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Research Article

Effect of Sex Steroid Hormones on the Clonal Growth of Female and Male Keratinocytes

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Abstract

The effect of male and female sex steroid hormones on the proliferation of both male and female epidermal keratinocytes was investigated in cells cultured in a completely defined serum-free medium. Growth was assessed by clonal growth assays. The clonal growth of male foreskin-derived keratinocytes was strongly inhibited at micromolar concentrations by both 17-β-estradiol and progesterone, and had only a moderate inhibitory effect on the clonal growth of female adult skin-derived keratinocytes. By contrast, clonal growth of either male or female keratinocytes was unaffected by micromolar concentrations of testosterone. Morphological studies provided additional support for the effect of female sex steroid hormones, showing drastic decrease in cell number, abnormal cell morphology and altered colony and cell arrangements. Evidence is provided for a specific and saturable 17-β-estradiol receptor present of the surface of male-derived suprabasal keratinocytes challenged by unlabelled competitor male and female sex steroid hormones: estradiol, estriol, norethistrerone, levonorgesterol and testosterone in a radio-labelled 17-β-estradiol binding assay.

Introduction

Sex steroids, also known as gonadal steroids, are steroid hormones that interact with vertebrate androgen or estrogen receptors [1]. Their effects are mediated by slow genomic mechanisms through nuclear receptors as well as by fast non-genomic mechanisms through membrane-associated receptors and signaling cascades [2]. The two main classes of sex steroids are androgens and estrogens, of which the most important human derivatives are testosterone and estradiol, respectively. Other contexts will include progestogens as a third class of sex steroids, distinct from androgens and estrogens. Progesterone, the most important and only naturally-occurring human progestogen, is an endogenous steroid hormone involved in the menstrual cycle, pregnancy, and embryogenesis of humans and other species. Progesterone is also a crucial metabolic intermediate in the production of other endogenous steroids, including the sex hormones. Topical application of both estradiol and progesterone creams appear to reverse signs of skin aging. In general, androgens are considered "male sex hormones", since they have masculinizing effects, while estrogens and progestogens are considered "female sex hormones" although all types are present in both sexes, albeit at different levels [1-7]. The review paper described estrogen effects on human skin and the potential mechanisms how estrogens can alleviate skin aging [8]. Other studies have shown that applying an estradiol cream to the skin of old males increased collagen production [9]. There is limited knowledge of the effects of testosterone on skin. Testosterone is actually converted in skin by an enzyme called 5-alpha reductase to Dihydrotestosterone (DHT), which controls the sebaceous glands in the skin and the production of sebum [10].

From previous studies, it is widely known that human skin is the second most active body site for the metabolic interconversions of sex steroid hormones [10]. Nevertheless, little is known about the direct effect of sex steroid hormones such as testosterone, estradiol and progesterone on the growth and differentiation of both male and female human epidermal keratinocytes. There have been several conflicting reports on the stimulatory effect of estradiol on human keratinocytes. An early report found that 17-β-estradiol stimulated the clonal growth of epidermal keratinocytes in culture [11]. Stimulation of newborn foreskin keratinocyte proliferation by 17-β-estradiol was reported to occur at sub-nanomolar concentrations, but required several days of exposure in Serum-Free Culture Medium (SFM) at suboptimal (0.06mM) calcium concentration [12]. Estrogen and progesterone were also reported to stimulate human keratinocyte proliferation in a serum-containing culture medium at nanomolar concentrations and were inhibitory at micromolar concentrations [13]. Finally, Treatment with 10nM 17-β-estradiol produced a slight but significant increase in cell proliferation in a serum-containing medium [7].

In view of these considerations and because living reformed human epidermis [14] is an ideal model for assaying the effect of sex steroid hormones, it was important to reassess their effects on testosterone, estradiol and progesterone on normal basal epidermal keratinocytes derived from both

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a male source, neonatal foreskin, and a female source, adult breast skin, cultured in a completely defined SFM. Moreover, we examined Reformed Human Epidermis (RHE) produced in serum-free culture as a model system to assess the effect of a wide variety of contraceptive hormones. Of immediate interest is to test the use RHE, as a living skin substitute for the transdermal delivery of contraceptive hormones. To answer this question, we first evaluated whether RHE has specific and saturable estradiol binding sites.

