Korean J. of Pharmacology
Vol. 31, No. 1, pp. 85-93, 1995

Effect of Testosterone on the Growth of Primary Rabbit Proximal Tubule Cells in Serum-Free Medium

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ABSTRACT

In order to examine the effect of testosterone of the cell growth, using a primary rabbit kidney proximal tubule cell culture system, we observed the effect of 3 growth factors and testosterone supplementation on the growth of primary rabbit kidney proximal tubule cells in the serum-free medium. 1 nM of testosterone showed a potentiation of the effect on the growth of the proximal tubule cell in serum-free medium, but higher concentration (>10 nM) of testosterone indeed inhibited the growth. In the absence of hydrocortisone as a growth supplement in serum-free medium, testosterone caused to potentiate the growth of the cell. In the presence of hydrocortisone, testosterone also potentiated the grwoth of the proximal tubule cells. According to the Northern analysis, testosterone increased significantly the level of β-actin mRNA in proximal tubular cells of rabbit kidney. Consequently we may suggest that growth stimulatory effect of testosterone on the primary rabbit kidney proximal tubule cell in serum-free and hormonally defined media ascribed to increase the synthesis of β-actin, which is an important protein consisting of cellular microfilament.

Key Words: Testosterone, Cell growth, Primary rabbit kidney proximal tubule cell, Serum-free medium, Growth factor

INTRODUCTION

Growth supplements have been identified that permit a number of established animal cell lines to grow in serum-free medium. In many cases the cells can grow at the same rate as that obtained with serum supplementation(Hayashi and Sato, 1976; Hutchings and Sato, 1978; Mather and Sato, 1979; Taub *et al.*, 1979). Long-term growth with periodic subculturing can be obtained with a number of established animal cell lines when using the appropriate hormone-supplemented serum-free medium (Ambesi-Impiombato *et al.*, 1980; Hayashi *et al.*, 1978; Hutchings and Sato, 1978; Mather, 1980; Taub *et al.*, 1979). In a number of cases clonal growth of animal cells can be obtained serum-free (Bettger *et al.*, 1981; Kaighn *et al.*, 1989; Rizzino, 1987; Taub and Sato, 1980).

Of particular impotance is the fact that the hormonally defined media developed for a number of established animal cell lines often permit the maintenance and/or growth of primary cultures of differentiated cells derived from the same tissue (Bottenstein *et al.*, 1979; Darmon *et al.*, 1981; Mather, 1984; Orly *et al.*, 1980; Taub *et al.*, 1979).

A primary rabbit kidney epithelial cell-culture system has been developed in serum-free medium. In this system, the epithelial cells express the functions such as proximal tubule functions (Chung *et al.*, 1982). Growth-stimulatory factors for primary rabbit kidney proximal tubule cells include insulin, transferrin, hydrocortisone and

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FGF (Wolfe *et al.*, 1980). A hormonally defined medium for the LLC-PK1 cell line, which also exhibits proximal-tubule functions, was also developed, but some growth requirements differ from those of primary rabbit kidney cells (Chunman *et al.*, 1982). This difference may possibley be related to the observation that LLC-PK1 cells possess some hormone responses typical of distal tubule cells rather than proximal tubule cells.

Hormonally defined media have been developed for the growth of several tumorigenic human epithelial cell lines, including HeLa (Hutchings and Sato, 1978), MCF-7 (Barnes and Sato, 1979), ZR-75-1 (Allegra and Lippman, 1978) and T84 (Murakami and Masui, 1980).

It has been reported that primary rabbit kidney cells grow without fibroblast overgrowth in serum-free medium supplemented with insulin, transferrin and hydrocortisone (Chung *et al.*, 1982; Jung *et al.*, 1992). Confluent monolayers of this culture cell could retain such proximal tubule transprot systems as a sodium/glucose cotransport system (Chung *et al.*, 1982; Sakhrani *et al.*, 1984), a sodium dependent phosphate transport system (Waqar *et al.*, 1988), and p-aminohippurate transport system (Yang *et al.*, 1988).

