Pharmacological effects of sex steroid hormones on lipogenesis in Meckel's chondrocytes *in vitro*

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Abstract : Chick serum (CKS) contains factors that stimulate adipocyte induction in Meckel's chondrocytes *in vitro*. In the present study, we examined whether sex hormones as shown by the analysis of hormone level in CKS have the capacity to induce lipogenesis in Meckel's chondrocytes *in vitro*. Cultures were treated with different sex hormones at physiological and pharmacological concentrations, including testosterone and 17 β-estradiol (E_2). Lipogenesis was evaluated by sudan staining. Testosterone and E_2, which were the two most effective hormones, were selected for the present study and their effects were further examined by histological and immunohistochemical methods, including immunolocalization of adipocyte markers. BrdU-incorporation in cells exposed to physiological concentration of E_2 and testosterone was increased compared to control cultures, but there was no significant difference in BrdU-incorporation in cultures treated with E_2 and testosterone. However, at pharmacological concentrations, these hormones induced dose-dependent lipogenesis in Meckel's chondrocytes. Almost all the lipid droplet-containing cells were positive for leptin, α-glycerophosphate dehydrogenase (GPDH), and CCAAT-enhancer binding protein (C/EBP α), as shown by immunoperoxidase staining. Testosterone and E_2 had no capacity to induce lipogenesis in Meckel's chondrocytes at physiological concentrations, but our results suggest that they have the potential for lipogenesis as a pharmacological effect.

Key words : Meckel's chondrocytes, lipogenesis, sex hormones, cell culture, immunohistochemistry

Introduction

Adipose tissue has been considered as an inactive and fat-storage organ, but a recent study has found that adipose tissue is an important endocrine organ for the secretion of adipocytokines such as leptin and TNF-α [1~3]. Adipocytes composing adipose tissue are triglicerid-stored uniocular cells and derive from undifferentiated mesenchymal cells. Although these mesenchymal cells can also differentiate into osteoblasts and myoblasts[4~6], it is unclear that adipocyte differentiation is regulated by any factors. Under the *in vitro* conditions, adipogenesis has been analyzed by the addition of a variety of growth factors and hormones to adipogenic cell lines such as 3T3-F442A

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cells[7], clonal RCJ 3.1 cells[4], and mouse calvaria 2 T 3 cells[8]. Xu and Björntorp[9] reported that the progesterone stimulates the terminal differentiation in rat preadipocytes. Dieudonne et al.[10] demonstrated that in rat preadipocytes exposed to sex hormones *in vitro*, androgens elicited an antiadipogenic effects, whereas estrogens acted as proadipogenic hormones. Dang et al.[11] reported that the exposure of KS 483 cells to estrogen enhanced osteogenesis and inhibited adipogenesis. Furthermore, it has been demonstrated that such functions of estrogen were mediated via the estrogen receptor-α (ER α) and β (ER β) in adipose tissue[12,13] and that ER α knockout mice brought an increase of adipose tissue[14,15]. Although various adipogenesis-stimulating factors were estimated at sequential preadipocytes and adipocytes *in vitro*, the involvement of sex hormones on adipogenesis is poorly understood.

Recently, we found that chick serum (CKS) contains an adipogenesis-stimulating factor[16,17]. When Meckel's chondrocytes were cultured in alpha-modified essential medium (α-MEM) plus 10% chick serum, they lost their chondrocytic phenotype and transformed into adipocytes containing abundant lipid droplets. Analysis of the hormone levels of CKS by radio immunoassay indicated high concentrations of estrogen and insulin in comparison with fetal bovine serum (FBS)[16], and there was a possibility that these hormones could be involved in the phenotypic transformation in Meckel's chondrocytes. In the present study, therefore, we examined the involvement of sex hormones on the lipogenesis in Meckel's chondrocytes.

