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Tissue Culture Media Supplemented with 10% Fetal Calf Serum Contains a Castrate level of Testosterone

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Abstract

Background—Human prostate cancer cells are routinely maintained in media supplemented with 10% Fetal Calf Serum (FCS) to provide androgen. In the present study, total and free testosterone levels in 10%FCS supplemented tissue culture media were determined and compared to levels in intact and castrated human males. Dextran-coated charcoal stripped FCS (i.e., DC-FCS) is often used instead of FCS to minimize the level of androgen provided in 10% serum supplemented media. Therefore, total and free testosterone levels in 10%DC-FCS containing media were likewise determined.

Methods—Total testosterone, free testosterone, and total dihydrotestosterone (DHT) were determined on RPMI-1640 media supplemented with either 10%FCS or 10%DC-FCS by ELISA assays before and after exposure to LNCaP human prostate cancer cells in culture. The growth and PSA secretion by these cells was also determined.

Results—10%FCS supplemented media contains a castrate level of testosterone. However, even with this castrate starting level of testosterone, LNCaP cells concentrate and metabolize the testosterone to produce a physiologic (i.e. 10 nM) level of intracellular DHT which optimally stimulates the growth of these cells in vitro.

Conclusions—The present studies document that prostate cancer cells auto-regulate their androgen metabolism so that an optimal level of DHT for growth is maintained during both up and down fluctuations in the supply of testosterone. These results have significant implications for whether exogenous androgen should be added to the 10%FCS supplemented media to grow prostate cancer cells from intact vs. castrated patients.

Keywords

cell culture; testosterone; castration levels; auto-regulation

Introduction

The average serum total testosterone (T_T) level in a normal adult human male is 6.1 $\pm$ 1.9ng/mL, with a normal range of 3–10ng/mL [i.e. concentration range from 10 to 35nM], Table 1 (1). Successful androgen ablation is reached when the serum T_T is <0.2ng/mL [<0.69 nM] after surgical castration (1,2) or <0.5ng/mL [<1.73 nM] after chemical castration (3). Approximately 98% of T_T is protein-bound; the remaining 2% (122 $\pm$ 37pg/mL, with a range of 60 to 200pg/mL [0.2–0.7nM]) is free testosterone (T_F) (4). Protein binding prevents the rapid loss of testosterone via glomerular filtration. The level of serum T_F is dependent on 2 major factors: the affinity of testosterone binding to specific binding proteins

and the serum availability of these proteins. In the human serum, testosterone is bound to sex hormone-binding globulin (SHBG), corticosteroid-binding globulin (CBG), human serum albumin (HSA), and to a lesser extent progesterone-binding globulin (PBG) and α 1-glycoprotein (AAG) (i.e., ρ % of serum testosterone is PBG plus AAG protein bound) (4). SHBG has a high affinity ($\sim 10^{-9}$ for testosterone) and is present in a moderate serum concentration (~ 30 nM), so that it tightly binds only ~ 58 % of the serum T_T . HSA has a relative low affinity ($\sim 10^{-6}$ for testosterone) but due to its high serum concentration (~ 500 μ M), it loosely binds ~ 40 % of the serum T_T . Due to its high affinity, SHBG slowly releases its tightly bound testosterone and thus this SHBG bound fraction serves as a metabolic reserve for serum testosterone to replenish the pool of HSA bound testosterone. In contrast, due to its low affinity, HSA releases and rebinds serum testosterone continuously allowing this HSA pool of loosely bound testosterone to replenish the free testosterone lost from the blood via its diffusion into the extracellular tissue compartments stabilizing the serum level of T_F .

The combination of serum SHBG and HSA thus provides a “buffering system” for providing a continuously stabilizing source of T_F to the nonvascular compartments of the prostate even when there are episodic fluctuations in the release of testosterone from the testes. The importance of stabilizing serum T_F is that the level of T_F is the driving force for the diffusion of testosterone from the circulation into the extracellular fluid of the prostate and thus into the prostate stroma and parenchyma. Within these nonvascular compartments of the prostate, the free testosterone is metabolized by a variety of enzymes, the most important is 5- α reductase which produces dihydrotestosterone [DHT] (5,6). DHT is the most critical metabolite since it has the highest binding affinity other than testosterone for the androgen receptor (AR) [i.e., K_d for testosterone and DHT are 0.75 and 0.3 nM respectively (7)]. If there were no metabolic amplification via 5- α reductase dependent conversion to DHT, the maximal tissue level of testosterone produced by diffusion of the T_F from the serum of normal adult human males would be only ~ 0.2 – 0.7 nM which is just at the K_d for testosterone binding to AR and thus could produce only ~ 50 % occupancy of the receptor. Also once bound, the half life of the testosterone/AR complex is ~ 2.5 times shorter than for the DHT/AR complex making it less able to sustain efficient AR signaling (8). Thus, tissue AR induced signaling is dependent upon both the maintenance of adequate serum testosterone levels and local tissue production of DHT.

