Effects of Vitamins on the Differentiation of Preadipocytes from Hanwoo Cattle Adipose Tissues[a]

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ABSTRACT : The experiment was conducted to study the effects of water soluble vitamins and retinoic acid on the differentiation of preadipocyte from omental, subcutaneous, intermuscular and intramuscular adipose tissue of Hanwoo. Differentiation was assessed by the change in enzyme activity, glycerol-3 phosphate dehydrogenase in serum free cell culture system. Preadipocytes treated with biotin (10 μM) and pantothenic acid (100 μM) were significantly ($p<0.05$) less differentiated than those from the control in all adipose tissue depots except intramuscular tissue. Although there was no significance, vitamin C was shown to stimulate the adipocyte conversion in omental and subcutaneous, but not in intermuscular and intramuscular adipose tissues. Lower values of GPDH activity in intermuscular preadipocyte were interpreted to be caused by relatively higher amounts of protein. In this experiment vitamin C did not stimulate fat deposition in intramuscular adipose tissue but further experiments are needed on the role of vitamin C in preadipocyte differentiation. When treated with different levels of retinoic acids, differentiation of preadipocytes was significantly ($p<0.05$) reduced from the level of 0.5 μg/ml in omental and intermuscular, from 50 μg/ml in subcutaneous, and in intramuscular at 500 μg/ml, thus showing that intramuscular preadipocytes were least responsive to retinoic acid in differentiation. All-trans retinoic acid appeared to inhibit the differentiation in a dose dependent manner, regardless of adipose tissues type. *(Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 4 : 446-450)*

Key Words : Hanwoo Cattle Preadipocytes, Omental, Subcutaneous, Intermuscular, Intramuscular, L-Ascorbic Acid, Biotin, Pantothenic Acid, All Trans-Retinoic Acid, GPDH, Protein

INTRODUCTION

The development of cell lines and culture methods for primary adipocyte precursors has greatly aided the study of adipocyte development and has enabled the definition of factors which regulate differentiation. Green and Kehinde (1974, 1976) reported that preadipocyte cells were differentiated to mature adipocytes in a confluent monolayer culture showing its specific morphological and biochemical characteristics.

The spontaneous conversion of preadipocyte cells usually takes a month after reaching the confluence, but the addition of stimulators such as insulin, dexamethasone (DEX), 1-methyl-3-isobutyl-xanthine (MIX) greatly accelerates this process and causes the accumulation of fat within few days (Mackall et al., 1976; Russell and Ho, 1976; Rubin et al., 1978).

It has been widely known that some vitamins were essential for the lipid metabolism in ruminant adipose tissue as cofactors. Also, several nutritional agents are known to affect the preadipocyte differentiation. It was reported that retinoic acid and 1-α, 25-dihydroxy-cholecalciferol, vitamin D_3, inhibited the differentiation of adipocytes in 3T3-L1 cells (Sato et al., 1980; Sato and Hiragun, 1988; Ishida et al., 1988; Stone and Bernlohr, 1989). Recently, it has been reported that there was a significant negative correlation between serum vitamin A concentration and marbling in beef cattle (Kawada et al., 1990); they suggested that a low level of dietary fat soluble vitamins, especially, vitamin A and carotenoid, actively stimulated the development of adipose tissue and bovine marbling. Also, it has been shown that treatment with the fat soluble vitamins, retinoic acid or D_3, inhibits adipocyte differentiation during early stages of differentiation at normal levels (ED50=20-50 nM) of physiological concentrations (Sato et al., 1988; Ishida et al., 1988).

It has been thought that intramuscular adipocyte differentiation is related to the formation of marbling in beef. It has been suggested that marbling may be controlled by nutritional manipulation and there has been much work to manipulate fat deposition in animal muscles. Various hormones and nutrients have been shown to affect preadipocyte differentiation in vitro (Hiragun, 1984). This study was conducted to investigate the effects of vitamins on the differentiation of preadipocytes from omental, subcutaneous, intermuscular and intramuscular adipose tissue of Hanwoo when isolated and cultured *in vitro*.

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MATERIALS AND METHODS

Materials

Dulbeco's modified Eagles's medium, Medium 199, Hanks' balanced salt solution, new-born calf serum, penicillin, streptomycin and L-glutamine were from Gibco-BRL. Collagenase type II, bovine serum albumin, insulin, triiodothyronine (T3), Dexamethasone (DEX), 1-methyl-isobutyl-xanthine (Mix), lipids, dihydroxy acetone phosphate (DHAP), nicotiamide adenine dinucleotide, reduced form (NADH), L-ascorbic acid, biotin, pantothenic acid, all trans-retinoic acid were from Sigma Chemical Co., USA.