Materials and Methods

1. Cell culture

Meckel's chondrocytes from ddY strain mice were used in this study. Meckel's cartilage was dissected out on day 17 of gestation (vaginal plug=day 0) and chondrocytes were isolated with the following mixture of enzymes; 0.1% hyaluronidase (type I-S; Sigma Chemical Co., St. Louis, MO, USA) and 0.15% collagenase (type II; Worthington Biochemical Corp., Freehold, NJ, USA), as described previously[18,19]. Chondrocytes were inoculated at an initial density of 1 x 10^4 cells per Penicylinder cup (0.28 cm^2; Top Labo-ware, Osaka, Japan). Alpha-modified Eagle's medium (α-MEM; Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal bovine serum (FBS; ICN Biomedicals Inc., Aurora, OH, USA), 0.03 mg/ml *L*-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako Pure Chemical Industries Ltd., Osaka, Japan), 60 cm^2/ml kanamycin (Meiji Seika Co., Tokyo, Japan), and 2 mM β-glycerophosphate (β-Gly; Nacalai Tesque, Inc., Kyoto, Japan) was freshly prepared as a control medium. Sex hormones were prepared at concentrations as shown in Table 1 and were added to the medium. Cultures were maintained for up to 2 weeks in a humidified atmosphere of 5% CO_2 in air and the medium was changed every other day.

2. Histological and statistical analysis of cell proliferation

Cell proliferation was evaluated by cell density and bromodeoxyuridine (BrdU) incorporation per mm^2. For analysis of cell density, the cells were cultured for 2 days

at physiological concentrations of 17β-estradiol (E_2) and testosterone. The cells were fixed with 4% paraformaldehyde and stained with 0.1% toluidine blue. For analysis of lipogenesis, chondrocytes were cultured for one week with various concentrations of testosterone and were processed as described above prior to Sudan III staining[16].

For incorporation of BrdU, chondrocytes cultured for 2 days at physiological concentrations of E_2 and testosterone were exposed to 0.5 mg/ml BrdU for 30 min, fixed with 4% paraformaldehyde for 15 min, and processed for immunohistochemistry with a BrdU-detection kit (Oncogene Research Products, Cambridge, Mass, USA). Using a hemometer, the number of BrdU-positive cells per mm^2 was calculated from five different areas in 2 dishes, and the findings were expressed as the mean ± SD.

3. Indirect immunohistochemical staining

For immunoperoxidase staining, we used antibodies against leptin (Affinity Bioreagents, Golden, Colo., USA), α-glycerophosphate dehydrogenase (GPDH; Nordic Immunological Laboratories, Tilburg, Netherlands) and CCAAT-enhancer binding protein (C/EBPα; Santa Cruz Biotechnology, Inc. CA, USA), as markers of mature adipocytes. Chondrocytes treated with 50 μg/ml of testosterone were harvested after 2 weeks in culture and were fixed with 4% paraformaldehyde for 30 min at room temperature, washed with PBS, and then intrinsic peroxidase was inhibited for 15 min with 3% H_2O_2. After washing three times with PBS, samples were exposed to horseradish peroxidase (HRP)-conjugated antirabbit IgG for 1 h at 37℃. Afterthorough washing with PBS, specimens were mounted in a mixture of glycerol and PBS (9:1, v/v) prior to light microscopic examination. For control specimens the primary antibody was omitted and specimens were incubated directly with the secondary antibodies, and then processed as outlined above. No positive immunoreactivity was found in the controls (data not shown).

4. Electron microscopy

Samples for electron microscopy were processed by conventional methods. The cells were cultured for 2 weeks with the female sex hormones, E_2 (100 μg/ml) and progesterone (100 μg/ml), were fixed with cold 2.5% glutaraldehyde for 1 h, washed with cacodylate buffer (pH 7.2), and further postfixed with 1% OsO_4 for 1 h. They were dehydrated with a graded ethanol series and embedded in Epon 812. Ultrathin-sections were cut with a diamond knife on a microtome, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (Hitachi H-7000, Tokyo, Japan).

Results

1. Cell proliferation

Light microscopy revealed that with toluidine blue staining, there was a distinct difference between cells grown in control medium and cells grown in testosterone or E_2-containing medium (Fig. 1 a-c); the number of chondrocytes in the sex hormone-containing medium was much higher than that in control medium.

The proliferation rate was tested for testosterone- and E_2-treated chondrocytes (Fig. 2). The number of BrdU-positive cells in E_2-containing medium was 31.5 cells per

