Role of Testosterone, Estradiol, and Insulin in Diet- and Exercise-Induced Reductions in Serum-Stimulated Prostate Cancer Cell Growth In Vitro

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Abstract: Prostate cancer risk is associated with a high-fat diet and a sedentary lifestyle. Placing men on a low-fat diet-and-exercise intervention reduces serum hormones, including estradiol, insulin, and free testosterone, that may play a role in prostate cancer growth. Eight men participated in a low-fat diet-and-exercise program for a mean of 14.2 yr, and LNCaP cell growth in culture was measured in medium supplemented with 10% of each subject's serum as well as with testosterone, estradiol, and insulin added singly or in combination. These results were compared in the fetal bovine serum (FBS)-stimulated growth and cell growth in serum obtained from a control group of 14 overweight men. In separate tissue culture experiments, LNCaP and PC-3 cell growth was also measured in response to the addition of testosterone, estradiol, or insulin to steroid-stripped FBS. LNCaP cell growth in medium with subject serum was 40% less than in FBS-stimulated medium and 49% less than in medium with serum from control, overweight men. Addition of testosterone, estradiol, and insulin to serum from diet-and-exercise subjects significantly stimulated LNCaP cell growth in vitro but accounted for only about half of the difference between the control and diet-and-exercise subjects. Thus other serum changes must also account for the significant reduction in LNCaP cell growth observed using medium with serum from the diet-and-exercise subjects in the cell culture assay.

Introduction

Prostate cancer incidence has a large international variation, with men in Asian countries having the lowest rates and men in the United States having the highest reported rates in the world (1). Men who migrate from regions of low prostate cancer incidence to regions of high incidence increase their risk of developing prostate cancer, suggesting a role for environmental factors in prostate cancer etiology (2). As well, a recent study of 44,788 pairs of twins found that environmental factors account for >50% of the risk of developing prostate cancer (3). A high-fat diet has been implicated as a significant environmental factor in prostate cancer risk in several epidemiological and animal studies (4,5). A sedentary lifestyle may also contribute to an increased prostate cancer risk, inasmuch as men who are physically inactive have an increased risk for prostate cancer relative to physically active men, as recently reviewed by Thune and Furberg (6).

We previously demonstrated that the serum of men consuming a low-fat, high-fiber diet combined with exercise for 11 days or for 14.2 yr significantly reduced the growth of an androgen-responsive prostate cancer cell line (LNCaP) in cultures compared with serum from overweight men (7). However, this inhibitory effect was not seen when subject serum was incubated with an androgen-independent prostate cancer cell line (PC-3) (7). Placing men on a low-fat, high-fiber diet combined with exercise has been shown to significantly increase serum sex hormone-binding globulin (SHBG) and significantly reduce serum estradiol, insulin, and free testosterone (7,8). These changes in hormone levels observed in men undertaking the diet-and-exercise regimen may account for the reduced LNCaP cell growth in culture using subject serum, inasmuch as estradiol, insulin, and androgens are known to stimulate prostate cancer growth in vitro and in vivo (9,10).

The present study was designed to elucidate the endocrine changes in the serum of men undergoing the diet-and-exercise regimen that may be responsible for the reduction in serum-stimulated LNCaP cell growth in vitro. We hypothesized that addition of testosterone, insulin, and estradiol to serum of subjects who had been long-term compliers with the diet-and-exercise regimen would increase the serum-stimulated growth of LNCaP cells in cultures to the levels seen with fetal bovine serum (FBS) and serum obtained from control, overweight men.

Methods

Diet-and-Exercise Intervention

The study protocol was approved by the Human Subject Protection Committee at the University of California, Los
Angeles, and informed consent was obtained from each subject before the blood draw. Eight men (54 ± 4 yr of age) were compliant with the diet-and-exercise regimen for an average of 14.2 yr (range 6–20 yr). Twelve-hour fasting blood was drawn from these men and stored at −80°C until analyzed. The dietary guidelines followed by the subjects consisted of meals containing <10% of calories from fat (polyunsaturated-to-saturated fatty acid ratio = 1.24), 15–20% of calories from protein, and 70–75% of calories from carbohydrate, primarily in the form of vegetables, fruits, legumes, and whole grains. Dietary fiber was 35–40 g/1,000 kcal each day, and protein was derived from vegetable sources, nonfat milk, and small amounts of fish, chicken, or lean meat not to exceed 3.5 ounces/day. The diet was also low in cholesterol (<100 mg/day). The subjects participated in regular aerobic exercise 4–6 days/wk for up to 60 min each day. Serum was also obtained from 14 similar-aged overweight control men (60 ± 3 yr) who were on no special diet or exercise program. These men had a higher body mass index (BMI, 38±2 vs. 60 ± 3 yr) who were on no special diet or exercise program. Insulin was added at concentrations that are greater than normally seen when fasting but may be observed in the postprandial state. Controls of FBS-supplemented medium and CT-FBS-supplemented medium were run at the same time. Wells were plated at a predetermined density: 5 × 10⁴ for LNCaP and 4 × 10⁴ for PC-3.