As mentioned above, although there has been reported that insulin, transferrin, hydrocortisone, triiodothyronine, prostaglandin, EGF and FGF have a role as growth factors in the serum-free media for the growth of several cells such as kidney proximal tubular epithelial cells and tumorigenic human epithelial cell lines, there is yet no report to show the effect of testosterone on the growth of the renal tubular epithelial cells.

In order to examine the effect of testosterone on the cell growth, using a primary rabbit kidney proximal tubule cell culture system, we investigated the effect of testosterone on β-actin mRNA levels from primary kidney cell cultures, and also the effects of 3 growth factors and testosterone supplementation on the growth of primary rabbit kidney proximal tubule cells in the serum-free medium.

MATERIALS AND METHODS

Materials

Hormones, human transferrin, trypsin EDTA (10X) and other chemicals were purchased from Sigma Chemical Corp. (St. Loius, Mo.). Powdered medium and soybean trypsin inhibitor were from Life Technologies (Grand Island, NY). Class IV collagenase was from Worthington (Freehold, NJ). Gamma ^{32}P-ATP, alpha ^{32}P-dCTP (3,000 Ci/mmol) and random priming labelling kits were purchased from Dupont/NEN. Restriction endonucleases were obtained from Promega, 1 kb DNA ladder from Life Technologies, Inc., and Zeta Probe Blotting Membrane from Biorad. Liquiscint was obtained from National Diagnostics (Parsippany, NY). Iron oxide was prepared by the method of Cook and Pickering (1958). Iron oxide stock solutions in 0.9% NaCl were sterilized using an autoclave, and diluted with phosphate buffered saline (PBS, pH 7.4) prior to use.

Cell culture environment

Primary rabbit kidney proximal tubule cells were maintained in a 5% CO_2 humidified environment at 37℃ by a serum-free basal medium supplemented with testosterone and 3 growth supplements, 5 μg/ml insulin, 5 μg/ml transferrin and 5×10^{-8}M hydrocortisone (Chung *et al.*, 1982; Jung *et al.*, 1992) or 2 growth rabbit kidney proximal tubule cell culture (DME/F12) consisted of a 50:50 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 Medium containing 15 mM HEPES buffer, and 20 mM sodium bicarbonate. Immediately prior to the use of the medium, the 2 or 3 growth supplements and testosterone were added. Water utilized in medium preparation was purified by mean of a Millique deionization system.

Primary rabbit kidney promixal tubule cell culture

Primary rabbit kidney proximal tubule cell cultures were prepared by a modification of the method of Chung *et al.* (1982). To summarize, the kidneys of a male New Zealand white rabbit (2 to 2.5 Kg) were perfused via the renal artery, first with phosphate buffered saline (PBS), and subsequently with DME/F12 containing 0.5% iron oxide (wt/vol) till the kidney turned grey-black in color. Renal cortical slices were homogenized with 4 strokes of a sterile Dounce homogenizer (type A pestle), and the homogenate was poured first through a 253 μ and then a 83 μ mesh filter.

Tubules and glomeruli on top of the 83 μ filter were transferred into sterile DME/F12 medium containing a megnetic stir bar. Glomeruli (containing iron oxide) were removed with the stir bar. The remaining purified proximal tubules were briefly incubated in DME/F12 containing 0.124 mg/ml collagenase (class IV), and 2.5 mg% soybean trypsin inhibitor. The tubules were then washed with DME/F12 medium by centrifugation and resuspended in DME/F12 containing the 3 supplements, and transferred into tissue culture dishes. Medium was changed one day after plating and every two days thereafter.

Cell growth studies

Primary proximal tubule cell cultrues were grown in 35 mm dishes for cell growth studies. Periodically, cells were removed from the dishes using phosphate buffered saline containing 0.05% trypsin, and 0.5 mM EDTA. The cells were counted using a Coulter Model ZF particle counter. Values are expressed as a the average of triplicate determinations.

