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Anti-adipocyte scFv-Fc Antibody Suppresses Subcutaneous Adipose Tissue Development and Affects Lipid Metabolism in Minipigs

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Abstract Anti-adipocyte monoclonal antibody has been shown to reduce body fat mass in animals. Here, we investigated the effects of an anti-adipocyte antibody (single-chain variable fragment and crystallizable fragment, scFv-Fc) on pig subcutaneous adipose tissue development and lipid metabolism. The scFv-Fc antibody did not alter feed intake or body weight of treated pigs. It suppressed subcutaneous adipose tissue development by reducing the percentage of larger adipocytes, which led to a reduction in body fat mass and subcutaneous adipose tissue thickness. Body fat mass was reduced by reducing triglyceride biosynthesis and promoting triglyceride lipolysis in adipose tissue. There was an increase in lipoprotein lipase mRNA expression in adipose tissue and activity in blood and an enhanced transportation of circulating high-density lipoprotein, low-density lipoprotein, and free fatty acids. Blood concentrations of triglyceride, total cholesterol, glucose, insulin, and adiponectin and mRNA expression of adiponectin in adipose tissue remained unaffected. These findings suggest that anti-adipocyte scFv-Fc antibody may have an application for reducing body fat mass in obese subjects.

Keywords scFv-Fc antibody · Adipocyte · Body fat · Obesity · Minipigs

Introduction

Excessive fat deposition in domestic animals, especially in pigs, has been recognized not only as detrimental to lowering meat production costs but also as posing a health risk to human consumers. It is generally acknowledged that a high-fat diet can result in obesity. Obesity is a condition in which excess body fat has accumulated to such an extent that health may be negatively affected [1]. Excessive body weight is associated with various diseases, particularly cardiovascular disease, diabetes mellitus type 2, and certain types of

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cancer [2, 3]. Treatments for obesity are limited to dieting, physical exercise, and anti-obesity drugs [4–6]. Therefore, finding more effective methods to suppress excessive fat deposition in obesity cases is of utmost importance.

Various approaches have been attempted to solve the problem. Recently, some studies on the use of polyclonal and monoclonal antibodies to depress the development of adipose tissue have been reported with promising results. Flint et al. [7, 8] first demonstrated that polyclonal antibodies raised in sheep against rat adipocyte plasma membranes were cytotoxic in vitro and caused a decrease in rat body fat in vivo which suggested the potential application of these antibodies in treating diet-induced obesity with minimal side effects. Polyclonal antibodies raised against adipocyte plasma membrane proteins of different animals have been reported [9–15], and their efficacy in reducing body fat has been demonstrated. However, such polyclonal antibodies not only act on fat cells but also on other tissue cells, thereby producing undesirable side effects. To improve the specificity of anti-adipocyte antibodies, therefore, studies have been devoted to the production of monoclonal antibodies against fat cell surface proteins that displayed a significant effect on reducing fat mass weight without side effects [16–21]. These studies have indicated that an immunological approach using monoclonal antibodies against adipocyte plasma membrane proteins may provide an effective way to suppress exorbitant fat deposition in animals and may have potential for the treatment of human obesity.

The shortcomings of large molecule murine monoclonal antibodies have restricted their application for human clinical treatment. However, humanized small molecule antibodies, such as the fusion antibody of single-chain variable fragment (scFv) and crystallizable fragment (Fc) of human immunoglobulin G (IgG) heavy chain constant region (scFv-Fc), have been shown to possess clinical potential.

In our previous studies, a 40-kDa adipocyte-specific membrane protein was identified and prepared [22], and a monoclonal antibody of the IgG1 subclass was raised against this protein [23]. Animal experimentation revealed significant effects on the suppression of body fat in vivo and in vitro [24–26]. We have isolated the scFv gene from a hybridoma secreting this monoclonal antibody [27] and have constructed a phage antibody library displayed this scFv fragment [28]. Subsequently, scFv was fused with the Fc fragment of human IgG, and the scFv-Fc antibody was expressed in *Pichia pastoris* [29]. The objective of this study was to investigate the effect of this scFv-Fc antibody on suppressing subcutaneous adipose tissue development and influencing lipid metabolic factors in Banna minipigs.

