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Effects of Sex Hormones on the Growth and Collagen Metabolism of Human Gingival Fibroblasts

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INTRODUCTION

The increased prevalence and severity of gingival inflammation during puberty and pregnancy have been described by many investigators[1-5]. Increased permeability of microvasculature in the gingiva caused by sex hormones is considered to be responsible for the gingival inflammation observed during pregnancy[6].

Hyperplastic change of gingiva is one of the features observed in gingivitis at puberty and pregnancy. These changes might be attributed to increased or unbalanced secretion of sex hormones although the mechanisms of hyperplasia caused by them is not known yet. The possible changes which occur in the hyperplastic gingiva might be 1) overgrowth of gingival fibroblasts, 2) increased production of collagen and/or proteoglycans.

Many *in vivo* studies have been conducted to examine the influence of sex hormones on the gingival tissue. Hugoson et al. reported that sex hormones injected intramuscularly into dogs increased the vascular permeability of the gingiva but there were no differences in fibroblast activity or collagen fiber configuration within the gingival connective tissue[7,8]. Histological studies using rats treated with sex hormones did not show any difference in the orientation of collagen fibers[9]. Ruleright *et al.* conducted a study using castrated rabbits and compared the effects of sex hormones on genital mucosa and gingiva. Significant changes were observed in the uterus and vagina of the rabbits but no changes were detected in the gingiva[10].

On the contrary, there are few *in vitro* studies concerning the effects of sex hormones on the function of gingival fibroblasts. Engel *et al.* reported that human gingival fibroblasts produce types I and III collagen *in vitro*[11]. Type I collagen accounted for 70 to 95% of the total collagenous protein in human gingiva[12]. Huamn gingival fibroblasts also produce a latent type collagenase *in vitro* which is activated by PCMB[13]. Thus we investigated the effects of sex hormones on the growth and type I collagen metabolism of the gingival fibroblasts *in vitro* by using a serum free culture medium.

MATERIALS AND METHODS

Cell culture

Human gingival fibroblasts were grown from biopsies of gingival connective tissue obtained from a 17 years old male with healthy gingiva. The cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 0.29 mg/ml of L-glutamine, 1.3 mg/ml of $NaHCO_3$ and 250 μg/ml of gentamycin. They were incubated at 37°C in a humidified atmosphere of 5% CO_2. FBS used in this experiment was treated with dextran coated charcoal to eliminate the indigenous sex hormones. Serum free medium ASF301 (Ajinomoto, Japan) was used for testing the effects of sex hormones on the cell growth and collagen metabolism[14]. The final concentrations of sex hormones in the medium were 0.04, 0.4, 1, 2 and 20 ng/ml for estradiol and 0.04, 0.2, 2, 20 and 200 ng/ml for progesterone.

Effects of sex hormones on the growth of gingival fibroblasts

The cells were plated on 35 mm tissue culture dishes

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using ASF301 medium which contains estradiol or progesterone. The initial density of the cells were 1.8×10^5 cells per dish for estradiol and 2.1×10^5 cells per dish for progesterone. Six dishes were used for each condition. The cells were detached after 24, 72 and 120 hrs of incubation using 1 ml of 0.25% trypsin solution and the number of the cells were counted using a hemocytometer under a microscope.

Effects of sex hormones on collagen metabolism of the gingival fibroblasts

The cells were plated on 25 cm^2 culture flask using MEM plus 10% FBS and cultured for 7 days. The initial density of the cells were 2.8×10^5 cells for estradiol and 2.2×10^5 cells for progesterone. After 7 days the media were changed to ASF301 which contains the designated concentration of sex hormones and incubated at 37°C. Six flasks were used for each condition. After 96 hrs, the culture medium was collected and dialyzed against PBS at 4°C overnight and used for the assay of collagenolytic activity and type I collagen determination.

Collagenolytic activity was determined by modification of the solution method using fluorescence isothiocyanate (FITC) collagen (Collagen Gijutsu Kenshukai, Japan) as a substrate[15]. Briefly, one volume of 0.1% FITC collagen, dissolved in 0.01 M acetic acid, was mixed with an equal volume of 0.1 M Tris-HCl buffer pH 7.6, containing 0.4 M NaCl and 10 mM $CaCl_2$. The resulting solution (0.2 ml) was incubated at 35°C for 2 hrs with 0.2 ml of culture medium containing 1 mM of p-chloromercuribenzoic acid (PCMB). After arresting the reaction with o-phenanthroline, the denatured product was extracted by adding 0.4 ml of 70% dioxane in 0.17 M Tris-HCl buffer pH 9.5. After the reaction mixture was centrifuged at $3,000\times g$ for 10 minutes, the supernate was assayed for fluorescence intensity by means of fluorescence spectrophotometer (Excitation 495 nm, Emission 520 nm). One unit of collagenolytic activity was defined as the amount of enzyme which degrades 1 μg of collagen per minute under the conditions employed.