Methods and Materials

Materials

Insulin, Epidermal Growth Factor (EGF), testosterone, estradiol, tri-iodothyronone (T_3), and progesterone were all purchased from Sigma Chemical Company (St. Louis, MO). Tissue culture wares were obtained from Corning (Corning, NY). Type IV collagenase (Dispase, 20U/ml) was from Boehringer- Mannheim (Los Angeles, CA). Radio-labelled 17-beta-estradiol (160 Ci/mM) was obtained from New England Nuclear Corp. (Boston, MA). The female sex hormones, estriol, levonogestrol and noresthisterone were a gift from Southern Research Institute (Birmingham, AL).

Methods

(a) Cell culture: Neonatal foreskin epidermal keratinocytes (NF) and adult human breast epidermal keratinocyte were prepared as previously described [14,15]. Primary and secondary passage cultures were all propagated in a modification of MCDB 153 SFM prepared from frozen stocks of ingredients as previously described [15].

(b) Clonal growth assays, microscopy and photography: The effect of sex steroid hormones of the growth and differentiation of normal male and female keratinocytes was assessed by clonal growth assays as previously described [14]. Briefly, for male neonatal undifferentiated basal keratinocytes 500 cells and for female adult breast keratinocytes 1,000 cells were seeded into 35cm^2 sterile plastic dishes and fed MCDB153 Serum-Free Medium (SFM) supplemented with insulin (5µg/ml) and EGF (5ng/ml). On the fourth day of culture the old medium was aseptically removed and 3.0 ml of fresh SFM containing insulin and EGF was added to all dishes. Then, 30µl of ethanol was added to duplicate dishes that served as the control, 30µl of 1000X testosterone stock in ethanol was added to duplicate dishes to make a final concentration of 3.7 x 10^{-6}M, and 30µl of 1000X stock of 17-β-estradiol in ethanol was added to duplicate dishes to make a final concentration of 4 x 10^{-6}M, and finally, 30µl of 1000X stock of progesterone in ethanol was added to duplicate dishes to make a final concentration of 3.7x 10^{-6} M. Ten days later, all treated dishes were fixed and stained with 0.2% crystal violet stain. The stained clonal dishes were photographed without magnification, and the stained colonies were counted using a (Artex) image analysis system. The stained dishes were also observed at 150X power using a Nikon inverted phase microscope and photographed with a Cannon camera.

(c) 17-β-Estraadiol receptor assay: The binding of radio-labelled 17-β-Estradiol to replicate samples of cultured Reformed Human Epidermis (RHE) from a single genetic source was determined as follows: RHE was formed by culturing neonatal foreskin basal epidermal keratinocytes in replicate 24-well cluster dishes and converting a confluent monlayer to a 3-dimensional sheet of histologically-complete stratified keratinized epidermis [15]. The culture medium was aseptically removed and 0.5 ml of Solution A (10mM glucose, 3mM KCL, 140mM NaCl, 1mM Na2HPO4, 22mM Hepes buffer) containing 10-50 mM of radiolabelled 17-β-estradiol added to each well. The concentration of the estradiol was fixed at half-maximal saturation to assure effective competition with unlabelled identical or analog steroid hormones over a wide range of competitor concentrations. The steroid competitors tested in the binding assay were 17-β-estradiol, testosterone, estriol, levonorgesterol and norethisterone. At the end of 24 hours incubation at 4°C, the radio-labelled solutions were removed, the surface of the RHE samples rinsed gently with 1.0ml of ice-cold Solution A and 0.5ml Dispase added to each well to enzymatically release the intact RHE. The RHE samples from each well were transferred to respective vials with scintillation spectrometry fluid and the contained radioactivity counted in a scintillation spectrometer.

Results

Figure 1 shows the effect of three different sex steroid hormones on the clonal growth of both male and female keratinocytes as investigated by the technique of clonal growth assay. Testosterone had a negligible effect on either male or female keratinocytes. By contrast, both progesterone and estradiol completely inhibited the clonal growth of male keratinocytes, while significantly inhibiting the clonal growth of adult female keratinocytes. The effects might be attributed solely to gender differences. However, the male cells were derived from newborn foreskin and the female cells were isolated from adult breast skin. This possibility was not excluded as equivalent age-matched tissues were not readily available. Further studies on mice might resolve this question.