Northern analysis of cellular RNA

Total RNA was isolate by the guanidinium isothiocyanate/cesium chloride method (Chirgwin *et al.*, 1979). RNA was isolated from primary rabbit kidney proximal tubule cells grown to confluency either in DME/F12 supplemented with insulin, transferrin, hydrocortisone and testosterone, or in DME/F12 supplemented with testosterone, in addition to the other 2 growth factors (minus hydrocortisone). RNA (10 μg/sample) was fractionated by electrophoresis in formaldehyde gels containing 0.8% agarose, and was tranferred to Zeta Probe Blotting Membranes. Duplicate RNA samples on the gel were stained with ethidium bromide to verify the quality of the RNA. A restriction fragment containing mouse β-actin cDNA obtained from plasmid pBR322(Boot-Handford 1987) were utilized for making labelled probes. The restriction fragment was radiolabelled with alpha ^{32}P-dCTP by the random primer method, and were utilized for hybridization following the method of Church and Gilbert (1984). Standard stringent hybridization conditions were utilized.

RESULT

Effect of testosterone on the growth of the rabbit kidney primary proximal tubule cells in serum-free medium

Fig. 1, 2 and 3 showed the effects of different concentrations of testosterone on the growth of primary rabbit kidney proximal tubule cells, whose growth was determined after 4, 8 and 12 days, respectively. In the 0.1 and 1 nM testosterone contaning medium, cell growth was augmented, but cell growth was inhibited with more than 10 nM concentration of testosterone. Therefore 1 nM of testosterone was thought to be a proper concentration for the growth of the proximal tubule cell. When we determined the effects of tes-

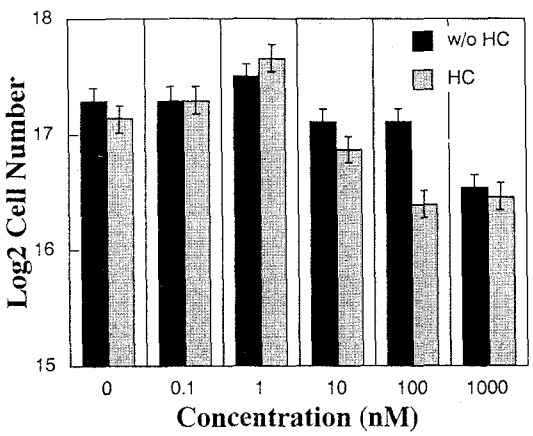

Fig. 1. Effect of testosterone on growth of primary rabbit kidney proximal tubule cells by different concentration of testosterone.
The cells were cultured in DME/F12 supplemented either with 5 μg/ml insulin, 5 μg/ml transferrin and 0-1000 nM testosterone (■), or with an additional 5×10^{-8}M hydrocortisone (□). The cell number present in each growth conditions was determined after 4 days in culture using a Coulter Counter. Values are the average (± Std. deviation) of triplicate determinations.
w/o HC: Without hydrocortisone in culture medium.
HC: With hydrocortisone in culture medium.

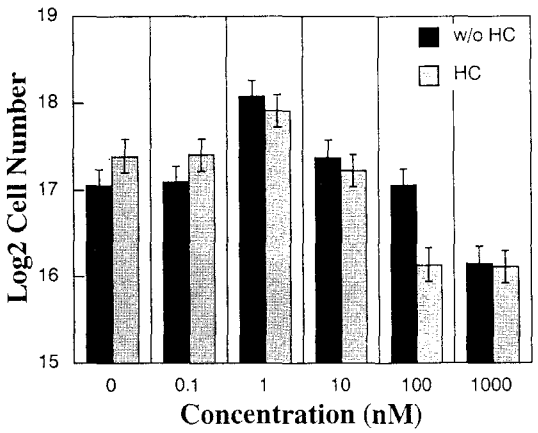

Fig. 2. Effect of testosterone on growth of primary rabbit kidney proximal tubule cells by different concentration of testosterone.
The cells were cultured in DME/F12 supplemented either with 5 μg/ml insulin, 5 μg/ml transferrin and 0-1000 nM testosterone(■), or with an additional 5×10^{-8}M hydrocortisone (□). The cell number present in each growth conditions was determined after 8 days in culture using a Coulter Counter. Values are the average (±Std. deviation) of triplicate determinations.

