Human sebocyte-based assay system for the screening of compounds to lower the lipid synthesis in sebaceous gland

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Key words: human sebaceous gland cell line (SZ95 cell), acne, 13-cis-retinoic acid, spironolactone, lipid

Summary

SZ95 cell is an immortalized human sebaceous gland cell line that shows the morphologic, phenotypic and functional characteristics of normal human sebocytes. Sebocytes may play crucial parts in the pathophysiologic processes and disorders of the pilosebaceous unit. The secretory activity of the sebaceous gland is remarkably species-specific and acne is an exclusively human disease. Thus, this SZ95 cells offer possibilities for investigations on the physiology of the sebaceous gland and its role in sebum-associated skin disease such as acne. In this study, we investigated the effects of 13-cis-retinoic acid (13-cis-RA) and spironolactone, frequently used as therapeutic agents of acne, on the lipid synthesis and proliferation of human sebocytes. Cell proliferation was determined by MTT assay and cytoplasmic lipid droplets was shown by Oil-red O staining. Total lipid levels were biochemically estimated by the sulfo-phospho-vanilline reagent. 13-cis-RA and spironolactone significantly inhibited proliferation and lipid levels in a dose-dependent manner. Combined treatment with testosterone and 13-cis-RA or spironolactone resulted in a lower total lipid levels than that with androgen alone. These observations indicate that 13-cis-RA and spironolactone are potent inhibitors of both cell proliferation and lipid synthesis in human sebocytes. We will provide experimental evidence that this human sebocyte cell line serves as an adequate tool for evaluating the anti-lipogenic activity of various compounds potentially useful for the bioactive cosmeceutical ingredients on acne skin, and studying the intracellular biochemical markers depending on the types of compounds from various sources.

Introduction

Acne vulgaris is the most common skin disease in man and affects more than 80% of the population to varying degrees. The etiology of acne is multifactorial, with seborrhea, follicular hyperkeratinization, bacterial colonization, and cutaneous inflammation all playing a role in the pathogenesis of this disorder[1]. There is increasing evidence that sebocytes may play crucial parts in the pathophysiologic processes and disorders of the pilosebaceous unit, especially in acne[2].

To date, much of our understanding of the physiology and pathophysiology of the sebaceous gland stems from experimental animal models[3] but no animal model was found predictive in assessing the effects of anti-acne drugs in human beings[4]. Thus, the changes of isolated human sebaceous glands or cells would be a highly desired goal for the investigation into the pathophysiology of acne. The changes of human sebaceous cells is also of relevance to drug discovery, especially in light of recent findings that indicate that for some compounds whole-animal models are not predictive for antiacne activity[5]. The facts that acne is an exclusively human disease and that the secretory activity of the sebaceous gland is remarkably species-specific[6] led to the search for human models.

SZ95 cell is an immortalized human sebaceous gland cell line transfected with a PBR-322-based plasmid containing the coding region for the Simian virus-40 large T antigen. SZ95 cells present several characteristics of nontransfected human sebocytes, such as the presence of cells with different sizes, a polymorphous epithelial appearance with numerous lipid droplets in their cytoplasm, abundant cytoplasmic organelles as well as structures indicating lipid synthesis, and synthesis of the characteristic sebaceous lipid squalene and wax esters[7].

In recent studies, the clinical benefits of 13-cis-RA in individuals with acne were well documented. Spironolactone is an aldosterone antagonist that has been clinically used as an antihypertensive and diuretic drug. Spironolactone has also been successfully used for treatment of acne, hirsutism, and androgenic alopecia[8].

In order to establish human sebocyte-based assay system for the screening of compounds to anti-acne drugs, we investigated the effects of 13-cis-retinoic acid (13-cis-RA) and spironolactone, frequently used as therapeutic agents of acne, on the lipid synthesis and proliferation of SZ95 cells as well as its possible modification of testosterone effects.

