Effects of Hormones on the Proliferation of Stromal Vascular Cells from Hanwoo Cattle Adipose Tissues[a]

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ABSTRACT : This study was designed to determine the effects of the insulin-like growth factor (IGF-1) and estradiol 17-β on the *in vitro* proliferation of stromal vascular cell from Hanwoo omental, subcutaneous, intermuscular and intramuscular adipose tissues. Cells were cultured in M199+20% newborn calf serum and the proliferation of cells was measured by direct microscopic cell counting and change of genomic DNA amount. Cell numbers increased slightly over the first 72 hour of culture and then increased greatly, regardless of adipose tissue depots. In IGF-1 treatment, the number of omental preadipocytes maintained highest level from the beginning to the 20th day of culture. However, in estradiol-17β treatment, those tended to be lower than the control from the beginning of culture and significantly lower at the 24th day. When IGF-1 was added to subcutaneous preadipocytes, the numbers of cells were higher from 11th day than those from other treatments, although there was no statistical significance. For intermuscular preadipocytes treated with IGF-1, its numbers were significantly ($p<0.05$) higher at 11th day, and in the other days it showed a similar tendency to those of the subcutaneous tissue. In this experiment, preadipocytes were taken from 24 month old fully matured steers and the highest proliferation rate was shown in intramuscular tissue followed by those of subcutaneous preadipocytes. Addition of 5 μM estradiol-17β to the growth medium failed to promote the replication of Hanwoo preadipocytes, as indicated by direct cell counts and total genomic DNA content. As the culture period proceeded, the amounts of DNA were increased, but the patterns of increment were not consistent with the results of cell numbers. (***Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 2 : 161-166***)

Key Words : Hanwoo Cattle Preadipocytes, Omental, Subcutaneous, IGF-1, Intermuscular, Intramuscular, Proliferation Estradiol 17-β

INTRODUCTION

Much interest is focused on highly marbled beef production in Hanwoo industry because intramuscular fat is believed to influence the eating quality of beef. To this end, it seems prerequisite to understand the mechanism of adipocyte development.

In mammals, adipose tissue occurs as a number of depots, some located in the abdominal cavity (omental), some under the skin (subcutaneous), some within the musculature (inter and intramuscular depots) (Pond, 1992). Accretion of adipose tissue occurs through both hypertrophy of adipocytes and adipocyte hyperplasia (Cryer and Cryer, 1990; Ailhaud et al., 1992; Flint and Vernon, 1993). Adipocytes do not divide, but rather are formed from precursor cells which proliferate and differentiate into mature adipocytes (Cryer and Cryer, 1990; Ailhaud et al., 1992; Flint and Vernon, 1993). These precursor cells are found in the stromal vascular fraction of adipose tissue. The capacity for adipocyte hyperplasia *in vivo* depends on a population of this fibroblast-like adipose precursor cells.

The hyperplasic development of these various depots in growing animals is asynchronous (Leibel et al., 1989; Bjorntorp, 1991; Ailhaud, 1992; Ailhaud et al., 1992; Kissebah and Krakower, 1994). In ruminant animals, abdominal depots are thought to mature earlier than carcass depots (Hammond et al., 1971) and hyperplasia of abdominal adipocytes appears to greatly slow down, at a time when proliferation of carcass adipocytes (subcutaneous and inter- and intra-muscular) is still very active (Broad et al., 1980; Vernon, 1986). The basis of these depot specific differences in hyperplasia have not been resolved.

Adipose precursor cells can be induced to proliferate and differentiate *in vitro* in chemically defined media (Cryer and Cryer, 1990; Ailhaud et al., 1992; Flint and Vernon, 1993). This has allowed investigation of the factors which regulate the production of adipocytes in a number of species including humans, rodents, ruminants, pigs and birds (Cryer and Cryer, 1990; Ramsay et al., 1992; Ailhaud et al., 1992; Flint and Vernon, 1993; Butterwith, 1994). It is possible that some growth factors might be involved in this regulation during a mitogenic phase of development and may have an autocrine and

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paracrine role in adipocyte development. Studies have investigated the role of growth factors added singly to cells in culture, whereas cells *in vivo* are exposed to a combination of factors. It is necessary to observe the proliferation of preadipocytes under conditions more like those *in vivo*.

Most studies have concentrated on the regulation of differentiation in cell lines, and the metabolic and endocrine control of lipid accumulation of pre-adipocytes is fairly well documented. However, little is known about the control of preadipocyte multiplication and the factors which might regulate their growth. Among growth factors, IGF-1 has been generally known to have a mitogenic effect on cells. It has been shown that IGF-1 can induce a mitogenic response for the 3T3 L1 (Morikawa et al., 1984) and the Ob17 preadipocyte cell lines (Grimaldi et al., 1983).