Materials and Methods

All experimental procedures were performed according to the Guide for Animal Care and Use of Laboratory Animals, Institutional Animal Care and Use Committee of Yunnan Agricultural University. The experimental protocol was approved by the Departmental Animal Ethics Committee of Yunnan Agricultural University.

Determination of Effects on Subcutaneous Adipose Tissue Development

Twenty-four Banna minipigs (average body weight 15±1.32 kg) were randomly divided into four treatment groups (six pigs per group, half each male and female). Three treatment groups were given an intraperitoneal injection of 0.1, 0.5, or 1.0 mg/kg purified scFv-Fc antibody in 10 ml phosphate buffered saline (PBS), and a control group was injected

intraperitoneally with 0.5 mg/kg of purified mouse IgG in 10 ml PBS as a nontarget antibody.

Pigs were housed individually. They had free access to water from nipple drinkers and to their regular diets ad libitum. They were weighed at the start and end of the treatment, and feeders were weighed every week for calculation of the average day feed intake. Blood samples were collected every week for 1 month postinjection for determination of scFv-Fc antibody titers.

Pigs were killed 3 months following the injection. Blood samples were collected for analysis of lipid metabolic factors. Subcutaneous adipose tissue was sampled immediately. Approximately 20-g samples of subcutaneous adipose tissue were taken from four back locations and freeze-sectioned immediately for adipocyte size measurement. Other samples from the same locations were stored frozen at −80 °C for later analysis of enzyme activity and gene expression. Sectioning of the subcutaneous adipose tissue was carried out as described by Seveus and Johannessen [30], and adipocyte size was determined as described by de Clercq et al. [19]. Body fat mass was measured after a 20-h chill at 2 °C from the left side of the carcass as described by Higbie et al. [31]. Measurements included fat mass weight, lean meat weight, and back fat thickness.

Detection of Lipid Metabolism-Related Gene Expression

Fatty acid synthase (FAS), hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), and adiponectin mRNA from subcutaneous adipose tissue were assayed by real-time quantitative reverse transcriptase polymerase chain reaction (PCR) of RNA samples previously treated with DNAse (DNA free, Promega, USA). One microgram of each sample was reverse-transcribed using an M-MLV reverse transcriptase kit (Promega, USA), and cDNA was synthesized in a final volume of 50 μl. The relative levels of FAS, HSL, LPL, and adiponectin mRNA were quantified in real time, using SYBR Green Supermix (Bio-Rad, USA) in an iCyler iQ Real-Time Detection System (Bio-Rad, USA). The β-actin gene was used as an internal control. The primers used were FAS, 5′-AGCCTAACT CCTCGCTGCAAT-3′ (forward) and 5′-TCCTTGGAACCGTCTGTGTTC-3′ (reverse); HSL, 5′-GCTCCCATCGTCAAGAAT C-3′ (forward) and 5′-TAAAGCGAATGCGGTCC-3′ (reverse); adiponectin, 5′-GAA GTAGACTCTGCTGAGATGG-3′ (forward) and 5′-TATCAGTGTAGGAGGTCTGT GATG-3′ (reverse); LPL, 5′-AAC TTG TGG CTG CCC TAT-3′ (forward) and 5′-GACCCTCTGGTGAAT GTG-3′ (reverse); and β-actin, 5′-ACTGCC GCATCCTCTTCCTC-3′ (forward) and 5′-CTCCTGCTTGC TGATCCACATC-3′ (reverse). Changes were expressed in twofold increments as described previously by Zhao et al. [32]. The activities of FAS and HSL were determined as described by Martin et al. [33] and Brewster and Matsumura [34], respectively.

Analysis of Blood Lipid Metabolic Factors

Serum titers of scFv-Fc antibody were determined as described by Zhao et al. [25]. Serum glucose, triglyceride (TG), free fatty acids (NEFA), total cholesterol (CHO), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and LPL analyses were performed using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) in a Rili-7170A automatic biochemistry analyzer. Serum insulin analyses were performed using a commercial kit (Tianjin Jiuding Bioengineering Ltd., China) with an SN-682 radio-immunity γ-events per unit time meter. Serum adiponectin was quantified using a specific enzyme-linked immunosorbent assay kit (Linco Research, UAS). All kit procedures were carried out according to the manufacturers' instructions.

