds (Meishan and Minzhu), that is, the Chinese allele. But, another study (Stancekova et al., 1999) reported that CC

genotype could be detected in European breeds (Large White, Large White × Landrace). In this study, a three-way cross breed (Duroc × Yorkshire × Landrace) was used and these different results could be due to genetically heterogeneous populations.

The effects of PIT-1 genotypes on the expression of growth factors have not been studied. In this study, the comparison of growth factor concentrations with PIT-1 genotypes revealed no difference in IGF-I, IGF-II, and TGF-β1 expression (table 2). However, it is of interest that a highly significant difference in EGF expression was detected ($p<0.05$). Jacob et al. (1999) suggested that the proximal PRL promoter is responsive to EGF and contains the response element for the cell type specific factor PIT-1. Thus, EGF and PIT-1 have similar properties to regulate PRL gene transcription, suggesting that the transcription mechanism may be affected by genotypes. Although IGF-I expression had nothing to do with PIT-1 genotypes in this study, a possibility of IGF-I involvement in carcass traits exists because IGF-I is known to mediate GH action for muscle development and PIT-1 reportedly regulates GH genes affecting muscle growth and carcass traits (Ingraham et al., 1988; Florini et al., 1996).

DHEA-S and cortisol concentrations in serum were analyzed to investigate their relationship (table 3). When samples were divided into two groups by average cortisol concentration (table 5), cortisol showed a negative correlation with DHEA-S, which was consistent with the report by Parker (1999). In pigs affected by stress, the level of cortisol secreted from adrenal cortex increases to stimulate the metabolism of glucose, lipid, and cholesterol in blood as a countermeasure about stress and correlatively DHEA-S concentration decreases (Parker et al., 1985).

Like other cytokines, the expression of DHEA-S and cortisol was also analyzed depending on PIT-1 genotypes, but differences were not found. This suggests that the expressions of these hormones are affected by the pituitary, not by genotypes. A report by Latimer et al. (1993) that cortisol levels decreased in hypophysectomized pigs suggested a possibility that the expression of cortisol is related to PIT-1. But our results indicated that cortisol is independent of PIT-1 genotypes. Also, in IL-2 and –6 expressions, significant differences between two genotypes were not detected. These suggest that IL-2 and –6 have no relationships with carcass traits in pigs.

The present report implies that PIT-1 expression can be associated with cytokine expression, especially EGF. However, further studies are needed to draw a definite conclusion because carcass traits depending on PIT-1 genotypes in this study were different from those in previous reports (Yu et al., 1995; Stancekova et al., 1999). Yu et al. (1995) suggested that pigs with CC genotype were fatter than those with CD or DD genotypes. But Stancekova et al. (1999) reported that DD genotype was significantly associated with greater average backfat thickness and lower percentage of lean content than CC and DD genotypes. Since these facts could be due to breeds, the analysis of PIT-1 polymorphism in our population needs to be done. Although this study suggested the possibility that EGF could be used as a marker for carcass traits, if the relationship among PRL, EGF, and PIT-1 is further established, it may be utilized to elucidate cytokine action on muscle development or carcass traits.

IMPLICATION

Several cytokine expressions and PIT-1 genotypes in pigs were monitored in order to investigate possible associations of cytokine expression with PIT-1 genotypes. Among those, a significant difference in EGF between CD and DD PIT-1 genotypes was detected, indicating that the EGF expression is associated with PIT-1 genotypes and implicating EGF as a possible marker for carcass traits. If further studies elucidate the association of cytokine concentrations with PIT-1 genotypes, those cytokine levels could be used as standard data to predict carcass traits in pigs.

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