Materials and Methods

Cell cultures

SZ95 cells were maintained as adherent cultures in a standard medium constituted of modified DME medium/Ham's F 12 medium (1:1) (Biochrom, Germany) with 2mM N-acetyl-L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and 50 μg gentamicin per ml (Gibco-BRL, Germany) at 37℃ in a humidified atmosphere containing 5% CO_2. Culture medium

was replaced every 2-3 d.

Treatment with Testosterone, 13-cis-RA, and Spironolactone

SZ95 cells were seeded in culture plates and were left to attach for 2 d at 37℃ with 5% CO_2 in culture medium. The medium with testosterone (10^{-8}-10^{-5} M) (Sigma), 13-cis-RA (10^{-8}-10^{-5} M) (Sigma), or spironolactone (10^{-8}-10^{-5} M) (Sigma) or their combination was added to culture plates at each concentration. The compounds were added to medium as a 0.1% dimethyl sulfoxide (DMSO; MERK) solution. Medium with 0.1% DMSO was concomitantly added to another culture plates serving as controls. Medium with and without compounds was changed every 2 d.

Cell Proliferation

Cell cultures were seeded in 96 well culture plates at densities of 4×10^3 cells per well. Cell proliferation was assessed by the 3-(4,5-dimethyl thiazo-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Oil Red staining

Cells grown in chamber slides (Nunc) were incubated either with 0.6% Oil Red solution (Sigma) in 60% isopropanol for 15-120 min at room temperature. The cultures were then observed under a light microscope.

Lipid analysis

Cell cultures were seeded in 10 cm dish (Nunc) at densities of 1×10^6 cells. Sebaceous lipids were extracted using the Folch-Lees extraction method[9] as modified by Ways and Hanahan[10]. Extracted lipids were stored prior to analysis in chloroform pre ml under oxygen-free nitrogen at -20℃. Total lipid content was assessed by the sulfo-phospho-vanillin colorimetric method.

Results

In order to characterize the lipids produced by cultured SZ95 cells, lipid was examined by histochemical and biochemical techniques. By staining with 0.6% Oil Red solution, numerous red droplets were detected in cytoplasm of cultured SZ95 cells. As a control, HaCaT cells, a human keratinocyte cell line, were not stained with Oil Red solution in the same experiment (Fig. 1). To determine the quantitative differences, total lipid content was estimated by sulfo-phospho-vanillin method. SZ95 cells markedly produced lipid as compared with HaCaT cells (Fig. 2). The lipid production of SZ95 cells was stimulated by testosterone in a dose-dependent manner after 5 d of treatment (Fig. 3). This stimulatory effect in HaCaT cells, however, was markedly weaker than the effect of testosterone on the lipid production of SZ95 cells (Fig. 2).

We determined, therefore, the effects of 13-cis-RA and of SP on the lipid synthesis of SZ95 cells.

Both 13-cis-RA and spironolactone inhibited the lipid synthesis of SZ95 cells in a significantly and dose-dependent manner after 5 d of treatment. The inhibitory effect of 13-cis-RA was 35% at 10^{-6} M ($p<0.01$) (Fig. 4) and the inhibitory effect of spironolacone was 40% at 10^{-6} M ($p<0.01$) (Fig. 5).

Testosterone stimulated the lipid synthesis of SZ95 cells, the effect being 112% at 10^{-5} M. 13-cis-RA significantly inhibited the stimulatory effect of testosterone on the lipid synthesis of SZ95 cells. Inhibitory effects were 43% at 10^{-7} M ($p<0.05$) and 82% at 10^{-6} M ($p<0.01$) of 13-cis-RA when added together with testosterone (10^{-5} M) (Fig. 4). In a similar way, the stimulatory effect of testosterone on the lipid synthesis of SZ95 cells was dose-dependently reduced by spironolactone. Inhibitory effects were 59% at 10^{-7} M ($p<0.01$) and 116% at 10^{-6} M ($p<0.01$) of spironolactone when added together with testosterone (10^{-5} M) (Fig. 5).