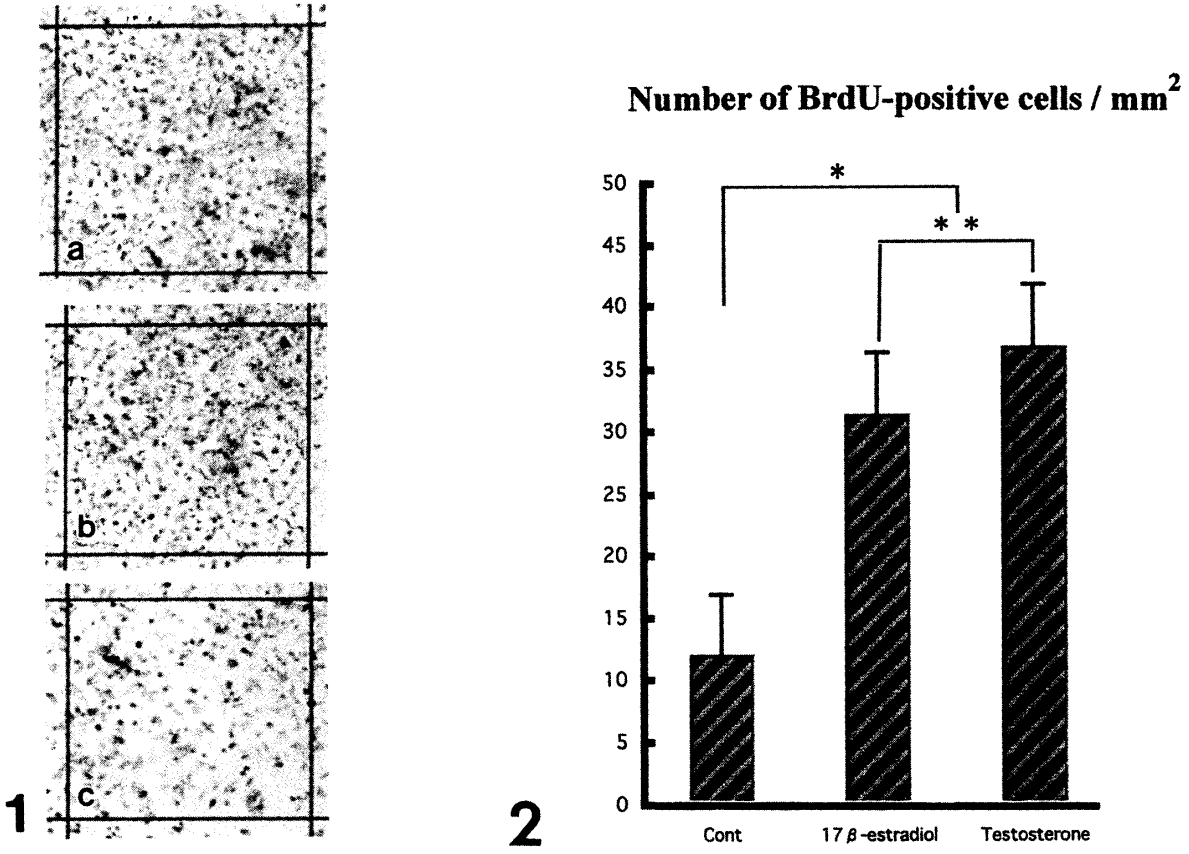

Fig. 1. Over view of cell proliferation, as revealed by toluidine blue staining. 17β-estradiol (E_2) (a) and testosterone (b)-treated chondrocytes proliferate more densely than control (c). Frame area shows 1 mm^2.

Fig. 2. Comparison of the proliferation rate of E_2 and testosterone by BrdU incorporation. The cells were cultured for 2 days at physiological concentrations. The number of BrdU-positive cells in the control group is 12.0 cells, while that in the E_2 and testosterone treated group is 31.5 and 36.8 cells. There is no significant difference ($p>0.05$) between these hormones, but there is a significant difference between controls and sex hormones ($p<0.01$).

mm^2, while in testosterone-treated cells it was 36.8 cells per mm^2. Statistical analysis indicated that this difference was not significant ($p = 0.6757$). In contrast, BrdU-labeled cells in control cultures were less dense than in cultures where the test medium contained sex hormones. Statistical analysis showed that the number of BrdU-labeled cells in the control medium was significantly different from that in sex hormone-treated cultures ($p=0.0011$).