The amount of type I collagen in the medium was determined by enzyme linked immunosorbent assay (ELISA). A 96 well microtiter plate was coated with standard type I collagen or dialyzed sample and incubated at room temperature. Rabbit antihuman type I collagen polyclonal antibody (Chemicon International, USA) was added and incubated for 2 hrs. Then biotinylated secondary antibody (Dako Corporation, USA) was added and incubated for 1 hr. The horseradish peroxidase conjugated streptoavidin biotin complex (Dado Corporation) was added and incubated for 30 min. Then freshly prepared 0.02% H_2O_2 0.4% phenylene diamine was added and incubated for 1 hr. The reaction was arrested with 8 N H_2SO_4 and optical density at 492 nm was determined by using a microplate reader (Toso, Japan).

Statistical analysis

The effects of sex hormones on the production of type I collagen and collagenase were statistically analyzed by unpaired t-test.

RESULTS

Effects of sex hormones on the growth of gingival fibroblasts

The number of the cells incubated with 1.0, 2.0 and 20 ng/ml of estradiol for 120 hours was significantly less compared to the control (Fig. 1). Progesterone at the concentration of 2.0, 20 and 200 ng/ml also showed inhibitory effects on the growth of gingival fibroblasts (Fig. 2).

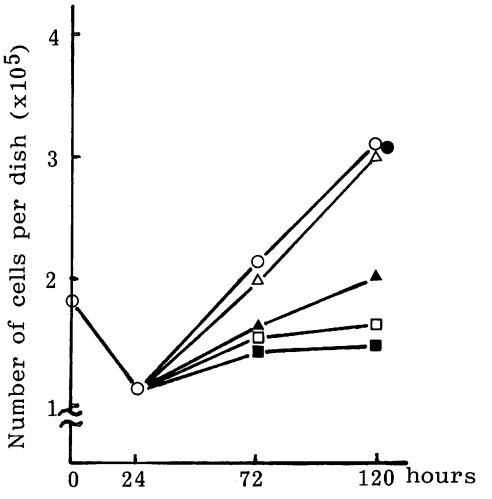

Fig. 1 Effects of estradiol on the growth of gingival fibroblasts. Concentrations of estradiol; ○ 0, ● 0.04, △ 0.4, ▲ 1.0, □ 2.0, ■ 20, (ng/ml).

Effects of sex hormones on collagen metabolism of the gingival fibroblasts

Both estradiol and progesterone showed similar effects on collagenolytic activity and type I collagen production. Table 1 shows the effects of estradiol on collagenolytic

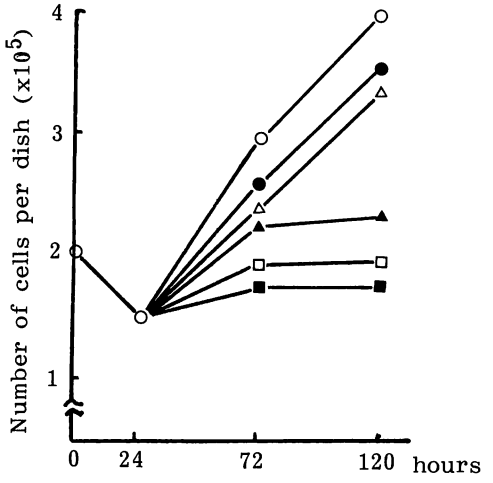

Fig. 2 Effects of progesterone on the growth of gingival fibroblasts. Concentrations of progesterone; ○ 0, ● 0.04, △ 0.2, ▲ 2.0, □ 20, ■ 200, (ng/ml).

activity and type I collagen production of gingival fibroblasts. Collagenolytic activity was enhanced at 0.4 ng/ml of estradiol and inhibited at 1.0, 2.0 and 20 ng/ml of estradiol. On the contrary, the production of type I collagen was reduced with 0.04 and 0.4 ng/ml of estradiol while it was enhanced with 2 and 20 ng/ml of estradiol. The effects of estradiol on collagen production and collagenolytic activity at low concentration were reversed at high concentration of estradiol. Progesterone inhibited the collagenolytic activity at the concentration of 2, 20 and 200 ng/ml. However the production of type I collagen was enhanced at 0.2, 20 and 200 ng/ml of progesterone (Table 2). It was shown that both estradiol and progesterone had reverse effects on collagenolytic activity and type I collagen production from gingival fibroblast.