In addtition, we determined the effects of 13-cis-RA and spironolactone on the proliferation of SZ95 cells in vitro. After 5 d of treatment, both 13-cis-RA and spironolactone slightly inhibited SZ95 cell proliferation in a dose-dependent manner. The inhibitory effect of 13-cis-RA was 15% at 10^{-5} M ($p<0.05$) and the effect of spironolacone was 19% at 10^{-5} M ($p<0.05$) (Fig. 6). The antiproliferative effect of spironolactone was slightly stronger than the effect of 13-cis-RA.

Discussion

Sebum production is generally accepted to be one of the major factors involved in the ethiology of acne. The presence of large lipid droplets within sebocytes, as well as the production of sebum-specific lipids, indicates that differentiation towards a mature sebaceous cell has occurred. Androgens are the best-known stimulators of the sebaceous gland, enhancing both mitosis and lipogenesis of human sebocytes in vivo[11]. The resulting seborrhea promotes the formation of acne lesions. In this study, numerous cytoplasmic lipid droplets were detected in SZ95 cells by Oil Red staining. Their lipid content was increased when cultured with testosterone.

Antisebocytic activities of retinoic acid have been reported Zouboulis et al.[12] using human sebocyte cultures and by Guy et al.[13] using isolated sebaceous glands. Spironolactone produces antiacne effects and has been shown to inhibit 5α- Dihydrotestosterone (DHT) receptors in human sebaceous gland [14]. We confirmed, therefore, the effects of 13-cis-RA and spironolactone on the lipid synthesis as well as its possible modification of testosterone effects using a recently established human sebocyte line (SZ95 cell) [7]. Both 13-cis-RA and spironolactone inhibited the lipid synthesis of SZ95 cells in a significantly and dose-dependent manner.

Androgens cause hyperactivity of sebaceous glands, with increased sebum secretion. It is generally accepted that testosterone, the major circulating androgen, is converted intracellularly by 5α-reductase to 5α-Dihydrotestosterone (DHT), which is the most potent androgen in tissue[15]. In this study, 13-cis-RA and spironolactone significantly inhibited the stimulatory effect of testosterone on the lipid synthesis of SZ95 cells. These observations indicate that 13-cis-RA and spironolactone antagonize testosterone, inhibiting its stimulatory influence on the lipid production of SZ95 cells.

Previous reports have shown that 13-cis-RA[4] and spironolactone [8] are potent inhibitors of cell proliferation in human sebocytes in vitro. In evaluating the antiproliferative effects of 13-cis-RA and spironolactone, we found that these compounds are inhibitors of SZ95 cell proliferation in vitro.

In conclusion, 13-cis-RA and SP may produce its antiacne effect by directly inhibiting the proliferation and the lipid production of SZ95 cells and by acting antagonistically on the stimulation of sebaceous gland by testosterone at the cellular level. Because 13-cis-RA and SP have been shown to be effective in acne, models of cultured SZ95 cells in vitro may serve as a useful tools for screening the anti-acne properties of new compounds as they are obviously superior to animal models.

Conclusions

In order to establish human sebocyte-based assay system for the screening of compounds to anti-acne drugs, we investigated the effects of 13-cis-retinoic acid (13-cis-RA) and spironolactone on the lipid synthesis and proliferation of human sebocyte line. 13-cis-RA and spironolactone inhibited cell proliferation and lipid levels of SZ95 cell in a dose-dependent manner. Combined treatment with testosterone and 13-cis-RA or spironolactone resulted in a lower total lipid levels than that with androgen alone. This observations indicate that 13-cis-RA and spironolactone are potent inhibitors of both cell proliferation and lipid synthesis in human sebocytes. Thus, we provide experimental evidence that this human sebocyte line serves as an adequate tool for evaluating the anti-lipogenic activity of various compounds potentially useful for the bioactive cosmeceutical ingredients on acne skin, and studying the intracellular biochemical markers depending on the types of compounds from various sources.

Refrences

[1] Cullen SI: Introduction. In: Cullen SI (ed.). Focus on Acne Vulgaris. Royal Society of Medicine Services, International Congress and Symposium Series No. 95. Royal Society of Medicine Services Limited, London, 1985, pp 1

[2] Gollnick, H., Zouboulis, ChC., Akamatsu, H., Kurokawa, I., Schulte, A., Pathogenesis and pathogenesis related treatment of acne, *J. Dermatol.,* **18** (1991) 489-499.