Animals

Five Hanwoo steers were fed and managed at a feeding barn in the Livestock Research Institute under a high quality beef production program. Eight steers were castrated at 3 months old and slaughtered at 24 months of age.

Cell culture

Immediately after stunning and exsanguination, the muscle and fat portions between the 10th to 12th ribs were removed and the subcutaneous, intermuscular and intramuscular fat depots were sampled from this rib section aseptically and were kept in sterile saline (0.154 M NaCl, 37℃) for recovery of stromal vascular cells. The stromal-vascular fraction of adipose tissue which contains the precursor cells was prepared as described by Cryer et al. (1987). Tissue was sliced and cells were released by collagenase digestion in Krebs Ringer bicarbonate (KRB) buffer (1.22 mM $CaCl_2$) containing 25 mM Hepes (pH 7.4), 5.5 mM glucose, 2 mM acetate, 2 mg/ml collagenase and 40 mg/ml bovine serum albumin (BSA) for 1 h at 37℃ with constant agitation. The digested tissue was filtered through a nylon mesh screen to separate cells from undigested tissue fragments and debris. The filtrate was collected in a sterile 50 ml centrifuge tube and the top layer containing the mature adipocytes was removed by aspiration and discarded. The remaining infranatant was centrifuged at 2,500 rpm for 5 min at room temperature. The pellet was washed twice by centrifugation (2,500 rpm, 5 min) with HBSS. The final pellet was resuspended in HBSS and filtered through a 20 μm mesh screen. The cells were counted using a haemocytometer, centrifuged at 2,500 rpm 5 min, resuspended in medium consisting of M199 supplemented with 20% new-born calf serum, 2 mM acetate, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) and seeded in 60 mm culture dishes at a density of approximately 2,500 cells/cm^2. The cells were incubated at 37℃ in 5% CO_2 in air. After 24 h, attached cells were washed twice with HBSS and fresh medium was added. Culture medium was changed every second day allowing the cells to proliferate to confluence (about 10 days). For differentiation, the cells were exposed to differentiation-induction media: DMEM-F12 medium plus penicillin (100 U/ml) and streptomycin (100 μg/ml) supplemented with combinations of triiodothyronine (T3, 2nM), insulin (5 ng/ml), dexamethasone (10 nM), Mix (0.1 μM), lipids supplement (10 μl/ml) and L-ascorbic acid (50 μM), biotin (10 μM), pantothenic acid (100 μM), all trans-retinoic acid (0.5, 5, 50, 500 μg/ml). Hormones and other additions were present for the whole of differentiation period, unless otherwise stated. Differentiation media were replaced with fresh media twice during the differentiation period.

Glycerol 3-phosphate dehydrogenase activity

Differentiation of the cells was monitored by the activity of the marker enzyme glycerol 3-phosphate dehydrogenase (GPDH). Briefly, cells were washed twice with ice-cold phosphate-buffered saline; 0.5 ml of assay buffer (100 mM triethanolamine, 2.5 mM EDTA, 0.1 mM mercaptoethanol, 0.5% NP40) was added and the cells were scraped from the wells mechanically. The cell suspension was transferred to a 1.5 ml centrifuge tube and lysated by drawing the suspension into a 1 ml syringe fitted with a 27 gauge needle. The lysate was rotated for 15 min at 4℃ and then centrifuged at 14,000 g in a microfuge for 10 min at 4℃. The supernatant was removed and immediately assayed for GPDH activity as described by Wise and Green (1979). GPDH activity was measured on 10th day of differentiation. Protein content was determined by the Bradford (1976) method using BSA as standard protein.

Statistical analysis

All data were analysed by analysis of variance. The differences of means between treatments were compared by Duncan's multiple range test, using General Linear Model (GLM) procedures of SAS package (1989).