Statistical Analysis

Results were expressed as means±standard error of the mean (SEM). Statistical significance of difference between means was assessed by the least squares method (GLM procedure, version 4.18; SAS, Cary, NC, USA). Differences were considered significant if $P<0.05$.

Results

Feed Intake and Body Weight

Treatment with different doses of scFv-Fc antibody had no effect either on feed intake or body weights of pigs ($P>0.05$; Fig. 1a, b).

Subcutaneous Adipose Tissue Development

Frozen section micrographs of subcutaneous adipose tissue revealed that adipose tissue cells from pigs injected with 0.5 or 1.0 mg/kg of antibody were clearly smaller than that of the control; however, cell size following treatment with 0.1 mg/kg of antibody did not differ significantly from the control (Fig. 2a–d). Measurement of cell diameters by planimetry after collagenase digestion and cell osmication confirmed that cell diameters in treatment groups of 0.5 or 1.0 mg/kg antibody were significantly smaller ($P<0.05$) than those of the control group (Fig. 3).

Body Fat Mass

The scFv-Fc antibody reduced back fat thickness and body fat weight (Table 1). At death, i.e., 3 months following treatment, back fat thickness was lower in both 0.5-mg/kg ($P<0.05$, 11.18% lower) and 1.0-mg/kg ($P<0.05$, 14.60% lower) treatment groups. No difference was found with 0.1-mg/kg treatment ($P>0.05$). Body fat weights were also reduced in both 0.5-mg/kg ($P<0.05$, 5.35% lower) and 1.0-mg/kg ($P<0.05$, 8.12% lower) treatment groups. On the other hand, the antibody increased body lean meat, with significant differences being observed in both 0.5-mg/kg ($P<0.05$, 10.65% higher) and 1.0-mg/kg ($P<0.05$, 16.43% higher) treatment groups (Table 1).

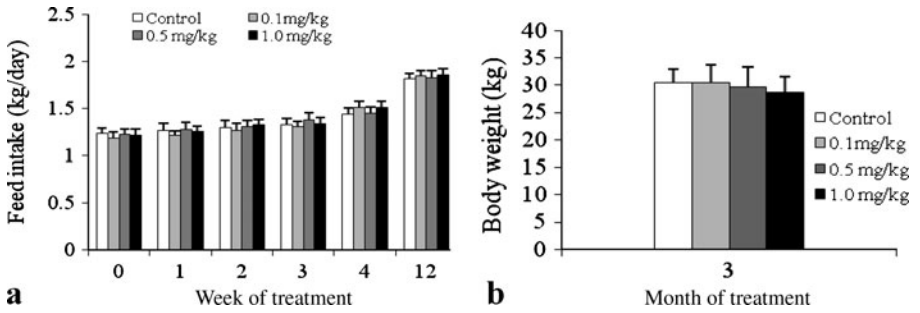


Fig. 1 Feed intake and body weight of pigs treated with different dosages of scFv-Fc antibody ($n=6$). **a** Feed intake in the first month and at the last week of treatment; **b** body weight at the end of treatment after 3 months