[3] Pochi, P., Sebaceous gland assay. In: Lowe N., Maibach, H., (eds), *Models in Dermatology,* Vol., 2. Basel: Karger, (1985) 70-75.

[4] Geiger, J.M., Retinoids and sebaceous gland activity, *Dermatology,* **191** (1995) 305-310.

[5] Boris, A., Hurley, J., Wong, C.Q., Comai, K., Shapiro, S., Sebum-suppressing activity of the nonpolar arotinoid Ro 15-0778 in rodents, *Arch. Dermato. Res.,* **280** (1988) 246-251.

[6] Nikkari, T., Compartive chemistry of sebum, *J. Invest. Dermatol.,* **62** (1974) 257-267.

[7] Zouboulis, C.C., Seltmann, H., Neitzel, H., Orfanos, C., Establishment and characterization of an immortalzed human sebaceous gland cell line (SZ95), *J. Invest. Dermatol.,* **113** (1999) 1011-1020.

[8] Akamatsu, H., Zouboulis, ChC., Orfanos, C.E., Spironolactone directly inhibits proliferation of cultured human facial sebocytes and 5α-Dihydrotestosterone *in vitro*, *J. Invest. Dermatol.,* **100** (1993) 660-662.

[9] Folch, J., Lees, M., Stanley, G.H.S., A simple method for the isolation and purification of total lipids, *J. Biol. Chem.,* **226** (1957) 497-509.

[10] Ways, P., Hanahan, D.J., Characterisation and quantification of red cell lipids in normal man, *J. Lipid Res.,* **5** (1964) 318-328.

[11] Thody, A.J., Shuster, S., Control and function of sebaceous glands, *Physiol. Rev.,* **69** (1989) 383-416.

[12] Zouboulis, C.C., Korge B., Akamatsu, H., Xia, L., Schiller, S., Gollnick, H., Orfanos C.E., Effects of 13-cis-Retinoic acid, all-trans-Retinoic acid, Acitretin on the proliferation, lipid synthesis and katin expression of cultured human sebocytes in vitro, *J. Invest. Dermatol.,* **96** (1988) 792-797.

[13] Guy, R., Ridden, C., Kealey, T., The improved organ maintenance of the human sebaceous gland: modeling in vitro the effects of epidermal growth factor, androgens, estrogens, 13-cis retinoic acid, and phenol red, *J. Invest. Dermatol.,* **106** (1996) 454-460.

[14] Akamatsu, H., Zouboulis, ChC., Orfanos, C.E., Control of human sebocyte proliferation in vitro by testosterone and 5-alpha-dihydrotestosterone is dependent on the localization of the sebaceous glands, *J. Invest. Dermatol.,* **99** (1992) 509-511.

[15] Takayasu, S., Wakimoto, H., Itami, S., Sano, S., Activity of testosterone 5α-reductase in various tissues of human skin, *J. Invest. Dermatol.,* **74** (1980) 187-191.