Figure 2 presents further clonal growth assays showing that doubling the concentration of female sex steroid hormones (2µg/ml), for both estradiol and progesterone produces an even greater inhibition of clonal growth of male keratinocytes, while doubling the concentration of testosterone has a lesser effect on male derived keratinocytes.

As a secondary control, we examined the effect of a non-steroidal hormone, triiodothyronine or T_3, a hormone made by the thyroid gland and responsible for regulating oxidative metabolism in vertebrate animals. The effect of T_3 (1x 10^{-9}M) on the clonal growth of

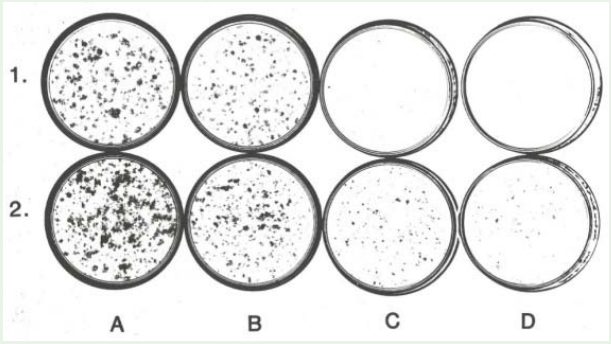

Figure 1: Effect of testosterone, progesterone and estradiol on the clonal growth of human epidermal keratinocytes cultured in standard MCDB 153 medium. Top row (1): new born foreskin keratinocytes. Bottom row (2) adult breast skin keratinocytes. A, no hormone; and 1 µg/ml each of: B, testosterone; C, progesterone and D, 17-β-estradiol.

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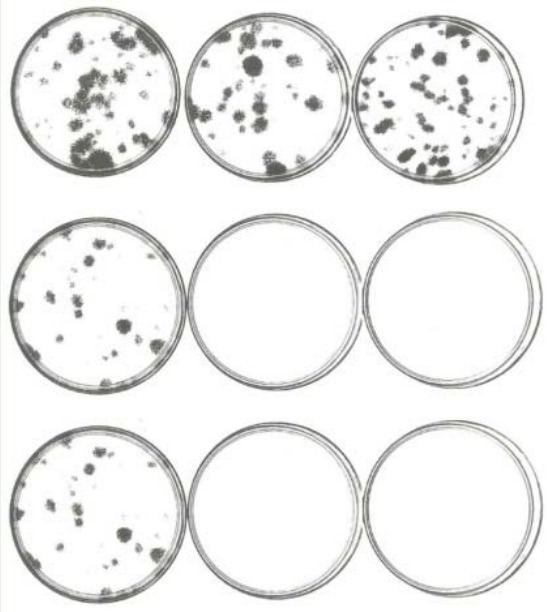

Figure 2: Clonal growth assays conducted on male keratinocytes testing testosterone (top row) at 0, 1µg/ml and 2µg/ml, progesterone (middle row) at 0, 1µg/ml and 2µg/ml, and estradiol (bottom row) at 0µg/ml, 1µg/ml and 2µg/ml.

male-derived keratinocytes was minimal with only a 22% inhibition of growth. No changes in cell morphology were noted (data not shown).

Table 1 presents numerical data for two sets of experiments, comparing the results between untreated controls and sex steroid treatments. The male-targeted sex steroid hormone, testosterone, had no significant inhibitory effect on female-derived keratinocytes in either experiment 1 or experiment 2, but did have a minor inhibitory effect on male-derived keratinocytes in both experiment 1 (12%) and experiment 2 (18%). By contrast, treatment of male keratinocytes with estradiol, a female targeted sex steroid hormone, had a drastic inhibitory effect in both experiment 1 and experiment 2 of 76% and 80%, respectively. However, treatment of female keratinocytes with estradiol gave only a moderate but significant inhibition (31%) in experiment 1 and no significant inhibition in experiment 2. Treatment of male keratinocytes with female targeted sex steroid

Table 1: Effect of Testisterone, Estradiol, Progesterone.