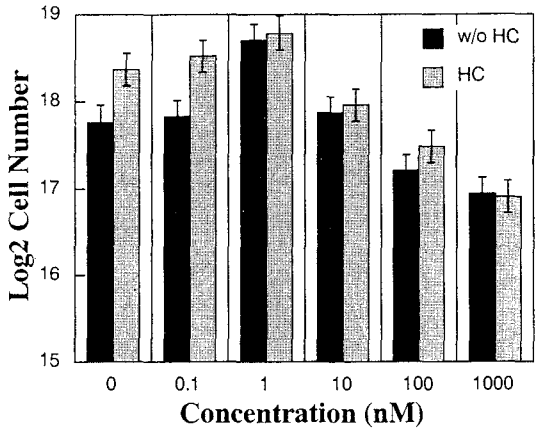

Fig. 3. Effect of testosterone on growth of primary rabbit kidney proximal tubule cells by different concentration of testosterone.
The cells were cultured in DME/F12 supplemented either with 5 μg/ml insulin, 5 μg/ml transferrin and 0-1000 nM testosterone (■), or with an additional 5×10^{-8}M hydrocortisone (□). The cell number present in each growth conditions was determined after 12 days in culture using a Coulter Counter. Values are the average (± Std. deviation) of triplicate determinations.

tosterone on the cell growth in the absence and presence of the hydrocortisone, no significant difference between two groups were observed.

The effect of testosterone on growth of primary rabbit kidney proximal tubule cells in in serum-free medium without hydrocortisone

Fig. 4 showed the effects of testosterone on the growth of the rabbit kidney primary proximal tubule cell in serum-free medium without hydrocortisone. As shown in the figure 4, 1 nM testosterone significantly potentiated the cell growth after 4, 8 and 12 days in culture supplemented with two growth factors, insulin and transferrin when we compared the growth without testosterone.

Effect of testosterone on growth of primary rabbit kidney proximal tubule cells in additional hydrocortisone medium

Fig. 5 showed the same result as figure 4 except that in figure 5 we represented the effect of testosterone on the cell growth in the presence of hydrocortisone. As illustrated in figure 5, 1 nM of testosterone was also cell growth stimulator in serum-free DME/F12 medium supplemented with insulin, transferrin and hydrocortisone.

We compared the difference of effect of testosterone on the cell growth in serum-free medium supplemented either with two growth factors (insulin and transferrin) of with three growth factors (two factors and hydrocortisone).

Although we already observed the stimulatory effect of testosterone on the cell growth in serum free medium with or without hydrocortisone, there was no significant difference of stimulatory actions between hydrocortisone free and containing medium. Therefore testosterone was thought to be a growth stimulatory factor whether hydrocortisone is present in the culture medium or not (Fig. 6).

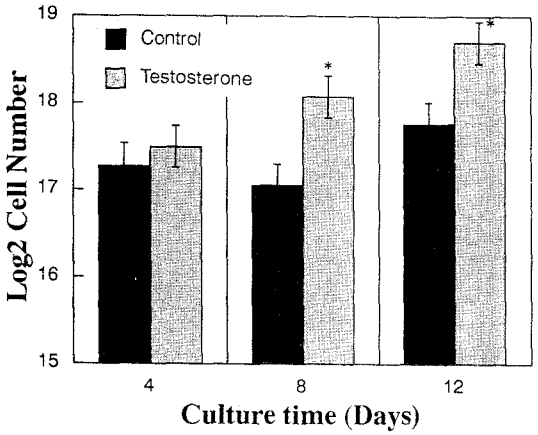

Fig. 4. Effect of testosterone on growth of primary rabbit kidney proximal tubule cells in serum-free medium without hydrocortisone.
Primary proximal tubule cells were grown in 35 mm dishes containing either testosterone free DME/F12 or DME/F12 supplemented with 1.0 nM testosterone. Both the testosterone free and testosterone containing culture medium was further supplemented with 2 growth factors, insulin and transferrin. Cells were counted periodically over a 12 day time interval. Values are the average (± Std. deviation) of triplicate determinations.
*Significantly different from the group of without testosterone, respectively($P<0.05$)