Attempts to grow human malignant prostate cancer cell as permanent cell lines in culture have been remarkable for their lack of success. In fact, only a handful of human cancer lines have been established with essentially all of these having been derived from an androgen ablated host (9,10). When successful, the media for growing human prostate cancer cells are routinely supplemented with 10% Fetal Calf Serum (FCS). The supplementation with FCS is to supply additional nutrients like essential fatty acids and cholesterol plus endocrine factors [e.g., androgen, tri-iodothyronine (T3), serum growth factors like insulin, insulin-like growth factors (IGF), epidermal growth factor (EGF)] and cell attachment proteins like fetuin (11). In particular, the inclusion of the 10% FCS is assumed to provide a physiological level of serum testosterone comparable to the level in intact human males for the prostate cancer lines.

The reported testosterone concentration in bovine serum (often measured in calves in the period between birth and 1 year of age) is between 1.2 ng/mL – 7.5 ng/mL [4 – 25 nM] (12–15) which are slightly lower than those in normal human adult males. Testosterone levels in fetal calf serum (FCS) have not been reported. In the previous reports, bovine serum testosterone levels were measured using radio-immuno (RIA) assays after ether extraction and are thus a measure of total testosterone levels. Although these research groups describe the testosterone binding mechanism of several binding proteins involved, no mention is made

about the percentage bound and free testosterone in bovine serum. SHBG-like protein is present in bovine serum (16), but there is approximately 10% less SHBG bound testosterone in bovine serum compared to human serum samples (17). These findings are explainable by the fact that the affinity constant for the binding of testosterone to bovine SHBG is 10 fold lower than the affinity constant for the binding of testosterone to human SHBG (i.e., 1.1×10^{-8} and 1.2×10^{-9} respectively (18). Although no concentrations are reported for SHBG in bovine serum, albumin serum levels are significant lower in bovine serum when compared to human serum, with bovine albumin concentrations mentioned in the literature between 29–32gr/L and human values between 32–41gr/L (19). These differences in the composition of bovine serum compared to human serum, as well as the differences in the binding affinity of testosterone for these binding-proteins have a significant impact on the concentrations of bound and free testosterone in bovine serum and in cell-media supplemented with 10%FCS additions. Thus, in the present study, the total and free testosterone levels in 10%FCS containing tissue culture media was determined and compared to these levels in intact and castrated human males. Dextran-coated charcoal stripped FCS (i.e., DC-FCS) is often used instead of FCS to minimize the level of androgen provided in 10% serum supplemented media. Therefore, total and free testosterone levels in 10%DC-FCS containing media were likewise determined.

Material and Methods

Fetal calf serum (FCS) and dextran charcoal stripped fetal calf serum (DC-FCS), RPMI-1640 media and Trypsin-EDTA 1x was purchased from Gibco/Invitrogen Carlsbad (CA) USA. Penicillin-Streptomycin solution (100x) and 1x PBS was purchased from Cellgro Manassas (VA) USA. Standard cell media was made by adding 10% serum (either FCS or DC-FCS) to 500mL RPMI-1640, together with pen/strep and L-glutamine. Cell growth was measured by a MTT assay as previously described (20)

Steroid Measurements

Testosterone was measured by a testosterone-ELISA kit purchased from BioCheck Inc., Fostercity (CA) USA. The sample size for this kit is 10 μ L. The minimal detectable testosterone concentration is 0.5pg. Classic ether extraction (3:1) was used to determine the presence of bound-testosterone, and to concentrate samples when testosterone levels were below the detection threshold.

Dihydrotestosterone (DHT) levels were measured with a DHT-ELISA kit (Alpha Diagnostics International, San Antonio (TX) USA). The kit is a direct competitive ELISA for the measurement of DHT in the serum. The sample size for the ELISA is 50 μ L, the minimal detectable DHT concentration is 0.06pg.