On Clonal Growth of Normal Male and Female Keratinocytes				
Culture conditions	**Growth Response[1] (colonies/dish)**			
Control	**Male[2]**		**Female[3]**	
	Exp. 1	Exp. 2	Exp.1	Exp. 2
	299±13	427±40	577±8	417±109
Testosterone (1.0µg/ml)	262±2.5	352±6	593±10	410±40
Estradiol (1.0µg/ml)	71±12.5	87±9.5	395±32	409±7.5
Progesterone (1.0µg/ml)	42±16	102±8.5	357±46	324±14.5

[1] Values represent the results of duplicate determination;

[2] Male neonatal foreskin keratinocytes were seeded at 500 cell per dish;

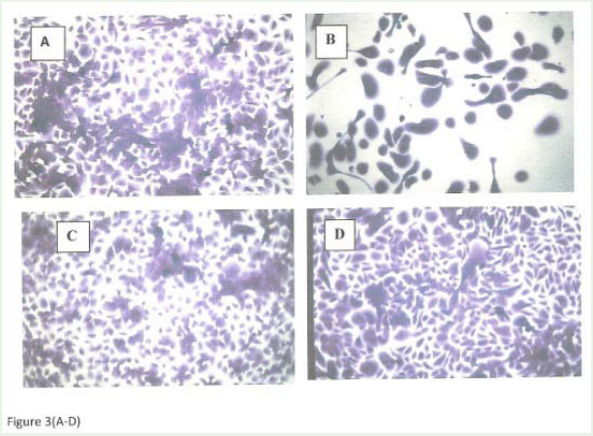

Figure 3: Images of (A) untreated male keratinocytes, (B) male keratinocytes treated with 17-β-estradiol, testosterone (C) and progesterone (D). Clonal growth assays, and staining procedures are described in the Methods. Total magnification, 300X.

hormone, progesterone, again produced a drastic inhibitory effect in both experiment 1 and 2 of 82% and 86%, respectively. By contrast, treatment of female keratinocytes with progesterone gave only a moderate but significant inhibitory effect in experiments 1 and experiment 2, of 3% and 18%, respectively (Table 1, Figure 1).

Morphological correlates of sex steroid treatments of male-derived keratinocytes

Figure 3 is photomicrographs showing the morphological effects of sex steroid treatments on newborn foreskin keratinocytes. Figure 3A (top left) shows a typical colony of untreated cells fixed and stained with crystal violet stain after 14 days of clonal growth. The majority of the monolayer cells are small and undifferentiated basal keratinocytes that remain solitary. Note the several foci of associated suprabasal cells as expected for culture in SFM containing low calcium (100µM). Figure 3B (top right) shows a photomicrograph

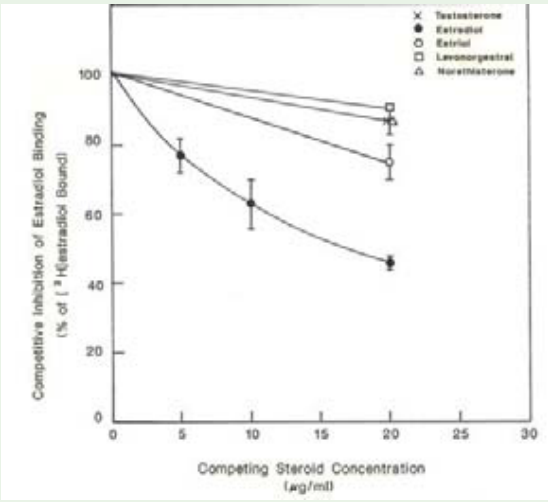

Figure 4: Competitive inhibition estradiol binding assay using [3H]-radiolabeled estradiol and unlabeled competitor sex steroid hormone: testosterone (x), estradiol (●), estriol (○), levanogestrol (□) and noresethisterone (Δ).

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of the effect of estradiol (1μg/ml) on clonal growth. The colony cells are highly dispersed and the cells are abnormally large and distorted in shape consistent with the inhibitory effect documented in Table 1. Figure 3C (bottom left) show a typical colony of testosterone treated keratinocytes. The majority of cells in the monolayer are small undifferentiated basal keratinocyted that are slightly smaller than the control undifferentiated basal cells, and again there are several foci of suprabasal type cells undergoing early stages of stratification as expected for culture in low calcium (100μM) SFM. Figure 3D (bottom right) shows a typical colony of progresterone-treated keratinocytes. The small cells in the monolayer appear typical of undifferentiated basal keratinocytes but they are arrayed in the monolayer in a twisted spiral arrangement unlike that seen in colonies of untreated control cells. There is also some evidence of decreased number of foci with fewer numbers of suprabasal cells per focus.