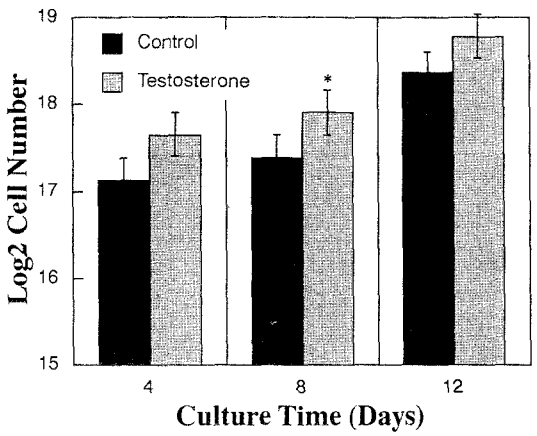

Fig. 5. Effect of testosterone on growth of primary rabbit kidney proximal tubule cells in additional hydrocortisone medium.
Primary proximal tubule cells were grown in 35 mm dishes containing either testosterone free DME/F12 or DME/F12 supplemented with 1.0 nM testosterone. Both the testosterone free and testosterone containing culture medium was further supplemented with 3 growth factors, insulin, transferrin and hydrocortisone. Cells were counted periodically over a 12 day time interval. Values are the average (±Std. deviation) of triplicate determinations.
*Significantly different from the growth of without testosterone, respectively ($P<0.05$)

The effect of testosterone on β-actin mRNA contents

β-actin mRNA was transfered to Zetabind boltting membrane and hybridized sequentially to β-actin specific radiolabeled probe. EtBr-stained RNA samples (Fig. 7-a) established minimal variation in sample recovery; as judged by the bands representing the major ribosomal RNA species (28 S and 18 S), there was negligible breakdown.

The effect of exogenous testosterone on the level of β-actin chain mRNA in primary rabbit kidney proximal tubule cells was examined by Northern analysis using mouse cDNA and nick-translated cDNA probes. Fig. 7-b. shows that the level of β-actin mRNA was elevated in primary cultures in medium containing 1 nM testosterone (Fig. 7-b; C, D), as compared with testosterone free medium (Fig. 7-b; A, B).

DISCUSSION

Several different hormonally defined media are available for kidney epithelial cells culture. The cell line can grow at the same rate in serum-free medium supplemented with insulin, transferrin, triiodothyronine, hydrocortisone and prostaglandin E_1 as the rate obtained in serum supplemented medium (Taub *et al.*, 1979). Both the long-term growth of MDCK cells and clonal growth can be obtained in serum-free medium supplemented with insulin, transferrin, T_3, hydrocortisone and prostaglandin E_1. The hormone-supplemented

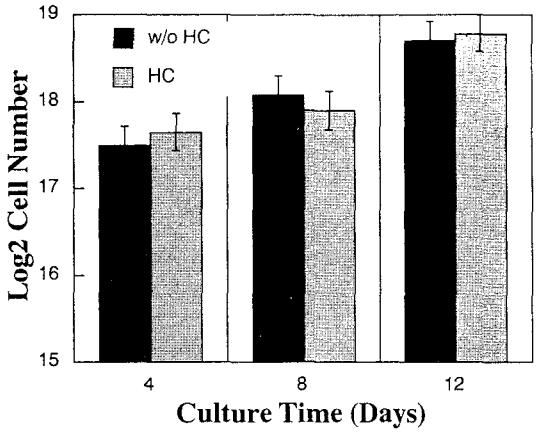

Fig. 6. Effect of testosterone on growth.
Primary proximal tubule cells were grown in 35 mm dishes containing either hydrocortisone free DME/F12 or DME/F12 supplemented with 5×10^{-8}M hydrocortisone. Both the hydrocortisone free and hydrocortisone containing culture medium was further supplemented with 2 growth factors, insulin and transferrin. Cells were counted periodically over a 12 day time interval. Values are the average (±Std. deviation) of triplicate determinations.

serum-free medium for MDCK cells has also been used for primary cultures of baby-mouse kidney epithelial celsl without fibroblast overgrowth (Taub *et al.*, 1979; Taub and Sato, 1980). The primary baby-mouse kidney epithelial cells exhibit functional properties like MDCK cells, but lack sodium-dependent sugar transport which is an unique function of a proximal tubule function.