Free-testosterone (T_F) in serum containing media was measured by equilibrium dialysis using a bench-top dialysis-cassette method (Slide-A-Lyzer 2000 MWCO 0.5–3.0mL capacity Pierce Biotechnology Rockford (IL) USA). Dialysis cassettes were filled with 2.5mL of PBS(1x), and submerged in a beaker with 550mL RPMI-10%FCS or RPMI-10%DC-FCS with slow stirring at 4°C overnight. Samples of the dialysate and the buffer were collected, ether-extracted, concentrated and testosterone levels were determined with the use of the testosterone-ELISA kit.

For the testosterone metabolism experiment LNCaP-cell culture (passage 43) were used. LNCaP cells were plated (2×10^6 cells) in RPMI-1640 media supplemented with 10%FCS and fresh media was replaced after 3 days. After another 24 hours this media was collected for PSA, testosterone and DHT levels. The cells were counted, pelleted and extracted with

ether for testosterone and DHT analysis. The LNCaP cells had an average cell-diameter of 14 μm and a single-cell volume of 1.437 femtoliter.

Western Blot Analyses of Steroidogenic Enzymes

LNCaP (p46–49) was cultured in vitro using standard media (RPMI-10%FCS, with Pen-Strep) in a 75cm² flask until confluent. Cells were trypsinized and pelleted. Cell pellets were resuspended in 300 μL of fresh ice-cold lysis buffer and left on ice for 15 minutes. Lysis buffer: 0.14M NaCl, 1% NP-40, 0.25mM Na-deoxycholate, 1mM EDTA, 0.02M Tris, complete miniprotease inhibitor (Roche Molecular Biochemicals), phosphatase inhibitor (Roche Molecular Biochemicals) and 1mM DTT. After the 15 minutes on ice the samples were spun on a benchtop centrifuge, at 4°C and 14,000 RPM for 15 minutes and the supernatant was collected. Protein levels were determined using a Bradford analysis. Equal amounts of protein were loaded on the gels for Western Blot analysis. Western blotting was performed following standard protocol. Adrenal tissue was used as a positive control. Adrenal tissue was homogenized in fresh ice cold lysis buffer as described. Serial dilutions (1:1, 1:10, 1:100 and 1:1000) were made using the lysis buffer. Each Western Blot was run using at least 2 of the adrenal dilutions as positive control and signal-strength reference. The following antibodies were used: mouse anti-StAR (H00006770-M01 Abnova) 2 $\mu\text{g}/\text{ml}$, goat anti-CYP11a1 (sc-18040 Santa Cruz) 1:500 and rabbit anti-CYP17 (sc-66849 Santa Cruz) 1:1000. The optimum dilution for each antibody was determined using the serial dilutions of the adrenal tissue.

PSA Determinations

PSA analysis was conducted by the JHMI Clinical Chemistry Laboratory.

Results and Discussion

The driving force for the diffusion of testosterone from the circulation into the extracellular fluid of human tissues is the serum level of free testosterone (i.e. 200–700pM), table 1. For comparison, Table 1 summarizes the results of the measurement of the total and free testosterone levels in tissue culture media supplemented with either 10% unstripped-FCS or 10%DC-FSC serum. The results document that 10%FCS supplemented tissue culture media contains levels of total testosterone and free testosterone which are more than a 100 fold lower than those in the serum from intact adult human males. In fact, the total testosterone value is even slightly lower than those in the serum from castrated adult human males, table 1. Appropriately 90% of the total testosterone is protein bound in the tissue culture media with the level of free testosterone in the serum containing media being essentially equal to that in the serum from castrated adult human males. The use of 10% dextran coated charcoal stripped FCS additionally lowers the total testosterone in the tissue culture media by ~80% placing it well below that in the serum from castrated adult males. In contrast to the total testosterone, the level of free testosterone remains the same as in the media supplemented with 10% unstripped-FCS, although now only ~50% of the total testosterone is protein bound in the media supplemented with 10%DC-FCS. This is reasonable since the level of total testosterone in the DC-FCS containing media is so low that it does not efficiently bind to SHBG or albumin.