For subject serum-stimulated cell growth, cells were added to wells of a six-well plate at predetermined densities (5 × 10⁴ for LNCaP) and allowed to attach to the plate over a 24-h period. Culture medium was then removed, and subject serum plus the given dose of hormone was added to assigned wells. The subject serum was added to RPMI 1640 at a 10% concentration, along with the regular amounts of penicillin-streptomycin and glucose. Each serum sample was run in triplicate, and all the samples of any given subject were run in one experiment to minimize interassay variability. A control group with FBS-supplemented medium (10%) was also run with each experiment to allow for comparison among experiments. At 48 h after the addition of subject serum, the cells were harvested by trypsinization and centrifugation. Each well of cells was then resuspended in medium, and trypan blue dye and a hemocytometer were used to count the cells.

**Statistical Analysis**

Statistical analysis was performed with the InStat statistical package for the personal computer (GraphPad, San Diego, CA). An analysis of variance was used to detect differences in the effects of hormone levels on cell growth. A Tukey-Kramer post test was then used to determine the source of statistical difference, if necessary. Values are means ± SE unless otherwise noted. P < 0.05 was considered statistically significant.

**Results**

Testosterone, estradiol, and insulin when added to CT-FBS significantly stimulated the growth of LNCaP cells in vitro compared with the CT-FBS control group (P < 0.01; Figs. 1–3). Neither testosterone nor estradiol at the concentrations used had any effect on the growth of PC-3 cells relative to the CT-FBS control. Insulin, however, significantly stimulated the growth of the PC-3 cell line relative to the CT-FBS control by 36 ± 2% at 300 pmol/l and by 45 ± 2% at 600 pmol/l.

LNCaP cell growth in cultures with media containing serum from men compliant with the diet-and-exercise regimen for a mean of 14.2 yr was 40% lower (P < 0.01) than growth in the FBS and 49% lower (P < 0.01) than growth in the serum from the control, overweight subjects (Fig. 4). The serum from the overweight subjects marginally increased the growth of LNCaP cells (109 ± 8%) compared with FBS (100%), although this was not significant.

Addition of testosterone or estradiol to the diet-and-exercise subject serum increased LNCaP cell growth in vitro by 12% and 16%, respectively (P < 0.01). Insulin added to
the diet-and-exercise subject serum stimulated LNCaP cell growth by 9% ($P < 0.01$). Addition of both testosterone and estradiol increased LNCaP cell growth by 18% ($P < 0.01$), although this increase was not significantly different from that observed with the addition of testosterone alone (Fig. 4). The further addition of insulin, along with testosterone and estradiol, to the diet-and-exercise subject serum increased LNCaP cell growth in vitro by 20% ($P < 0.001$; Fig. 4). This increase in LNCaP cell growth with the addition of all three hormones was still significantly below the cell growth observed with FBS and serum from control, overweight subjects (Fig. 4).

Discussion

The results of this study confirm our earlier observation that androgen-responsive prostate cancer cell growth in culture is reduced in medium containing the serum of men participating in a long-term diet-and-exercise regimen. The

LNCaP growth with serum from subjects on the diet-and-exercise regimen was reduced by 40% compared with FBS and by 49% compared with medium containing serum from control, overweight subjects. Reductions in insulin, estradiol, and free testosterone might account for approximately one-half of this reduction, inasmuch as adding these hormones to the diet-and-exercise subject serum increased LNCaP cell growth in vitro by one-half of the difference with the FBS control. Testosterone when added to CT-FBS stimulated the growth of the LNCaP cell line by 56% but did not affect the growth of the PC-3 cell line. The LNCaP cell line is well characterized and is responsive to androgens, including testosterone and dihydrotestosterone. In the prostate gland, testosterone is converted to dihydrotestosterone, which has a high relative affinity for the androgen receptor. It is possible that testosterone is also being converted to 17β-estradiol, which stimulates the LNCaP cell line, by the en-
zyme aromatase in prostate tumors. However, a recent study demonstrated that <3% of androgens in the LNCaP cell line are converted to estradiol (11). We have confirmed (unpublished data) that aromatase activity is very low in LNCaP cells.