2. Sudan staining of the testosterone-treated chondrocytes

In the present study, testosterone was the most effective hormone for lipogenesis among the used sex hormones (Table 1) and therefore, the results for chondrocytes cultured for one week at various concentrations of testosterone are described here (Fig. 3 a-d). After treatment at physiological concentrations (5 ng/ml), no significant accumulation of lipid droplets was seen in the chondrocytes (Fig. 3 a). This result was consistent with that of chondrocytes treated with control medium. Chondrocytes exposed to testosterone at a dose of 10μg/ml showed accumulation of granular lipids in the cytoplasm (Fig. 3 b). Relatively large lipid droplets were

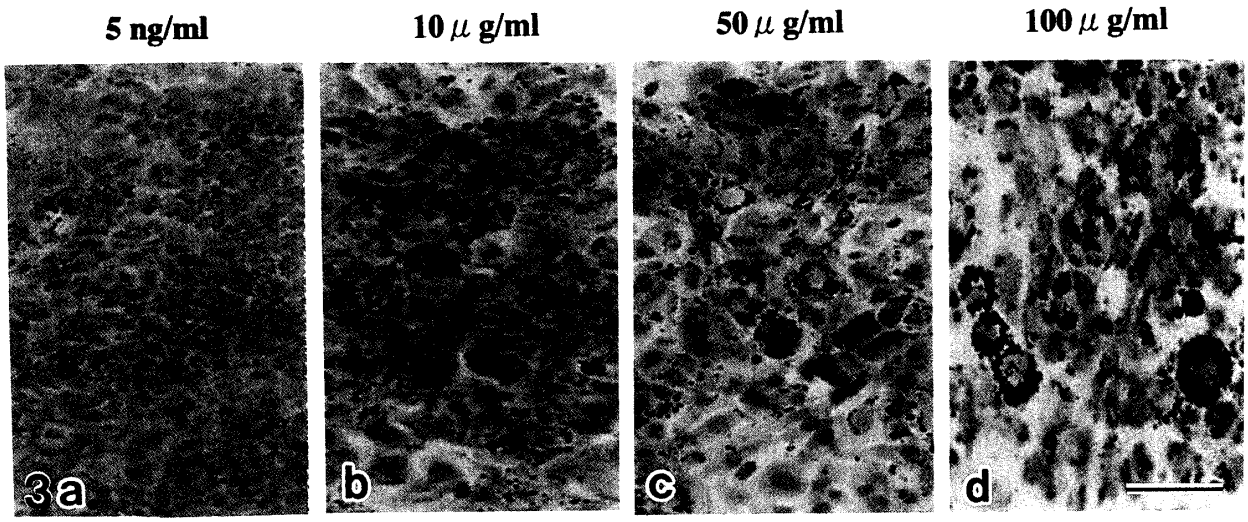

Fig. 3. Photomicrographs of chondrocytes treated with various concentrations of testosterone as shown by sudan staining after one week in culture. (a) Significant accumulations of lipid droplets are not seen at a physiological concentration of 5 ng/ml. (b) and (c) At a dose of 10 μg/ml and 50 μg/ml of testosterone, numerous granular lipid droplets appear in the nodular chondrocytes. (d) At a dose of 100μg/ml testosterone, predominantly sudan-positive cells filled with large lipid droplets appear. Scale bar=20 μm.