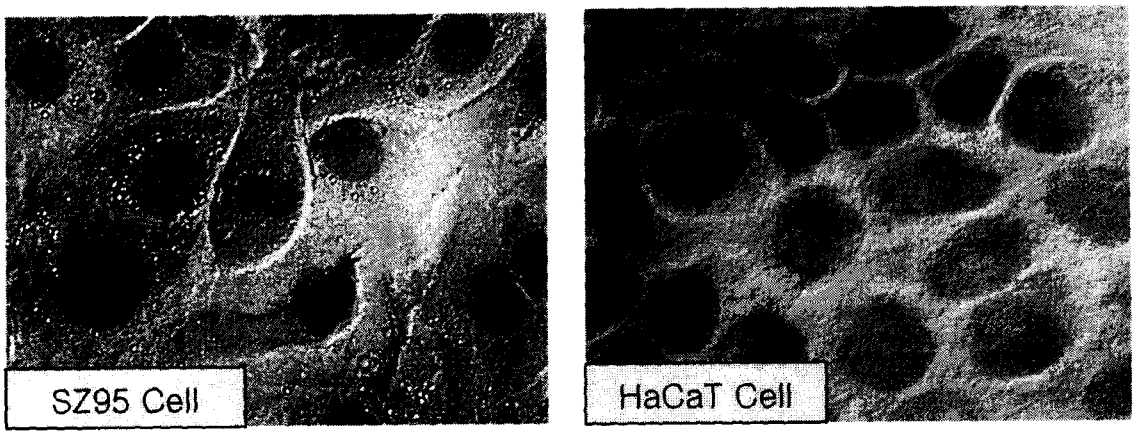

Fig. 1. Observation of cytoplasmic lipid droplets in SZ95 cells. The majority of the SZ95 cells positively labeled with Oil Red dye identifying lipids, while HaCaT cells negatively labeled with Oil Red dye. Stained cytoplasmic lipid droplets in SZ95 cell were observed.

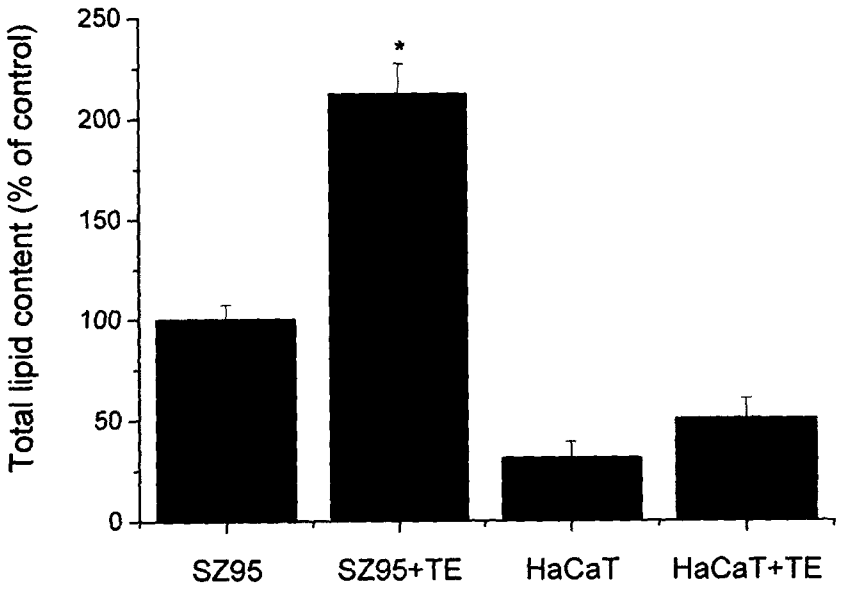

Fig. 2. Lipid synthesis of SZ95 cells. Cells were treated with or without testosterone (10^{-5} M) for 5 d. Total lipid content was assessed by the sulfo-phospho-vanillin colorimetric method. Values are mean ± SD and are presented as percent of controls. * $P < 0.01$, compared with controls.