In general, females have their own adipose development characteristics and this might be related to sex hormone secretion. At puberty, the adipose tissue of females is exposed to appreciably higher levels of estrogen; estradiol 17-β is reported to stimulate the replication of adipocyte precursor (Roncari et al., 1978).

It might be meaningful to examine the preadipocyte cell proliferation in relation to growth factors and sex steroid hormones as the effect of those hormones on the proliferation of preadipocytes have not been well characterized in Hanwoo. The purpose of the study was to examine the effects of IGF-1 and estradiol 17-β on the proliferation of stromal-vascular cells from each adipose tissue and compare the proliferation rate among adipose tissues in Hanwoo.

MATERIALS AND METHODS

Materials

Medium 199 (M199), Hanks' balanced salt solution (HBSS), new-born calf serum, penicillin, streptomycin and L-glutamine were from Gibco-BRL. Collagenase type II, bovine serum albumin, insulin like growth factor (IGF-1), estradiol-17β and 0.25% trypsin/EDTA in HBSS were purchased from Sigma Chemical Co. and Genomic DNA purification kit was from Promega.

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 old.

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 (Cianzio et al., 1982).

The stromal-vascular fraction of adipose tissue 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. 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 re-suspended in HBSS and filtered through a 20-μm mesh screen. The cells were counted using a haemocytometer, centrifuged at 2,500 rpm 5 min and re-suspended in medium containing 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 24 well 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 until confluence (about 10 days). For proliferation treatment, IGF-1 and estradiol 17-β were added to proliferation medium at the concentration of 100 nM and 5 μM respectively.

Cell count

On the day of cell recovery, cells were rinsed with HBSS and detached from the culture dish surface by exposure to 0.25% trypsin/EDTA in HBSS for a few minutes and then transferred to Eppendorff tubes after diluting with proliferation medium to inactivate the trypsin. Cell numbers were determined microscopically on days 1, 3, 5, 7 ,9, 11, 13, 16, 18, 20 and 24 of culture.

Total DNA analysis

Cells were removed from the culture dish by 0.25% trypsin/EDTA in HBSS, pelleted by centrifuging for 2 min and the supernatant was aspirated and discarded. For the determination of total genomic DNA, the pelleted cells were lysed and subjected to two cycles of freezing in liquid nitrogen. The amount of total DNA was measured by the procedure of

genomic DNA purification kit.

Statistical analysis

All data were analysed by analysis of variance; depending on days of culture and hormone treatment. 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

Changes of stromal vascular cell number from omental, subcutaneous, intermuscular and intramuscular adipose tissues are shown in table 1, 2, 3 and 4. Cell numbers increased slightly over the first 72 h of culture and then increased greatly regardless of adipose tissues used.

In IGF-1 treatment, the number of omental preadipocytes maintained highest level from the beginning to the 20th day of culture. However, in estradiol-17β treatment, these tended to be lower than the control from the beginning of culture and then significantly lower at the 24th day. Significant hormonal effect was shown at 13, 20, 24th days (table 1).

When IGF-1 was added to subcutaneous preadipocytes, the numbers of the cells were higher from 11th day than those from other treatments, although there were no statistical significances. For intermuscular preadipocytes treated with IGF-1, numbers were significantly higher at 11th day, and in the other days it showed a similar tendency to those from the subcutaneous preadipocyte (table 2 and 3).

There were significant hormonal effects in subcutaneous preadipocytes at 7th and in intermuscular cells at 11th day; such an effect was not seen in the

Table 1. Changes in stromal vascular cell number of omental adipose tissue during *in vitro* culture ($\times 10^4$ cells/ml)

Treatment	Days of culture											Day effect	
	1	3	5	7	9	11	13	16	18	20	24	L	Q
Control	3.23	3.57	9.30	13.33	16.77	30.55^{ab}	31.55^{ab}	35.10	41.73	44.37^{ab}	72.80^{a}	*	NS
IGF-1	2.97	4.40	9.84	18.97	27.37	33.80^{a}	38.60^{a}	41.40	45.00	55.40^{a}	54.30^{ab}	**	NS
Estradiol	2.20	4.13	7.80	12.27	14.17	21.25^{b}	24.30^{b}	26.10	34.00	33.40^{b}	41.60^{b}	**	NS
Hormone effect	NS	NS	NS	NS	NS	NS	*	NS	NS	*	*		

^{a,b} Means in the same column with different superscripts differ ($p<0.05$). * : Significant ($p<0.05$), ** : Significant ($p<0.01$), NS : Not significant, L : Linear effect, Q : Quadratic effect.