RESULTS AND DISCUSSION

Differentiation of preadipocytes was assessed by changes in the activity of the marker enzyme GPDH and by morphological changes, in particular the appearance of lipid droplets within the cells. GPDH activity is expressed/mg protein, but some treatments increased the amount of protein per culture well, so in some tables, protein content of the well is given also. Preparatory studies in which differentiation was induced by insulin, T_3 and lipids and monitored by the appearance of lipid droplets in the cells showed that droplets began to appear by day 3 of

Table 1. Effects of biotin, pantothenate and L-ascorbate on differentiation of omental, subcutaneous, intermuscular and intramuscular preadipocytes from Hanwoo

Sites	Cell components[1]	Treatments			
		Control	Biotin	Pantothenic acid	L-ascorbic acid
Omental	GPDH	139.86[a]	48.33[b]	53.14[b]	158.13[a]
	Protein	346.00[b]	360.00[b]	371.00[b]	573.00[a]
Subcutaneous	GPDH	30.65[a]	15.35[b]	21.55[b]	48.37[a]
	Protein	312.00[b]	363.00[ab]	443.00[a]	283.00[b]
Intermuscular	GPDH	133.78[a]	63.93[c]	56.65[c]	107.26[b]
	Protein	411.00[b]	358.00[b]	357.00[b]	949.00[a]
Intramuscular	GPDH	42.91[a]	30.81[a]	13.35[b]	26.97[a]
	Protein	189.00[a]	198.00[a]	184.00[a]	136.00[a]

[a,b,c,d] Means in the same row with different superscripts are significantly different ($p<0.05$).
[1] GPDH (nmol/min/mg protein), Protein (μg/well).

differentiation and reached a maximum by about day 10 of differentiation.

An initial study using lipid, T_3 and insulin showed that omission of insulin from the culture medium resulted in little or no differentiation. Further studies showed that the high concentration (1.6 μg/ml) of insulin used routinely in the cultures could be replaced by a lower (5 ng/ml) concentration. In this and subsequent studies, measurement were made of the GPDH activity of control preadipocytes maintained in differentiation medium with added lipid, T_3 and insulin for 10 days.

As shown in table 1, three water soluble vitamins were examined for their effects on adipocyte differentiation. Preadipocytes undergoing the adipose differentiation became more rounded in appearance and lost their fibroblast-like characteristics morphologically after differentiation treatment. Preadipocytes treated with biotin and pantothenic acid were significantly less differentiated than those from the control in all adipose tissues except intramuscular ($p<0.05$). Although there was no significance, vitamin C was shown to stimulate the adipocyte conversion in omental and subcutaneous, but not in intermuscular and intramuscular adipose tissues. A lower value of GPDH activity in intermuscular preadipocyte was interpreted to be caused by a relatively higher amount of protein, another parameter of differentiation. It showed quite similar results to Torii et al. (1995) who reported that vitamin C stimulated the differentiation of abdominal preadipocytes in adult sheep. Vitamin C had affected the GPDH activity at the level of 50 μM. Supplementation of the medium with 10 μM biotin 100 μM pantothenic acid had an inhibitory effect on adipose differentiation (table 1).

Kawada et al. (1990) reported that vitamin C was effective to adipocyte differentiation not at 1 μM, but at over 10 μM. Furthermore, he showed that the addition of a physiological concentration of vitamin C (200 μM) to Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum markedly enhanced the Triglyeride content and GPDH activity.

Hata et al. (1988) reported that the effect of vitamin C on the collagen synthesis had been for human skin fibroblasts. Furthermore, L-ascorbic acid phosphate, stable in solution, was reported to stimulate the synthesis and secretion of type IV collagen and other collagens both from 3T3-L1 preadipocytes and adipocytes and then accelerate the emergence of lipid droplets (Ono et al., 1989). Hata et al. (1988) postulated that one of the most important functions of vitamin C in adipogenesis might be its activity as a cofactor for the hydroxylation of proline and lysine residues in collagen, which is the most abundant protein of the extracellular matrix. It can therefore be presumed that the extracellular matrix plays an important role in the differentiation of preadipocytes.

Although it wasn't seen that vitamin C stimulated fat deposition in intramuscular adipose tissue in this study, there need to be further experiments to study the role of vitamin C in preadipocyte differentiation.

The effect of retinoic acid was investigated by using all trans-retinoic acid as vitamin A metabolite. Compared with those of the retinoic acid untreated, as shown in table 2, the differentiation of preadipocytes was significantly ($p<0.05$) lower from the level of 0.5 μg/ml in omental and intermuscular, from 50 μg/ml in subcutaneous, and in intramuscular at 500 μg/ml, thus showing that intramuscular preadipocytes were least responsive to retinoic acid in differentiation. Regardless of adipose tissues, all-trans retinoic acid appeared to inhibit the differentiation in a dose dependent manner.