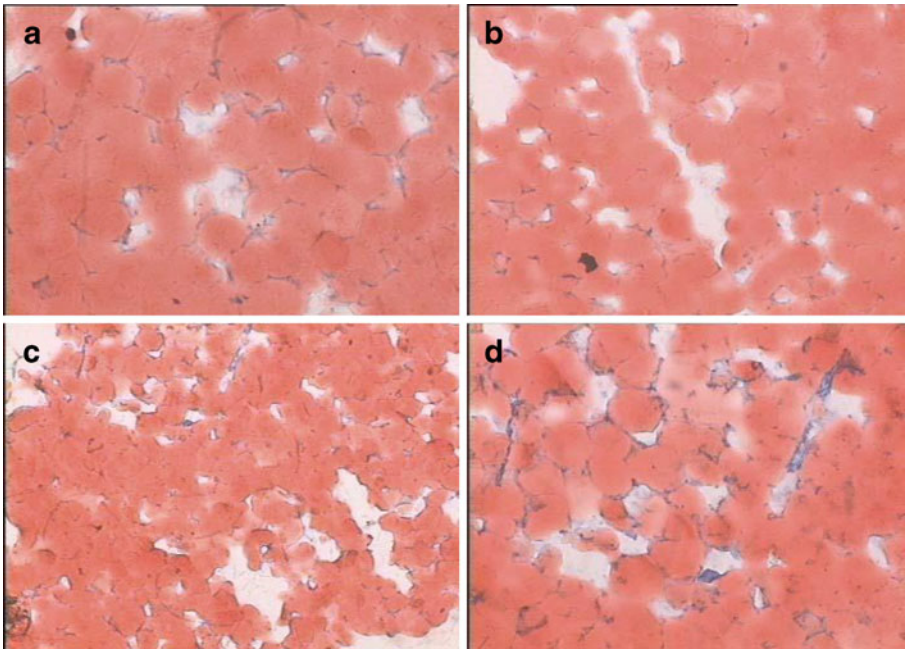


Fig. 2 Micrographs of frozen section of porcine subcutaneous adipose tissue. Magnification, ×200. Cells were stained with Oil Red O. Treatment: **a** 0.1 mg/kg of antibody, **b** 0.5 mg/kg of antibody, **c** 1.0 mg/kg of antibody, **d** 0.5 mg/kg of rabbit IgG (control)

Lipid Metabolism-Related Gene Expression in Subcutaneous Adipose Tissue

To further assess the effect of scFv-Fc antibody on lipid metabolism in adipose tissue, mRNA expression of FAS, HSL, LPL, and adiponectin and enzyme activities of FAS and HSL were examined. Results are shown in Fig. 4. The mRNA expression level and enzyme activity of FAS were reduced ($P<0.05$) in treatment groups 0.5 and 1.0 mg/kg. HSL activity was increased ($P<0.05$) in these two groups (Fig. 4a–d). Treatment with 1.0 mg/kg of scFv-Fc antibody also increased mRNA expression of LPL ($P<0.05$), but no differences

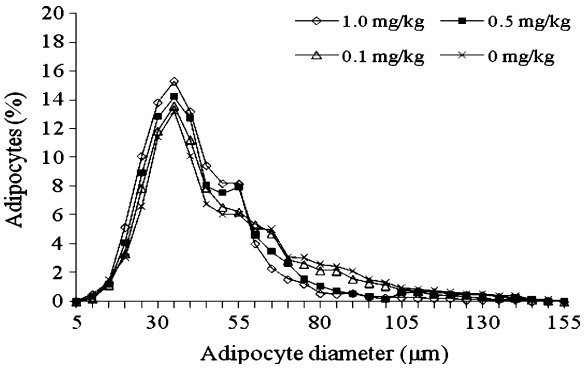


Fig. 3 Cell sizes in subcutaneous adipose tissue in control and treated groups. $P<0.05$ between control and treatment groups with 0.5 or 1.0 mg/kg antibody ($n=6$)

Table 1 Body fat mass of pigs in the four groups after 3 months treatment.

Group	Control	0.1 mg/kg	0.5 mg/kg	1.0 mg/kg
Back fat thickness (cm)	3.22 (±0.223)a	3.18 (±0.214)a	2.86 (±0.187)b	2.57 (±0.206)b
Fat mass weight (kg)	5.79 (±0.162)a	5.55 (±0.131)a	5.48 (±0.154)b	5.32 (±0.048)b
Lean meat weight (kg)	4.32 (±0.321)a	4.48 (±0.334)a	4.78 (±0.256)b	5.03 (±0.186)b

Date are shown as means±SEM (n=6). Means without common letter differ significantly (P<0.05)

were observed in the other groups (Fig. 4e). Treatment did not affect adiponectin mRNA expression (P>0.05; Fig. 4f).

Blood Lipid Metabolic Factors

Serum scFv-Fc Antibody Titers

After treatment, serum scFv-Fc antibody titers gradually decreased with time. By 3 weeks, titers of the treatment groups were the same as the control (Fig. 5).

TG, NEFA, and CHO

Serum TG levels were slightly, although not significantly, reduced after scFv-Fc antibody treatment (P>0.05; Table 2). Serum total CHO levels were also not significantly different in the four groups (P>0.05) although elevated slightly after treatment (Table 2). However, serum NEFA was lowered significantly (P<0.05, 22.45% lower) in the 1.0-mg/kg treatment group (Table 2).