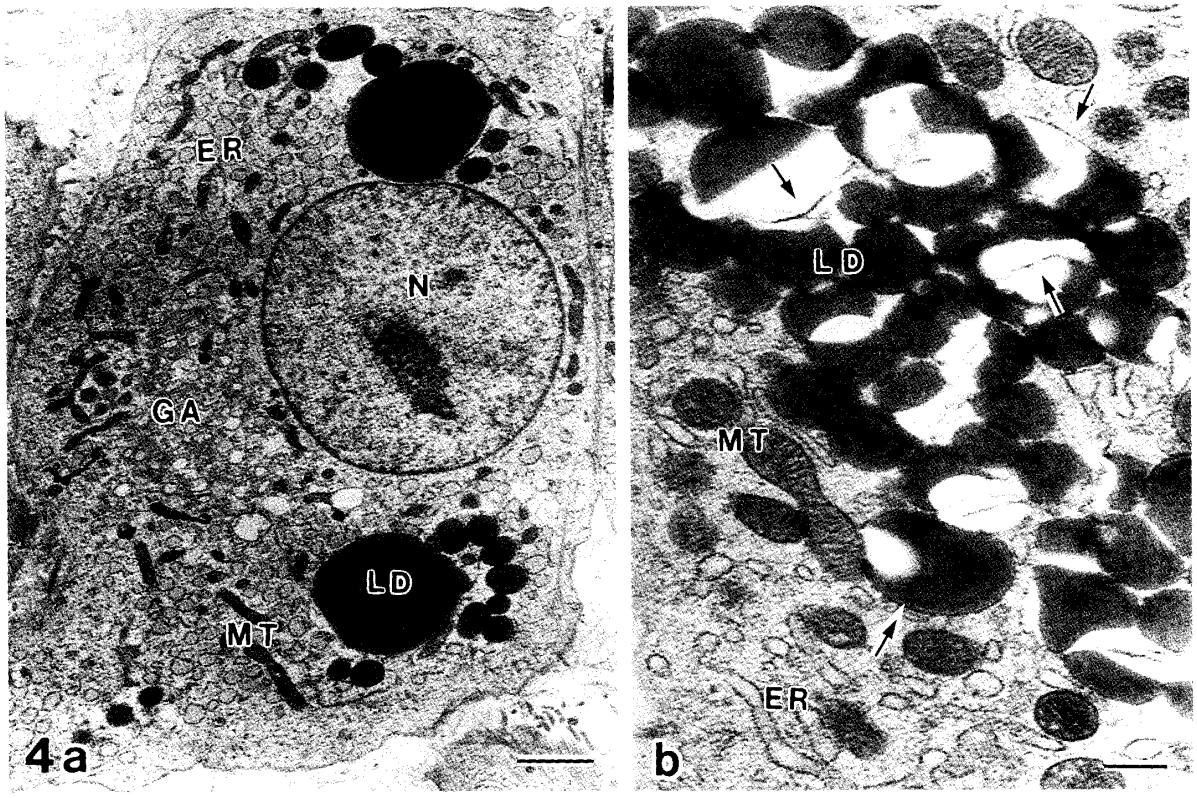

Fig. 4. Electron micrographs of the lipid-droplet-rich cells induced by the treatment of E_2 and progesterone for 2 weeks. (a) E_2-exposed cells contain various sizes of vesicular structures and a few large lipid droplets (LD). N=nucleus, ER=rough endoplasmic reticulum, MT=mitochondria, GA=Golgi apparatus. (b) Magnified electron micrograph of progesterone-treated cells. Cells contain membrane-bounded lipid droplets (arrows, LD), mitochondria (MT) and rough endoplasmic reticulum (ER). The content of some lipid droplets was lost and fused with each other. Scale bar=(a) 2 μm, (b) 1 μm.

observed abundantly in sudan-positive chondrocytes exposed to 50 μg / ml testosterone (Fig. 3 c). At a dose of 100μg/ml testosterone, lipid droplet-rich cells had been converted to round-adipocytic cells (Fig. 3 d).

3. Electron microscopy

The chondrocytes treated with E_2 at 2 weeks after culture were characterized by the appearance of various sizes of lipid droplets. Lipid droplet-rich cells contained many vesicular elements; a round nucleus with developed euchromatin and a prominent nucleolus, elongated mitochondria, a profile of rough endoplasmic reticulum, and Golgi-associated vacuoles (Fig. 4 a). The cytoplasm usually contained both large and small lipid droplets.

In contrast, progesteron-treated chondrocytes were characterized by aggregated small lipid droplets, small mitochondria, and rough endoplasmic reticulum (Fig. 4 b). In many cases, almost every lipid droplet was enclosed by a limiting membrane and coalesced with one another to increase in size. The lipid droplets consisted of homogenous material with high electron-density, but usually the contents were lost from central zone of lipid droplets.

4. Immunoperoxidase staining

When testosterone-treated chondrocytes were examined immunohistochemically after 2 weeks in culture, immunopositive granular products indicative of leptin, a marker of differentiated adipocytes, appeared in lipid droplet-rich chondrocytes (Fig. 5 a). Similarly, immunoreactivity for GPDH (Fig. 5 b) and C/EBP α (Fig. 5 c) was also high in adipocyte-like cells containing abundant lipid structures. These immunohistochemical findings revealed the sufficient features as mature adipocytes.