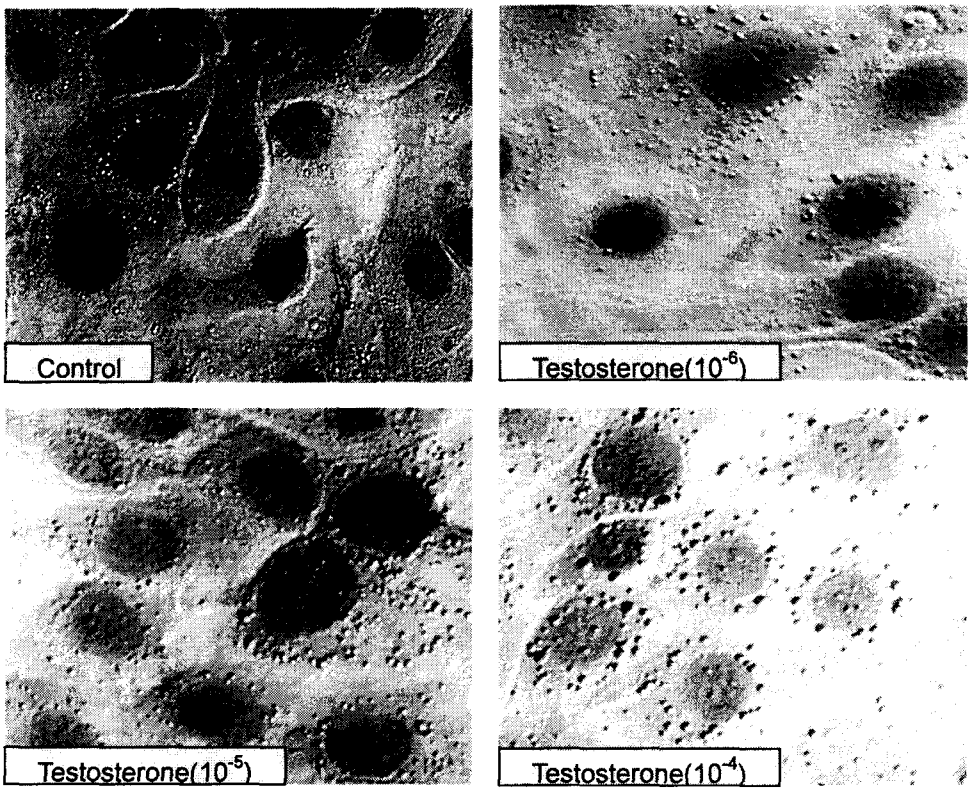

Fig. 3. Effect of testosterone on lipid synthesis in SZ95 cells. SZ95 cells were treated with or without testosterone (10^{-4} - 10^{-6} M) for 5 d and stained with Oil Red dye. Testosterone stimulated lipid droplet accumulation in SZ95 cells dose-dependently.

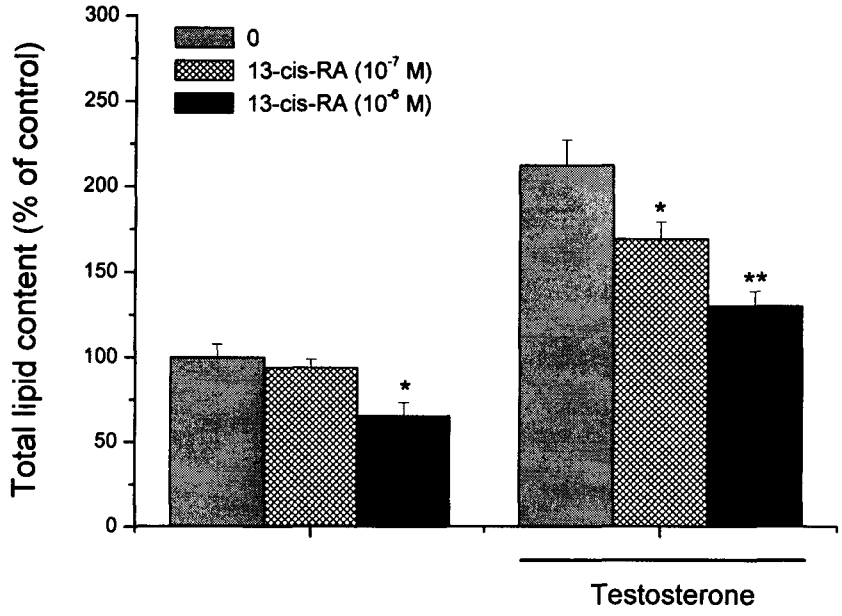

Fig. 4. Effects of 13-cis-RA on lipid synthesis in SZ95 cells. SZ95 cells were treated with 13-cis-RA (10^{-7} M or 10^{-6} M) or combined 13-cis-RA (10^{-7} M or 10^{-6} M) and testosterone (10^{-5} M). for 5 d. Total lipid content was assessed by the sulfo-phospho-vanillin colorimetric method. Values are mean ± SD and are presented as percent of controls. * $P < 0.05$, ** $P < 0.01$, compared with controls.