HDL, LDL, and LPL

Levels of both HDL and LDL were elevated (P<0.05, 24.41% and 19.81% higher, respectively) in treatment group 1.0 mg/kg. No difference was observed in the other three groups (P>0.05; Table 2). Treatment with 1.0 mg/kg of scFv-Fc antibody increased serum LPL activity (P<0.05, 24.79% higher), but no differences were observed in the other treated groups (Table 2).

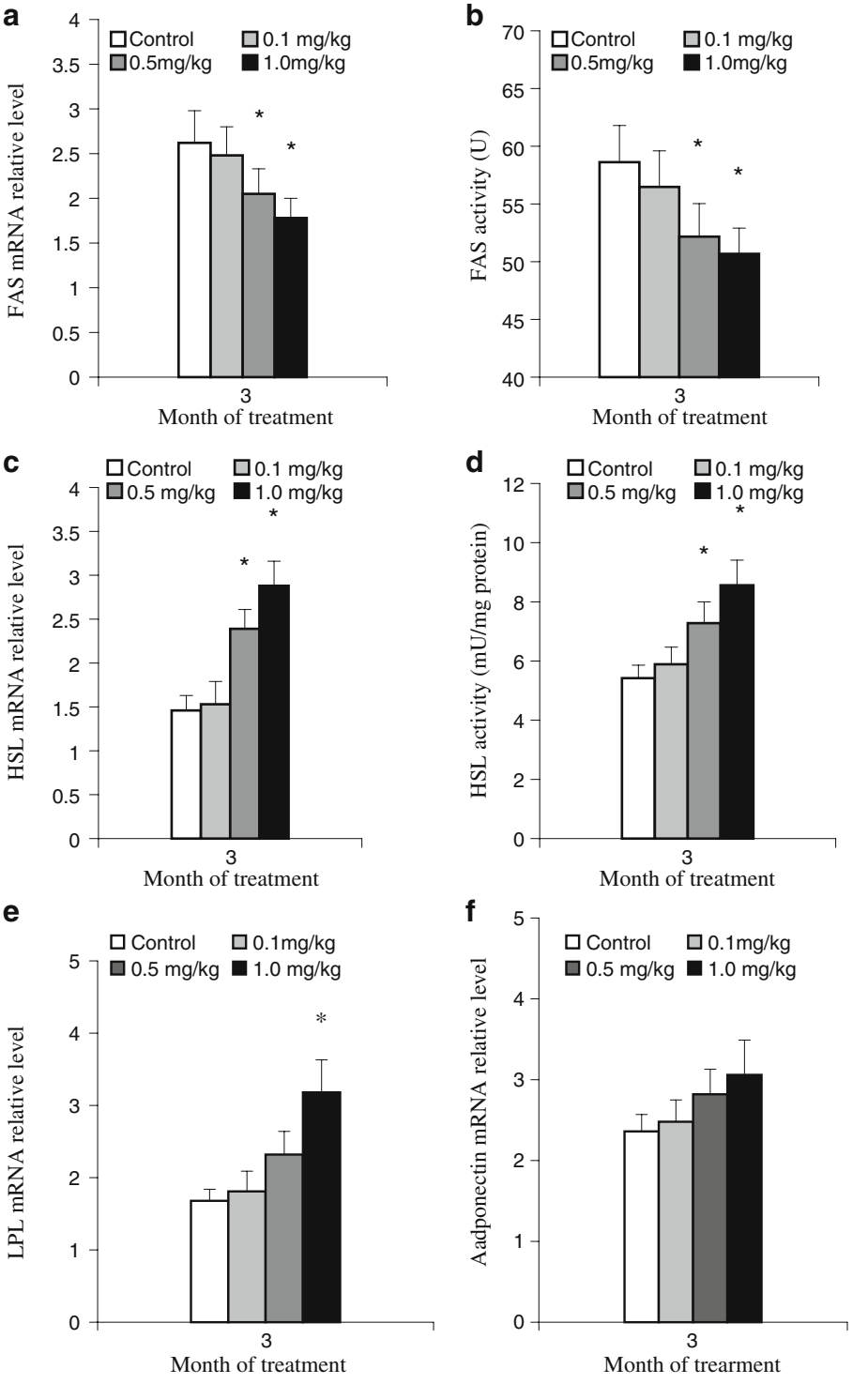
Glucose, Insulin, and Adiponectin

There were no differences in the blood levels of glucose, insulin, and adiponectin among the four groups (P>0.05; Table 2).