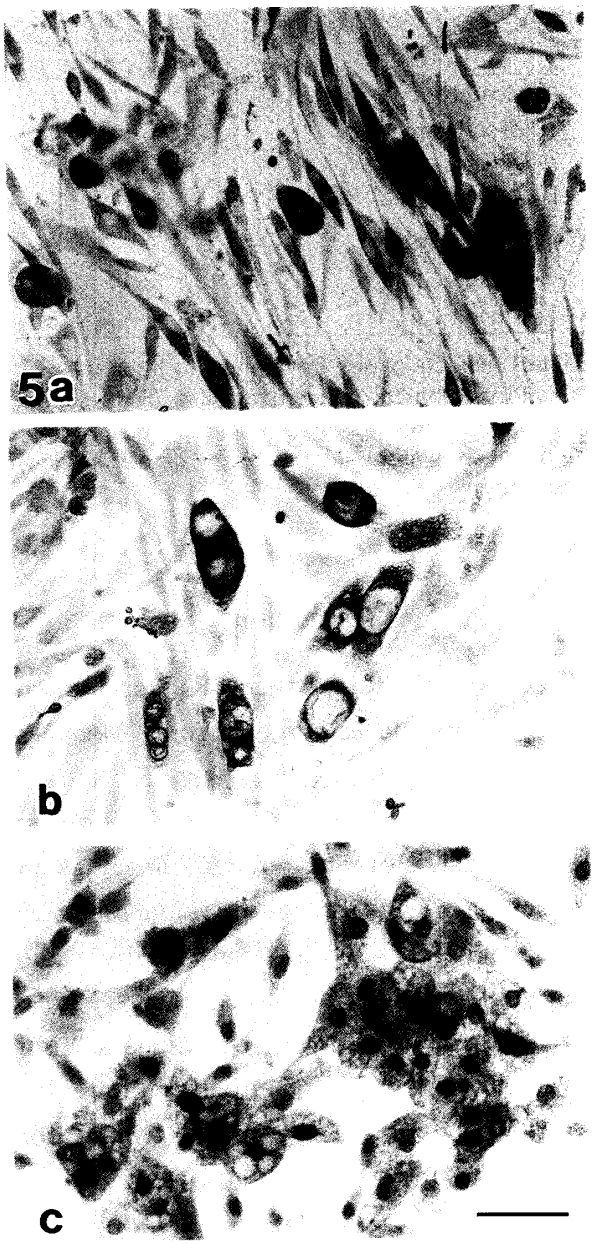

Fig. 5. Immunofluorescent staining with antibodies against mature adipocytes, leptin, GPDH and C/EBP α. Cultures were collected from cells exposed to testosterone for 2 weeks. (a) Leptin is positive in the lipid droplet-containing cells. (b) Large lipid droplet-containing cells are immunopositive for GPDH. (c) Positive immunoreactivity for C/EBP α is widely distributed in lipid droplet-rich cells. Scale bar=20 μm.

Hormones	Concentrations				
	PC	100 ng	1 μg	5 μg	10 μg(ml)
Testosterone propionate[1]	−	++	++	+++	+++
Androsteron[2]	−	−	−	−	+
Progesteron[3]	−	−	+	+	++
Estrogen					
17β-estradiol[4]	−	+	+	++	++
estrone[5]	−	−	−	−	−
estriol[6]	−	−	−	−	−

Table 1. Effects of sex hormones on lipogenesis in Meckel's chondrocytes *in vitro*.
PC : Physiological concentrations; [1](50 ng/ml), [2](2.5 ng/ml), [3](30 ng/ml), [4](0.5 ng/ml), [5](0.2 ng/ml), [6](50 ng/ml).
Lipogenesis was evaluated as, — : no lipogenesis, +: slight lipogenesis, ++: active lipogenesis, +++: more intensive lipogenesis.

Discussion

Recently, we found that CKS accelerated the phenotypic changes from Meckel's chondrocytes to adipocytes *in vitro*[16]. Furthermore, CKS factors induced adipogenesis not only in chondrocytes *in vitro* but also in splenic stromal cells *in vivo*[17]. When CKS was analyzed by radio immunoassay at hormone levels, since estrogen and insulin were contained at high concentrations[16], we supposed that sex hormones could be the initial trigger for lipogenesis. In the present study, we further examined whether lipogenesis is induced by such hormonal factors and showed that E_2 and testosterone at physiological concentrations stimulated proliferation of chondrocytes but at high concentrations, the chondrocyte cultures induced formation of sudan-positive lipid droplets that were also shown to be positive for adipocyte markers by immuneohistochemistry. Therefore, we suggest that sex hormones could play a role in adipocyte induction as a pharmacological effect, although E_2 and testosterone at physiological concentrations were not concerned with lipogenic activity.