DISCUSSION

Fukuda demonstrated that estrogens may stimulate the proliferation of gingival fibroblasts while progesterone inhibits it[16]. Maruotti also demonstrated the stimulating effects of estradiol on gingival fibroblast proliferation[17]. However the culture media they used in the studies were supplemented with bovine serum so that they could not exclude the influence of indigenous sex hormones and sex

Table 1. Effects of estradiol on collagenase and type I collagen production from gingival fibroblasts

Estradiol ng/ml	Collagenase		Type I Collagen	
	unit/ml	% of control	μg/ml	% of control
0	0.119±0.006		8.4±0.7	
0.04	0.116±0.003	97	5.3±0.6**	63
0.4	0.138±0.005**	116	3.6±0.6**	43
1.0	0.109±0.004*	92	8.7±1.1	102
2.0	0.108±0.002**	91	10.7±1.7*	127
20.0	0.080±0.008**	67	12.6±0.6**	149

(n=6) Mean±SD * $p<0.05$, ** $p<0.01$

Table 2. Effects of progesterone on collagenase and type I collagen production from gingival fibroblasts

Progesterone ng/ml	Collagenase		Type I Collagen	
	unit/ml	% of control	μg/ml	% of control
0	0.071±0.005		3.3±1.0	
0.04	0.065±0.010	92	2.1±1.4	63
0.2	0.064±0.010	90	5.4±1.0*	162
2.0	0.038±0.011**	54	4.6±0.6	137
20.0	0.045±0.013**	63	7.2±0.7**	215
200.0	0.041±0.010**	58	5.6±0.8**	168

(n=6) Mean±SD * $p<0.05$, ** $p<0.01$

hormone binding protein contained in the serum. It is very important to use a serum-free medium when studying the effects of sex hormones *in vitro* because it can exclude the effects of sex hormones and sex hormone binding proteins usually contained in the serum. The serum free medium ASF301 keeps the gingival fibroblasts alive and makes them proliferate. Thus the results we obtained in this experiment are free from the influence of the serum.

It has been shown that gingival tissue has steroid hormone receptors and it considered to be a target tissue for these hormones although the exact role of sex hormones in the regulation of the metabolism and cell proliferation in the gingiva is not known yet[18,19]. This is the first *in vitro* study which demonstrated that sex hormones affect the growth and collagen metabolism of the gingival fibroblasts. However, participation of the steroid hormone receptors in this action is not clear yet.

The concentrations of sex hormones used in this experiment were based on the physiologic concentrations in the plasma of adult male or female (including pregnant woman). The reported concentrations of estradiol and progesterone in plasma are 0.04–20 ng/ml and 0.2–200 ng/ml respectively[20].

The results coincide with the clinical features (i.e. hyperplasia of the gingiva) observed during puberty and pregnancy. It is interesting that sex hormones at high concentration in the culture medium inhibited the growth of the gingival fibroblasts while they enhanced the production of type I collagen. The hyperplastic change of the gingiva observed during puberty and pregnancy might be the result of increased production of type I collagen but it is not caused by the overgrowth of the gingival fibroblast.

It has been shown that sex hormones increase the permeability of microvasculature and enhance the gingival inflammation[2]. Morishita *et al.* reported that increased secretion of estradiol and decreased secretion of progesterone might be one of the factors which stimulate the progress of gingivitis at puberty[21]. An *in vitro* study showed that the chemotaxis of human PMN were inhibited by estradiol while they were enhanced by progesterone[22]. In these studies, estradiol and progesterone showed antagonistic effects on inflammation and PMN chemotaxis. However in the present study, the effects of estradiol and progesterone on the gingival fibroblasts were not antagonistic. Thus the effects of sex hormones on inflammation and hyperplasic change of the gingiva should be discussed separately.

The present study suggests that the gingival hyperplasia observed during puberty and pregnancy might be caused by the increased secretion of sex hormones through their action on the increased production of type I collagen but not by the overgrowth of the gingival fibroblasts.

SUMMARY

The purpose of this study was to evaluate the effects of sex hormones on the growth and collagen metabolism of human gingival fibroblasts *in vitro*. A serum free medium was used to exclude the effects of sex hormones and sex hormone binding proteins contained in the serum. The results demonstrated that both estradiol and progesterone inhibited the growth of gingival fibroblasts. The collagenase activity was enhanced at 0.4 ng/ml of estradiol while it was inhibited at 1.0, 2.0 and 20 ng/ml of estradiol and 2.0, 20 and 200 ng/ml of progesterone. The production of type I collagen was inhibited at 0.04 and 0.4 ng/ml of estradiol while it was enhanced at 2.0 and 20 ng/ml of estradiol and 0.2, 20 and 200 ng/ml of progesterone. It was suggested that the gingival hyperplasia observed during puberty and pregnancy might be caused by the increased secretion of both estradiol and progesterone.

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