Table 2. Changes in stromal vascular cell number of subcutaneous adipose tissue during *in vitro* culture ($\times 10^4$ cells/ml)

Treatment	Days of culture											Day effect	
	1	3	5	7	9	11	13	16	18	20	24	L	Q
Control	3.30	3.80	8.80	12.30	22.00	26.25	25.15	41.55	57.93	57.80	59.90	*	NS
IGF-1	2.00	3.43	9.07	16.60	21.47	34.90	40.50	42.80	45.30	60.30	76.40	*	NS
Estradiol	1.93	3.37	8.20	11.13	19.07	22.40	25.60	28.10	45.30	46.30	61.60	**	*
Hormone effect	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS		

^{a,b} Means in the same column with superscripts differ ($p<0.05$). * : Significant ($p<0.05$), ** : Significant ($p<0.01$), NS : Not significant, L : Linear effect, Q : Quadratic effect.

Table 3. Change in stromal vascular cell number of intermuscular adipose tissue during *in vitro* culture ($\times 10^4$ cells/ml)

Treatment	Days of culture											Day effect	
	1	3	5	7	9	11	13	16	18	20	24	L	Q
Control	2.07	2.90	7.63	10.30	16.73	22.30^{b}	28.85	31.50	35.10	39.53	60.35	NS	NS
IGF-1	2.07	3.63	8.37	15.47	17.63	36.30^{a}	43.80	43.80	55.00	55.10	56.00	**	NS
Estradiol	2.13	2.50	6.47	9.30	13.77	17.65^{b}	29.00	31.50	38.8	32.50	35.00	**	NS
Hormone effect	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS		

^{a,b} Means in the same column with different superscripts differ ($p<0.05$). * : Significant ($p<0.05$), ** : Significant ($p<0.01$), NS : Not significant, L : Linear effect, Q : Quadratic effect.

other days in these cells.

The numbers of intramuscular preadipocytes which were treated with IGF-1 were not statistically different from the control but those treated with estradiol-17β were significantly lower than that at 11th and 13th day (table 4).

Robelin (1981) reported that in intermuscular and internal adipose tissue, there was apparent proliferation at between 35 and 45% mature weight; in the subcutaneous adipose tissue this apparent proliferation appeared later, at 45-55% mature weight. Leat and Cox (1980) reported that in cattle, adipocytes occurred at 13 months in muscle and grew rapidly in the fattening period until slaughter as new-born fat tissue.

In this experiment, preadipocytes were taken from 24 months old fully mature steers and the highest proliferation rate was in intramuscular tissue which is late developing, youngest on a cellularity basis and the high and late hyperplasia, was followed by subcutaneous preadipocytes.

In vitro proliferation rates of the results above followed this characteristic of adipose tissue development.

The highest degree of replication in preadipocyte from intramuscular tissue seems to be a demonstration of genetic factors involved in tissue specific growth rate, since with *in vitro* conditions cells are removed from certain growth factors that might be circulating at elevated levels.

The results above showed that addition of IGF-1 at the level of 100 nM stimulated the proliferation of preadipocytes, a result supported by several reports. IGF-1 has been reported to have a mitogenic effect for the 3T3 preadipocyte cell line and to promote the proliferation and differentiation of the Ob17 cell line and rat preadipocytes (Djian et al., 1983; Grimaldi et al., 1983, 1984). Grimaldi et al. (1983) showed that IGF-I induced the proliferation and differentiation of cells derived from the stromal-vascular fraction of porcine adipose tissue in either serum-containing or serum-free media, but the role of IGF-1 has been examined only with limited species of domestic animals (Grimaldi et al., 1983; Morikawa et al., 1984).

The best-known effect of IGFs related to their long-term anabolic effects is to stimulate the DNA, RNA and protein synthesis and mitogenesis as indicated by increased cell proliferation. For cell proliferation, IGF-I has been labelled a progression factor, allowing a cell to traverse the Go/Gs cell cycle, following exposure to competence factors such as PDGF (Pledger et al., 1978). In this culture condition in which serum was added to the medium at 20%, those competence factors are considered to support the mitotic function of IGF-1.

The data presented in this study showed similar stimulation of mitogenic action of this growth factor in cell number and DNA increase, which is consistent with the idea that growth factors may have an autocrine or paracrine role in adipocyte development.

Addition of estradiol-17β to the growth medium failed to promote the replication of Hanwoo preadipocytes, as indicated by direct cell counts and total genomic DNA content, but Roncari et al. (1978) reported that estradiol-17β, the biologically most potent estrogen in humans, was found to promote the replication of adipocyte precursors. Roncari et al. (1978) reported that addition of estradiol-17β (50 ng/ml) at the beginning of growth in first subculture did lead to a significant increase 48 hours later in the number of human omental adipocyte precursors. These findings may explain in part the increase in fat cell number which occurs in some depots at puberty.