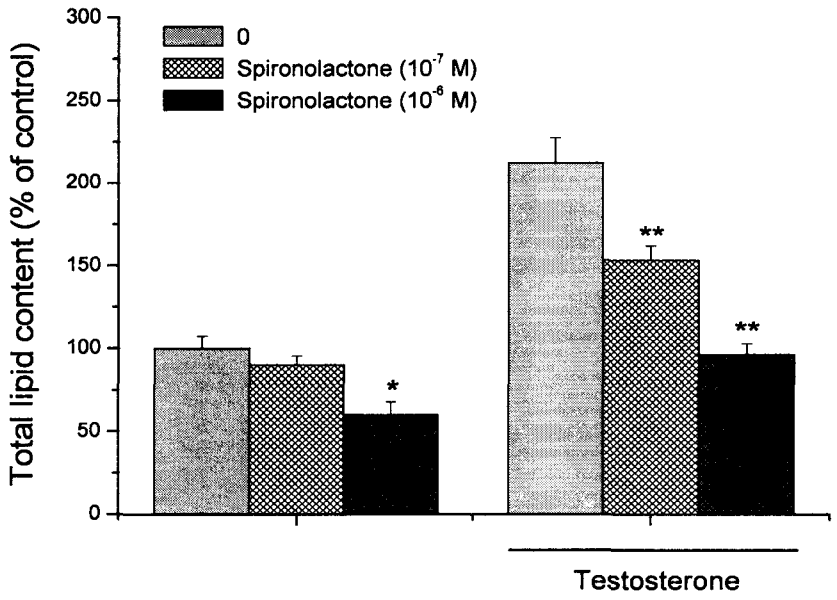

Fig. 5. Effects of spironolanctone on lipid synthesis in SZ95 cells. SZ95 cells were treated with spironolactone (10^{-7} M or 10^{-6} M) or combined spironolactone (10^{-7} M or 10^{-6} M) and testosterone (10^{-5} M). for 5 d. Total lipid content was assessed by the sulfo-phospho-vanillin colorimetric method. Values are mean ± SD and are presented as percent of controls. * $P < 0.05$, ** $P < 0.01$, compared with controls.

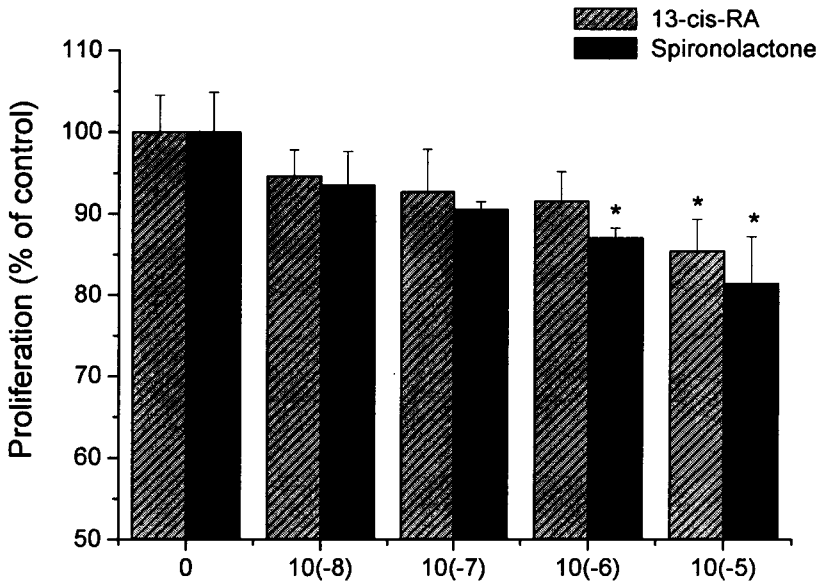

Fig. 6. Effects of 13-cis-RA and spironolactone on cell proliferation. SZ95 sebocytes were treated with 13-cis-RA (10^{-8} M or 10^{-5} M) or spironolactone (10^{-8} M or 10^{-5} M) for 5 d. The cell proliferation was measured by MTT assay. Values are mean ± SD and are presented as percent of controls. * $P < 0.05$, compared with controls.