These results document that cells placed in 10%FCS supplemented tissue culture media are exposed to a castrate, not physiologically normal, concentration of testosterone. This revelation is significant in that the human prostate cancer lines presently available are routinely maintained in such low testosterone 10%FCS supplemented tissue culture media (9,10). Human prostate cancer cells can respond to such low testosterone 10%FCS supplemented tissue culture media, however, with androgen induced AR dependent growth

stimulation. For example, the growth of the human LNCaP prostate cancer cells is profoundly stimulated when these cells are switched from media supplemented with 10% DC-FCS to media containing 10% non-stripped FCS, Figure 1. Likewise, the production and secretion of the AR dependent Prostate Specific Antigen (PSA) protein is increased by more than 10 fold (i.e., 2ng PSA protein secreted into the media/ 10^6 cells/ 24 hours in 10% DC-FCS vs. 210 ng PSA/ 10^6 cells/ 24 hours in 10% unstripped FCS media). These responses to the 10%FCS supplemented media are AR mediated as documented by the inhibition of both the growth response, Figure 1, and PSA secretion by the co-exposure to $10\mu\text{M}$ of the direct acting AR ligand antagonist, casodex.

The robust androgen dependent PSA and growth response of LNCaP cells to the castrate level of testosterone in the 10%FCS supplemented media is even more remarkable when it is realized that these cells have a mutation in the ligand binding domain of their AR gene which decreases their K_d [i.e., 5.4nM] for binding testosterone/DHT [i.e., K_d is 0.3nM for wild type AR] (7). Thus based upon mass action steady state diffusion, the intracellular concentration of testosterone driven by the level of testosterone in the 10%FCS media should only be able to reach at most 50–100pM. Based upon the its K_d and Langmuir Isotherm equation, this low amount of intracellular testosterone provided by 10%FCS media should only allow ~10–20% of the AR to be occupied. This raises the issue of how such a castrate level of testosterone provided by the 10%FCS supplemented media can efficiently stimulate such AR dependent PSA and growth stimulation in LNCaP cells. One possibility is that the LNCaP cells are capable of converting cholesterol and or precursor steroids into testosterone and or DHT. In order to accomplish this conversions, LNCaP cells would have to express detectable levels of the key steroidogenic enzymes including StAR, CYP11A1, and CPY17 (21). Therefore, western blot analyses were performed to evaluate this possibility. These analyses demonstrated that none of the key enzymes of testosterone synthesis are expressed at detectable levels in LNCaP cells grown in 10% FCS supplemented media.

A second possibility is that LNCaP cells concentrate the low testosterone supplied by the 10%FCS supplemented media and metabolic convert it via 5-alpha reductase to DHT which is then avidly retained. This possibility is supported by our earlier studies which documented that LNCaP cells express high levels of type I coupled with modest levels of type II 5-alpha reductase (20). To directly evaluate this second possibility, 10^6 LNCaP cells were incubated at 4 degree with 10%FCS containing media and then 20 minutes later, the media collected and analyzed for its testosterone level. These studies documented that by as little as 20 minutes, ~25 % of the testosterone is already retained by the cells. These studies were expanded in which not only the media but also the intracellular content of both testosterone and DHT were determined following 24 hour of exposure of LNCaP cells to the 10%FCS supplemented media. These studies document that by 24 hours, the intracellular testosterone concentration was 20nM and the intracellular DHT was 9.9nM even though the media concentration of testosterone was only $93.4 \pm 34.4\text{pM}$ and there was no detectable level of DHT in the media.

Previous rodent and human studies have documented that in intact adult hosts, physiological tissue concentration of DHT are maintained in the range of 5–18nM, regardless of whether these are normal or malignant prostatic tissues (20,22–27). Based upon the Langmuir binding isotherm, this range of physiological tissue DHT should allow >90% of the AR protein to be occupied by DHT. Thus the fact that the present studies documents that LNCaP cells concentrate the castrate level of testosterone provided by the 10%FCS supplemented media and converts it to maintain a ~10nM intracellular DHT concentration is highly significant. This is because at this ~10nM intracellular DHT concentrated level, at least 2/3 of the AR protein in the LNCaP cells should be occupied even with the ligand binding

domain mutation in AR of these in these cells. The ability of the LNCaP cells to maintain the intracellular level of DHT at a physiological level despite a castrate level of testosterone provided by the 10%FCS supplemented media is consistent with the fact that this cell line was originally established from a lymph node metastasis from a castration resistant patient. Thus, 10%FCS supplemented media provides essentially the same level of testosterone that these castration resistant cells were exposed to in vivo. This suggests that during their in vivo transformation, LNCaP cells adapted their level of AR protein and androgen metabolism to optimally grow in the presence of a castrate level of testosterone.