Receptor binding of 17-β-estradiol to male-derived RHE

Table 1 presents the results of experiments designed to examine the specificity of radiolabelled 17-β-estradiol binding to samples of in vitro reformed human epidermis, RHE, exposed several different unlabelled competitor sex steroid hormones including 17-β-estradiol, estriol, norethisterone, levonorgesterol, and testosterone. The results were compared with radiolabelled estradiol in the absence of any unlabelled sex steroid hormones. As expected, testosterone exhibited no competition against labeled estradiol, and unlabelled estradiol was the best competitor, reducing radio-labelled estradiol binding by 42%. The other unlabelled competitor female sex steroid hormone, estriol, norethisterone, and levongesterol exhibited correspondingly less competition ranging from 25%, 14% and 10% reduction in binding, respectively. The typical hyperbolic decrease in [^{3}H]-17-β-estrrading binding to RHE is presented as a function of increasing concentration of unlabelled estradiol (Figure 4).The use of RHE combined with radio-labelling demonstrates that keratinocytes organized in to a functional epidermis retain specific receptor binding properties observed for in vitro basal keratinocytes.

Discussion

Our studies show for the first time that female sex steroid hormones estradiol and progesterone at micromolar concentrations have a profound inhibitory effects on the proliferation of normal male-derived human keratinocyte, with a much less effect on female-derived keratinocytes. For estradiol, we found that the inhibitory effects are mediated by a specific 17-β-estra diol receptor present on the surface of keratinocytes in the RHE skin substitute. These results are consistent with results from previous studies on the use of RHE for assaying the percutaneous absorption of estradiol [16]. At present, we are unaware of any published reports demonstrating an inhibitory effect of female sex steroid hormone on male proliferating keratinocytes. By contrast, the male sex steroid hormone, testosterone, had minor or no effect on keratinocyte proliferation in either male or female keratinocytes. The significance of the lack of an inhibitory action of testosterone is unknown. Our binding studies showed that testosterone had no effect on estradiol binding. We are also not aware of any reports demonstrating an inhibitory effect of female sex steroid hormone on female proliferating keratinocytes or of any reports on the effect of male sex steroid hormone, testosterone, on proliferating keratinocytes. These findings are supported by the morphological correlates of inhibition of proliferation, which reveal abnormal cell shapes and other signs of cell and colony alterations. As a negative control, we examined the effect of the thyroid hormone, T_3. It was found to have a modest inhibitory effect on male keratinocytes, the significance of which is unknown.

There are several reports showing a modest stimulatory effect of estradiol on male human keratinocytes. For example, 17-β-estradiol stimulated the growth of epidermal keratinocytes in culture [13]. However, the stimulation was minimal and occurred under poorly defined growth factor and ill-defined SFM conditions, such as the use of bovine pituitary extract. In another report [12], stimulation of newborn foreskin keratinocyte proliferation by 17-β-estradiol occurred at sub-nanomolar concentrations. Significant stimulation was delayed until the third day of exposure, and occurred in SFM at suboptimal (0.06mM) calcium concentration, suggesting that estradiol may stimulate proliferation in slowly growing cultures. In a separate report, subnanomolar concentrations of both estrogen and progesterone stimulated human keratinocyte proliferation in a serum-containing culture medium, but were inhibitory at micromolar concentrations [11]. Unfortunately, the use of serum to support keratinocyte growth is a two-edge sword as it contains both growth-promoting and growth-inhibiting factors, which confound the effects of added sex steroid hormones.

As regards, the inhibitory effects of progesterone reported here, it has been reported that cultured human keratinocytes do express a cytoplasmic progesterone receptor as well as progesterone receptor mRNA transcripts [6]. The inhibitory progesterone effect may be due to supramaximal receptor affinity concentrations as appears to be the case with estradiol. Nevertheless, this does not account for the fact that female adult keratinocytes are less sensitive to female sex steroid hormone inhibition than are male keratinocytes, suggesting a difference either at the ligand binding receptors or in the downstream signaling cascade.

Conclusions

Female sex steroid hormones estradiol and progesterone have a profound inhibitory effect on clonal growth of male derived keratinocytes, but have a significantly less effect on female-derived keratinocytes. By contrast, testosterone, a male sex steroid hormone, had no effect on the clonal growth of either male or female keratinocytes. RHE, a model skin epidermis, possesses saturable 17-β estradiol receptor binding sites.

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