A particular strain of HeLa grows in serum-free medium supplemented with insulin, transferrin, EGF, FGF and hydrocortisone with serial subculturing, at the same rate as that obtained in serum supplemented medium (Hutchings and Sato, 1978). Hydrocortisone is the most critical factor for the long-term serum-free growth of these HeLa cells (Hayashi *et al.*, 1978). Hormonally defined media have been developed for two different established human mammary-tumor cell line, ZR-75-1 and MCF-7. Whereas ZR-75-1 cells require insulin, transferrin, FGF, hydrocortisone and triiodothyronine for serum-free growth (Barnes and Sato, 1979), MCF-7 cells require insulin, transferrin, EGF and prostaglandin F2α, as well as several attachment factors (Allergra and Lipp-

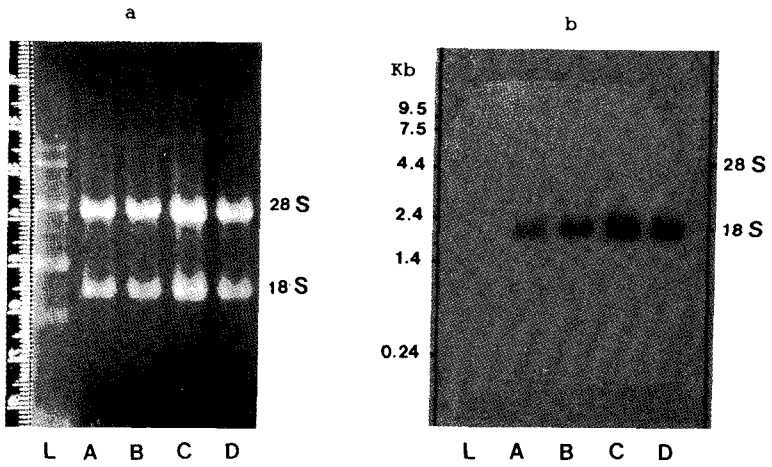

Fig. 7. Northern blot analysis of β-actin mRNA from primary cultures rabbit kidney proximal tubule cells.
RNA was isolated from primary cultures grown to confluency in DME/F12 with the 2 or 3 supplements, and testosterone at 1.0 nM. Total RNA (5 μg/lane) was subjected to electrophoresis in 0.8% agarose gel. Duplicate set of sample were run on gel. One set of sample from gel was transfered to Zetabind blotting membrane. Blot was hybridized with a β-actin chain cDNA probe in the case of gel. The blot was washed, and exposed to X-ray film for 24 hours. The result with the β-actin probe is illustrated in b. The other set of RNA sample on gel was stained with ethidium bromide, as illustrated in a. L: RNA ladder, A: Without hydrocortisone control, B: With hydorcortisone control, C: Without hydrocortisone, add testosterone, D: With hydrocortisone and testosterone.

man, 1978).

Growth-stimulatory factors for primary culture of rabbit kidney proximal-tubule cells such as insulin, transferrin and hydrocortisone has been well studied. A hormonally defined medium for the MDCK and LLC-PK1 cell-line, which also exhibits proximal-tubule transport functions, was also developed, but the growth requirements differ from those of primary rabbit kidney cells (Chunman *et al.*, 1982). This difference may possibly be related to the observation that LLC-PK1 cells possess some hormone responses typical of distal tubule cells, rather than proximal tubule cells.

Although many investigators already showed the effect of steroid hormones as growth sitmulator on cell growth, there is no report to reveal the capacity of testosterone in serum free medium as a cell growth factor in rabbit kidney primary proximal tubule cell culture.

There are several reports to show the possible role of steroid hormones as growth supplements in defined growth media: e.g. testosterone in human breast cancer cell line (Allegra and Lippman, 1978), hydrocortisone in HeLa cell (Hutchings and Sato, 1978), progesterone in rat neuroblastoma cell line (Bottenstein and Sato, 1979) and testosterone in mouse prostatic epithelial cell (Waymouth *et al.*, 1982).