In this experiment, however, estradiol-17β was added at the level of 5 μM and this high level of steroid hormone might affect the proliferation.

Changes of total genomic DNA by the hormone treatments and the different adipose tissues during *in vitro* culture are seen in figure 1 and 2. As the culture period proceeded, the amount of DNA increased, but the patterns of increment was not consistent with the results for cell number. There needs to be a more reliable and reproducible method to analyze the DNA synthesis such as the ^{3}H-thymidine incorporation technique.

Table 4. Change in stromal vascular cell number of intramuscular adipose tissue during *in vitro* culture ($\times 10^4$ cells/ml)

Treatment	Days of culture											Day effect	
	1	3	5	7	9	11	13	16	18	20	24	L	Q
Control	3.40	3.20	7.37	14.47	23.03	33.50	45.65	49.25	49.00	57.43	86.25	*	NS
IGF-1	3.05	4.43	9.11	12.60	22.43	39.30	45.60	56.00	59.00	70.10	90.80	**	NS
Estradiol	3.40	4.87	7.73	11.43	19.33	21.55	27.05	45.50	52.50	72.40	-	NS	**
Hormone effect	NS	NS	NS	NS	NS	NS	NS	NS	**	NS	NS		

[a,b] Means in the same column with different superscript differ ($p<0.05$). * : Significant ($p<0.05$), ** : Significant ($p<0.01$), NS : Not significant, L : linear effect, Q : quadratic effect.

Deslex et al. (1987) reported that addition of IGF-I to rat adipocyte precursors stimulated DNA synthesis.

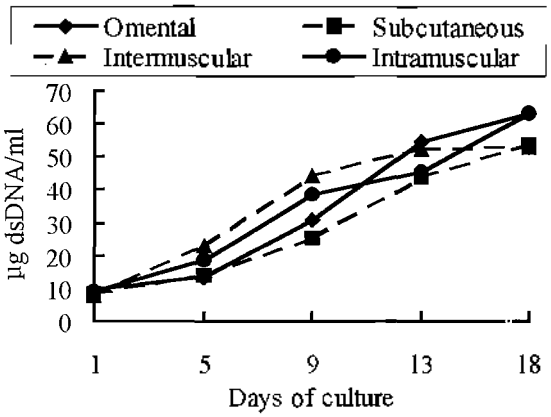

Figure 1. Change in total genomic DNA of stromal vascular cells from adipose tissues during *in vitro* culture (μ g/ml)

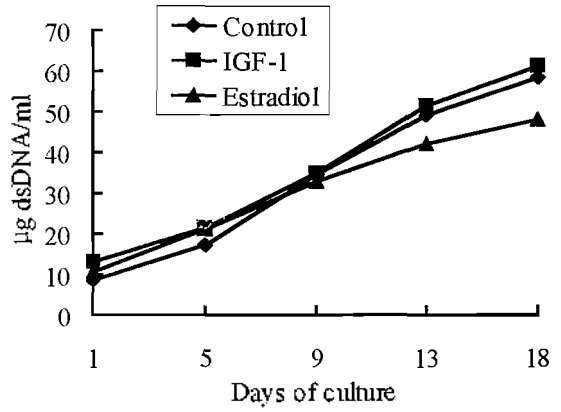

Figure 2. Effects of hormones on the changes in total genomic DNA of stromal vascular cells from adipose tissues during *in vitro* culture (μ g/ml)

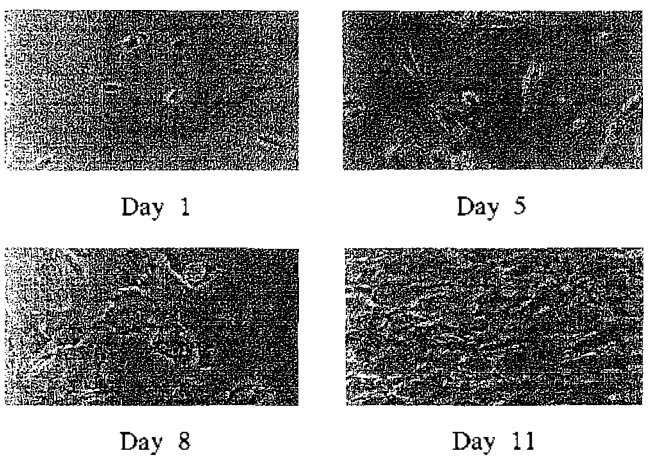

Figure 3. Proliferation of stromal vascular cells from Hanwoo

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