Of vitamin A derivatives, it was reported that retinol and retinoic acid, inhibited the adipocyte conversion of ST 13 cells at μM level (Sato et al., 1980). Also Stone and Bernlohr (1989) reported that retinoic acid inhibited the expression of adipose-specific genes during the differentiation of 3T3-L1. The concentrations of all trans-retinoic acid required to

Table 2. Effects of levels of all-transretinoic acid on differentiation of omental, subcutaneous, intermuscular and intramuscular preadipocytes from Hanwoo

Sites	Items[1]	Retinoic acid (μg/ml)				
		0	0.5	5	50	500
Omental	GPDH	146.37^{a}	56.34^{b}	41.98^{b}	26.59^{b}	12.45^{b}
	Protein	352^{a}	353^{a}	334^{a}	269^{ab}	220^{b}
Subcutaneous	GPDH	21.82^{a}	21.68^{a}	14.6^{ab}	7.89^{b}	7.45^{b}
	Protein	312^{ab}	370^{a}	397^{a}	251^{b}	290^{ab}
Intermuscular	GPDH	133.78^{a}	133.78^{b}	85.89^{bc}	67.36^{c}	15.36^{d}
	Protein	436^{a}	436^{a}	470^{a}	454^{b}	242^{b}
Intramuscular	GPDH	31.15^{a}	16.94^{ab}	16.38^{ab}	13.48^{ab}	7.25^{b}
	Protein	217^{a}	214^{a}	200^{a}	191^{a}	248^{a}

^{a,b,c,d} Means in the same row with different superscripts are significantly different ($p<0.05$).
[1] GPDH (nmol/min/mg protein), Protein (μg/well).

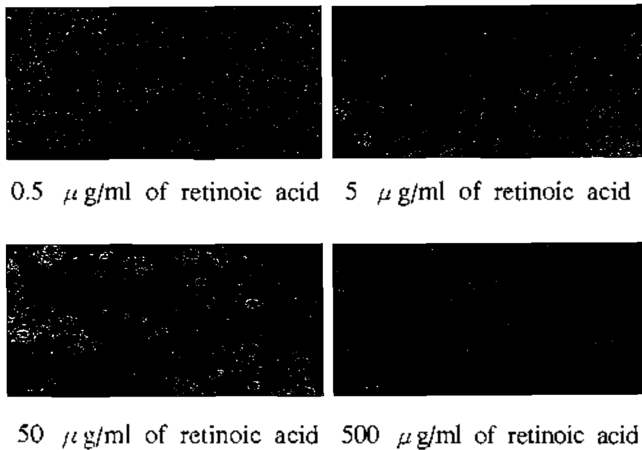

Figure 1. Preadipocytes differentiating to mature adipocytes which were treated with different levels of retinoic acid

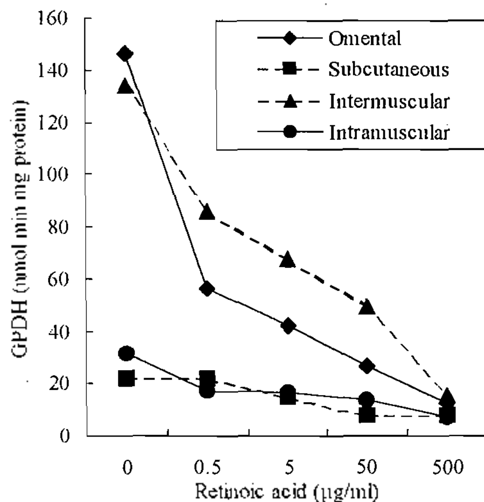

Figure 2. Effects of all-trans-retinoic acid on GPDH activity of Hanwoo omental, subcutaneous, intermuscular, intramuscular preadipocytes ($\times 10^4$ cells/ml)

inhibit differentiation were reported to be at supra-physiological levels (0.1~10 μM), whereas no or only minor effects could be seen below 10 nM. Kawada et al. (1990) reported that retinoids inhibited differentiation of Ob 17 cells when present at high concentrations. However, the same retinoids act, at concentrations below or close to their Kd values for the retinoic acid receptors, as positive effectors of adipose cell differentiation.

Through these results, it can be suggested that retinol present in blood circulation acts as one of the inhibitors of adipocyte differentiation *in vivo*. Kawada et al. (1990) reported that serum retinol concentration was negatively correlated with beef marbling performance. These results are supported by some work which has also demonstrated a negative correlation between serum retinol level and beef marbling score (Nakai et al., 1992; Oka et al., 1992). Our present data imply that retinoic acid level in blood during the fattening period could influence intramuscular fat deposition of beef cattle through its antiadipogenic action on preadipocytes present in muscle tissues, and that manipulation of the retinoic acid status of fattening beef cattle by nutritional or pharmacological treatment could be effective for improvement of beef quality.

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