Discussion

Pigs and humans share numerous physiological and phenotypic similarities in fat deposition [35], so pigs have been intensively used as a biomedical research model for various human

Fig. 4 **a**–**f** Quantitative expression mRNA levels by real-time PCR analysis and enzyme activities in porcine ▶ subcutaneous adipose tissue at the end of treatment with scFv-Fc antibody. Data are expressed as mean± SEM (n=6). *P<0.05


a
Control
0.1 mg/kg
0.5mg/kg
1.0mg/kg
FAS mRNA relative level
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
3
Month of treatment
b
Control
0.1 mg/kg
0.5mg/kg
1.0mg/kg
FAS activity (U)
70
65
60
55
50
45
40
*
*
3
Month of treatment
c
Control
0.1 mg/kg
0.5 mg/kg
1.0 mg/kg
HSL mRNA relative level
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
3
Month of treatment
d
Control
0.1 mg/kg
0.5 mg/kg
1.0 mg/kg
HSL activity (mU/mg protein)
12
10
8
6
4
2
0
*
*
3
Month of treatment
e
Control
0.1mg/kg
0.5 mg/kg
1.0 mg/kg
LPL mRNA relative level
5
4
3
2
1
0
*
3
Month of treatment
f
Control
0.1mg/kg
0.5 mg/kg
1.0 mg/kg
Aadponectin mRNA relative level
5
4
3
2
1
0
3
Month of trearment

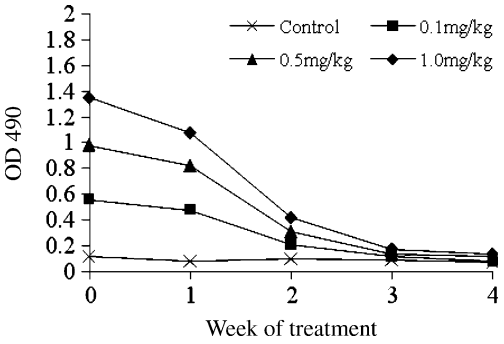


Fig. 5 Titers of serum scFv-Fc antibody of treated pigs treated with time (n=6)

physiological conditions such as obesity [36]. The Banna minipig is a traditional Chinese local breed in Yunnan, China. This breed of pig possesses the unique trait of accumulating more fat in subcutaneous adipose tissue than other Chinese local and commercial pigs. It is acknowledged as being a typical fatty pig in China and, as in this study, has been used as an animal model for obesity research.

Previous studies on monoclonal antibodies against adipocyte plasma membrane proteins have demonstrated that these had no influence on feed intake and body weight of pigs [19, 25]. Similarly, the present study showed that neither feed intake nor body weight was significantly altered by treatment with the scFv-Fc antibody, although a slight body weight loss was observed at the highest dose treatment. This indicates that the scFv-Fc antibody did not affect feed intake and growth of pigs. In addition, we did not observe any noticeable abnormalities of internal organs in treated pigs at slaughter, and no histopathological evidence was noted in the various organs (heart, liver, spleen, kidney, and pancreas) examined.

Previous studies have demonstrated that monoclonal antibodies against adipocyte membrane proteins suppressed subcutaneous adipose tissue development, producing a reduction in adipocyte cell numbers by complement-mediated adipocyte killing [19, 24, 25]. In the present study, we found a similar effect of the scFv-Fc antibody on subcutaneous adipose tissue

Table 2 Serum levels of metabolic factors in the four groups after 3 months treatment.

Group	Control	0.1 mg/kg	0.5 mg/kg	1.0 mg/kg
Glucose (mg/dl)	163.26 (±12.072)	164.68 (±12.156)	165.43 (±12.238)	168.15 (±12.302)
TG (mmol/l)	1.93 (±0.162)	1.87 (±0.083)	1.79 (±0.123)	1.71 (±0.162)
NEFA (mmol/l)	1.96 (±0.151)a	1.76 (±0.146)ab	1.71 (±0.131)ab	1.52 (±0.107)b
Total CHO (mmol/l)	2.54 (±0.093)	2.61 (±0.126)	2.68 (±0.115)	2.72 (±0.132)
HDL (mmol/l)	1.27 (±0.138)a	1.31 (±0.124)a	1.42 (±0.108)ab	1.58 (±0.116)b
LDL (mmol/l)	1.06 (±0.085)a	1.13 (±0.092)ab	1.21 (±0.103)ab	1.27 (±0.083)b
Insulin (μIU/ml)	4.36 (±0.184)	4.35 (±0.176)	4.32 (±0.124)	4.28 (±0.142)
Adiponectin (ng/ml)	13,920.68 (±563.43)	13,980.13 (±432.56)	14,180.22 (±385.23)	14,383.31 (±513.52)
LPL (U/ml)	31.46 (±1.231)a	32.72 (±1.115)ab	35.84 (±2.168)ab	39.26 (±3.023)b