The biological effects mediated by the androgenic steroids in the male reproductive system as well as in those tissues associated with the secondary sex characteristics are all believed to occur as a consequence of the association of the appropriate androgen with a cytoplasmic receptor in a given target tissue (Saartok *et al.*, 1984).

The androgen receptor is localized in the cytoplasmic nuclear protions of the target cell. After assoceation of the ligand with the protein receptor, the steroid-receptor complex associates with specific DNA domains and initiates specific gene transcription for proteins necessary for the biological response of the androgen in that particular target cell (Liao., 1977).

The most thoroughly studied system is the rat ventral prostate. The prostate cell nuclei have been shown to bind, on the average, 2000~6000 molecules of dehydrotestosterone (DHT) per cell nucleus. The DHT receptor has a mobility of both 7~12 S and 3~5 S in 5~20% sucrose gradients, suggesting a molecular weight for the oligomeric form of 270,000 and a subunet molecular weight of 70,000. The large units can be transformed into the smaller units by incubation at 20~30℃ (Chang *et al.*, 1988; Griffin and Wilson, 1989).

The anabolic actions of testosterone and its metabolites in non-reproductive tissue such as kidney, muscle, liver and bone are as yet not as thoroughly studied.

Connective tissues contain different species of collagens, glycorproteins, and proteoglycans (Bornstein & Sage, 1980; Kleinman *et al.*, 1986). These macromoleules form the matrix structures that contribute to the physical characteristics of tissues as well as provide unique substrates for the resident cells. Basement membranes contain type IV collagen (Kefalides, 1973; Orkin *et al.*, 1976), the glycoproteins laminin (Timpl *et al.*, 1979), entactin (Carlin *et al.*, 1981), and nidogen (Timpl *et al.*, 1983) and heparan sulfate proteoglycan (Kanwar and FArquhar, 1979; Hassell *et al.*, 1980, 1985). β-Actin is a major structural protein of cellular microfilaments, it is considered to be involved in cell motility and mitosis.

In this study we investigated the effect of testosterone on cell growth in serum-free medium supplemented with insulin, transferrin and hydrocortisone. Testosterone indeed increased the proliferation of rabbit proximal tubule cell whether hydrocortisone is present in the growth medium or not, and according to the Northern analysis mRNA level of β-actin was increased though there was no measurable change in alpha I (IV) collagen mRNA level.

Therefore we may conclude that growth stimulatory property of testosterone ascribed to increase the synthesis of β-actin protein, which is a primary component of cellular microfilament.

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=국문초록=

Testosterone이 토끼 근위 세뇨관 상피세포의 성장에 미치는 영향

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Testosterone이 serum-free medium에서 배양한 토끼의 신장 근위세뇨관 상피세포의 세포성장과 기능에 미치는 영향을 관찰한 바 다음과 같은 결과를 얻었다.

1. 토끼의 신장 근위세뇨관 상피세포는 testosterone 1 nM의 농도에서 유의한 세포 성장 촉진 효과를 나타내었고, testosterone 10 nM이상의 농도에서는 세포성장이 억제되었다.

2. Testosterone은 serum-free medium에서 성장촉진인자의 하나인 hydrocortisone을 growth supplement로 넣어준 serum-free medium에서 토끼 신장의 근위세뇨관 상피세포의 성장을 촉진시키었다.

3. Testostrone은 hydrocortisone을 growth supplement로 넣어준 serum-free medium에서 토끼 신장의 근위세뇨관 상피세포의 성장을 촉진시키었다.

4. Testosterone은 Northern blot analysis에 의하여 확인한 토끼 신장의 근위 세뇨관 상피세포의 β-actin mRNA level은 증가되었다.

이상의 결과로 미루어 보아, serum-free 그리고 hormonally defined media에서 testosterone이 토끼의 신장 근위세뇨관 상피세포의 성장 및 기능에 대하여 촉진적으로 작용하는 것은 cellular mecrofilament의 중요한 구성단백의 하나로 밝혀진 β-actin의 합성 증가에 기인하는 것으로 생각된다.