Adipocyte differentiation *in vitro* has been investigated in various established cell lines such as 3 T 3 -F442A cells, mouse calvariae 2 T 3 cells[7,8], and embryonal carcinoma-de rived C 1 cells[20~22]. However, it was essential to add dexamethasone, insulin, indomethacin, and growth hormone[20~22),23]. These studies suggested that the factor necessary for commitment to the adipocyte phenotype differs between cell lineages but a common inductive factor from undifferentiated mesenchymal cells to adipocytes has not yet been determined. Almost all cell lines as described above, are cells that are committed to adipocyte differentiation, and each of the inductive factors known so far encourages differentiation only. From an inferential point of view, the CKS factor for lipogenesis induction is a common inductive factor[24], and there was the possibility that hormone-like elements induced adipocyte formation.

The results of high levels of estrogen, including E_2 and of insulin by CKS analysis led us to investigate the involvement of hormonal elements in lipogenesis. Thus, we tried to analyze morphologically adipocyte

induction by sex steroid hormones. When the chondrocytes were exposed to E_2 and testosterone at physiological concentrations, both hormones accelerated cell proliferation of chondrocytes significantly, as followed by increased BrdU incorporation. Dieudonne et al.[10] demonstrated in rat preadipocytes in culture that an exposure to sex hormones androgens elicited an antiadipogenic effects, whereas estrogens behaved as proadipogenic hormones. Dang et al.[11] also reported that the exposure of KS483 cells to estrogen enhanced osteogenesis and inhibited adipogenesis. Some reports, including Dieudonne et al.[10], indicated that sex hormones stimulated cell proliferation in 3 T 3 -L1. These results are consistent with the result in Meckel's chondrocytes in the present study. It has been reported that estrogen enhances bone formation but inhibits adipogenesis at physiological concentrations[11] and that testosterone, one of the main components of androgens, enhances both bone formation and adipocyte differentiation. In the present study, since testosterone and E_2 stimulated cell multiplication rather than lipogenesis in chondrocytes, it was assumed that these hormones might play a role in the regulation of the number of adipocytic precursor cells.

Meckel's cartilage is derived from primitive cranio-facial mesenchyme, originating in the neural crest[25,26]. The chondrocytes have unique features such as adipocyte phenotypic changes *in vitro* by CKS elements[16] and fibroblastic transformation *in vivo*[27]. We implied that these features may origin-associated features of Meckel's cartilage derived from neural crest-ectomesenchyme. These phenotypic changes were also useful as a model for the analysis of lipogenic inductions.

The study of lipogenesis has been performed in the established preadipose cell lines, such as 3 T 3 -L 1 and F442A cells, that have committed to the adipocytes[28,29], and therefore, such cells have a more effective adipocytic conversion than Meckel's chondrocytes. Since the cells used in our study consist of pure chondrocyte population that had already acquired cartilage features, it seemed that lipogenesis in Meckel's chondrocytes was not affected by sex steroid hormones at the physiological concentrations. Generally, steroid hormones act via receptors. Although the receptors for estrogen and androgen were not confirmed in these chondrocyte cultures in preliminary experiments by immunocytochemical stainings (data not shown), it is necessary to further investigate whether the present results were due to steroid hormone receptors.

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培養メッケル軟骨細胞に対する性ホルモンの脂肪形成効果について

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抄録：ニワトリ血清（CKS）はメッケル軟骨細胞に脂肪形成を促進する因子を含んでいる。本研究では，CKSのホルモン分析の結果から得られたような高レベルの性ホルモンが培養メッケル軟骨細胞の脂肪形成にどの様に関わっているのか検討した。培養軟骨細胞は生理的ないし薬理的濃度のテストステロンと17β-エストラジオール（E_2）を含む数種の性ホルモンで添加培養し，脂肪滴形成能をズダン染色によって評価した。最も脂肪形成能の高かったテストステロンとE_2については，電子顕微鏡，免疫染色とBrdUの取込みから細胞増殖率を算定した。その結果，生理的濃度でのテストステロンとE_2は細胞増殖を促進したが，脂肪形成は促進しなかった。高濃度では両者のホルモンは脂肪形成を亢進した。形成された脂肪細胞はレプチン，GPDHやC/EBPα抗体による免疫染色では陽性であった。これらの結果から，テストステロンとE_2は薬理学的作用として脂肪形成能を有しているが，生理的条件下では脂肪形成そのものより未分化型細胞での細胞増殖を刺激している可能性が示唆された。

キーワード：メッケル軟骨細胞，脂肪形成，性ホルモン，細胞培養，免疫組織化学