Date are shown as means±SEM (n=6). Means without common letter differ significantly (P<0.05)

development. However, there was a difference; the effect on subcutaneous adipose tissue development by scFv-Fc antibody was due to a decrease in adipocyte size rather than number. Therefore, the action of scFv-Fc antibody resulted in thinner subcutaneous adipose tissue and lower body fat weight. Consequently, with the loss of fat, the lean meat weight was increased. This suggests that the dose-dependent effect of the antibody on reducing body fat mass was carried out by suppressing subcutaneous adipose tissue development. The increased lean meat may be attributed to more energy being provided to muscle tissue by NEFA released from adipose tissue, although the mechanism requires further study.

Analysis of lipid metabolism-related gene expression provided some insight into the mechanism by which the scFv-Fc antibody reduced body fat mass. Reduction in mRNA expression and FAS activity and increase in HSL activity in adipose tissue suggest that reduction of body fat mass by the antibody is likely associated with FAS and HSL expression leading to reduction in triglyceride biosynthesis and increased triglyceride lipolysis in adipose tissue.

The observation that a single dose of the scFv-Fc antibody remained in circulation for more than 2 weeks is an important one. It is essential for the antibody to play a long-term role in vivo, perhaps by changing and normalizing metabolic parameters in adipose tissue and blood.

Lipid metabolism in adipose tissue is reflected in the change of lipid metabolic factors in blood. Our previous study demonstrated the effect of monoclonal antibody on blood lipid profiles [26]. The present study has shown that scFv-Fc antibody reduced the level of NEFA and increased levels of HDL and LDL in blood, as well as LPL activity. However, no effect on blood concentrations of TG and CHO were observed in treated pigs. The reduction in blood NEFA indicates that more NEFA may be transported to other tissues for utilization, and the increase of blood HDL and LDL concentration reveals that the transportation of lipids in circulation was enhanced. The increase in mRNA levels of LPL in adipose tissue and in the activity of LPL in blood indicates that LPL expression in adipose tissue was upregulated by scFv-Fc antibody. LPL is the major enzyme responsible for hydrolysis of TG in circulating lipoproteins in the conversion to LDL and is considered the key factor determining lipid disposition among tissues [37]. Therefore, the increased LPL activity in circulation resulted in increased LDL and reduced NEFA in blood, indicating that scFv-Fc antibody reduced body fat mass by affecting fat mobilization in adipose tissue and lipid metabolism in blood.

Circulating concentrations of glucose and insulin are associated with insulin sensitivity in obese subjects. Recent studies have shown that adiponectin secretion from adipose tissue is downregulated in obesity, so blood concentrations of adiponectin are lower in obese than in lean subjects [38, 39]. The reduced adiponectin secretion in obesity is correlated with insulin resistance [40, 41]. Our present study has shown that blood concentration of glucose and insulin, mRNA expression in adipose tissue, and concentration in blood of adiponectin are not changed by the scFv-Fc antibody treatment. This indicates that although the scFv-Fc antibody affected lipid metabolic factors in adipose tissue and blood, it did not affect insulin sensitivity.

In conclusion, we have found that scFv-Fc antibody did not alter feed intake and body weight of pigs. It reduced body fat mass by suppressing subcutaneous adipose tissue development, reducing triglyceride biosynthesis, and promoting triglyceride lipolysis in adipose tissue. It enhanced transportation of circulating lipoproteins and NEFA without affected insulin sensitivity. These findings support the suggestion that scFv-Fc antibody has potential application for reducing body fat mass in obese subjects. However, the mechanism of action of this antibody requires further study.

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