The realization that growing LNCaP cells in vitro in 10%FCS supplemented media similarly maintains them in a low testosterone environment provides an explanation of their paradoxical response to exogenous androgen. It is well established that when exogenous androgen is added to the 10%FCS supplemented media, the growth of LNCaP cells is inhibited, Figure1, even though their secretion of PSA is increased by > 2 fold by such androgen addition (i.e., going from 210 to 697ng of PSA/10⁶ cells/24 hours in 10%FCS media with and without the addition of 500nM testosterone). The mechanism for such androgen induced growth inhibition has been recently demonstrated to be due to the fact that the AR in prostate cancer functions as a licensing factor for DNA replication needed for proliferation of prostate cancer cells (28–30). Thus, AR needs to be degraded in mitosis for re-licensing in the next cell cycle and therefore the prostate cancer cell must regulate (i.e., limit) the amount of DHT to allow such AR degradation. In the presence of too high a level of testosterone, the level of DHT becomes too high preventing AR degradation during mitosis and thus prostate cancer cell growth is inhibited (28–30). Since such AR degradation is not needed for PSA production, this explains how AR dependent transcriptional upregulation of PSA production can continue to increase in a dose response manner while growth is inversely inhibited.

Conclusion

The results of the present studies document that prostate cancer cells auto-regulate their androgen metabolism so that an optimal level of DHT for growth is maintained during both up and down fluctuations in the supply of testosterone. These results raise an important issue with regards to the successful culturing of human prostate cancer cells from patient material. If such malignant tissue is obtained from an intact patient, then attempts to grow these cancers in a castrate level of testosterone provided by 10%FCS supplemented media will not mimic the environment from which they were derived. Thus attempts to grow malignant cells from intact patients should include both 10%FCS supplemented media with and without the addition of exogenous androgen. If the synthetic androgen, R1881, is used for such exogenous androgen addition, a range of concentrations should be tested beginning at 0.01nM. This is because R1881 has a similar high affinity for the AR as testosterone/DHT but unlike testosterone/DHT, it is not bound by TEBG and is not metabolized (31). This last point is relevant for two reasons. First, it allows the R1881 containing media not to have to be changed as often as media supplement with exogenous testosterone. Second, since R1881 is not metabolized, however, too high a level of R1881 can not be regulated downward and thus will inhibit, not stimulate, the AR dependent growth of prostate cancer cells. This suggests that media addition with testosterone should also be tested. In contrast, if tissue is obtained from a castrated host that has already adapted to a low testosterone environment, then exogenous androgen should not be added to the 10%FCS supplemented media.

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Figure 1.

LNCaP cell (p46) counts. Two different cell-media (RPMI-10%FCS and RPMI-10%DC-FCS) are used. The RPMI-10%FCS is supplemented with 100nM testosterone, 500nM testosterone or 10µM Casodex (bicalutamide). Strong inhibition of cell proliferation is seen with RPMI-10%DC-FCS. Also the addition of 100nM testosterone is inhibitory on proliferation. Equal inhibition of proliferation is seen with the addition of 500nM testosterone and 10µM Casodex.

Tabel 1

Comparison of the Testosterone levels in Serum from Adult Human Male vs Fetal Calf Serum Supplemented Tissue Culture Media

SAMPLE	TOTAL TESTOSTERONE +/- SEM	FREE TESTOSTERONE +/-SEM
Serum from Intact Adult Human Male	3.0–10.0ng/mL (10 – 35nM)	60–200pg/mL (200 – 700pM)
Serum from Castrated Adult Human Male	<200pg/mL (<700pM)	<4pg/mL (<14pM)
RPMI Media Supplemented with 10% Fetal Calf Serum	22.0+/-6.1pg/mL (55.1 – 97.5pM)	2.0+/-0.29pg/mL (5.9 – 7.9pM)
RPMI Media Supplemented with 10% Dextran Coated Charcoal Stripped Fetal Calf Serum	5.0+/-0.49pg/mL (15.6 – 19.0pM)	1.8+/-0.48pg/mL (4.6 – 7.9pM)

Testosterone values. Molar concentrations are given in parenthesis.