In the present study, estradiol added to CT-FBS stimulated the growth of the LNCaP cell line in vitro by 96% but did not affect the growth of the PC-3 cell line. Estradiol treatment has previously been shown to result in a higher incidence of prostate tumors in a testosterone-induced model of prostate carcinogenesis in rats (12). Estradiol has also previously been reported to stimulate the growth of the LNCaP cell line (9). The LNCaP cell line has an androgen receptor with a point mutation in the steroid-binding region, which increases its binding affinity for estrogens, progestagens, and antiandrogens relative to the wild-type receptor (13). This mutated androgen receptor may not be typically present in men with newly diagnosed prostate cancer. However, the LNCaP cell line was derived from a patient with prostate cancer and is the most common cell line used to study androgen-dependent prostate cancer in vitro. The presence of estrogen receptors in LNCaP cells may also provide a mode of action for estradiol (9). It was previously reported that the diet-and-exercise program reduced total serum estradiol in men by 48% in 3 wk (14). Carruba and colleagues (15) reported that estradiol significantly inhibited the growth of PC-3 cells over 6 days, although our studies showed no significant effect of estradiol on PC-3 growth over 2 days. The difference in length of PC-3 exposure to estradiol may account for our observed difference in results.

In the present study, we found that addition of insulin significantly stimulated the growth of the LNCaP and PC-3 cell lines in vitro. Insulin is a potent mitogen that has previously been shown to stimulate the growth of a rat prostate cancer cell line in vitro (10). Insulin plays a dual role in cell growth, mediating glucose transport across the cell membrane to provide an energy source as well as directly activating cellular RNA and protein production via the mitogen-activating protein kinase pathway (16). Both effects of insulin may result in the observed increase in LNCaP cell growth. The amount of stimulation from insulin, however, may not be significant, inasmuch as the decrease in insulin did not affect the growth of PC-3 cells.

Testosterone was added to the CT-FBS in concentrations of 18.5, 22.5, and 30 pg/ml, and all concentrations stimulated growth to the same level. In the diet-and-exercise subject serum, free testosterone was only reduced by 4.3 pg/ml compared with control subjects (8). It is doubtful that this small reduction in free testosterone could be a major factor in the reduced LNCaP cell growth seen with the diet-and-exercise serum compared with control or FBS. In fact, when we added 30 pg/ml testosterone to the diet-and-exercise serum, LNCaP cell growth was only increased from 62.6% to 74.6% of FBS control growth. Binding of testosterone by SHBG and albumin may have reduced the bioavailable amount of added hormone, inasmuch as a large fraction of testosterone is normally bound by these proteins in the circulation (17). We assumed that, at the normal testosterone levels, these proteins may be saturated and any added hormone would be in the free state. In addition, the amount we added to the diet-and-exercise serum (30 pg/ml) was far greater than the 4.3 pg/ml reduction observed as result of the diet-and-exercise program (7). Estradiol stimulated LNCaP cell growth in vitro when added to the diet-and-exercise subject serum, although cell growth in subject serum with both testosterone and estradiol was not significantly different from growth with testosterone or estradiol alone. The finding that the effects of the two hormones were not additive might suggest that they stimulate LNCaP cell growth through the same mechanism, likely the mutated androgen receptor of the LNCaP cell line. Estradiol is also bound in the circulation by SHBG, potentially reducing growth stimulation by the hormone (17). Insulin levels in the subjects should be low due to the consistent diet-and-exercise regimen, which in turn should maintain SHBG at relatively high levels, as previously reported (8). Insulin has been reported to inhibit the production of SHBG by the liver (18).

The addition of all three hormones to the diet-and-exercise serum accounted for only 50% of the reduction in LNCaP cell growth, despite the fact that the amounts added were far greater than the amount of reduction observed after the diet-and-exercise program. This suggests that there may be other serum factor(s) changing as a result of the intervention that cause a reduction in LNCaP growth. Other potential serum changes include reductions in insulin-like growth factor-1 (IGF-I), directly or as a result of an increase in IGF binding proteins. IGF-I stimulates prostate cancer cell growth and is regulated in part by insulin (19,20). Insulin has been shown to be reduced in men undergoing a low-fat diet-and-exercise regimen, which may reduce circulating IGF-I (8). IGF binding protein-1 (IGFBP-1) binds IGF-I in the circulation and has been shown to be increased in men undergoing a diet-and-exercise intervention for 6 mo (21). IGFBP-1 may be increased in these subjects, reducing the amount of IGF-I available to stimulate prostate cancer cell growth. Antioxidants may play a role in reducing prostate cancer growth, inasmuch as a recent study showed that vitamin E supplementation in SCID mice fed a high-fat diet reduced the growth of LNCaP tumors to the level of the low-fat dietary group (22). Serum antioxidants have been found to increase in men undergoing this diet-and-exercise regimen for 3 wk, potentially reducing the growth of the LNCaP cell line (23).

In conclusion, the results of this study confirm our findings that tissue culture media containing serum from men participating in a long-term diet-and-exercise regimen reduce LNCaP cell growth in vitro compared with tissue culture media containing FBS or serum from control, overweight men. Reductions in estradiol, insulin, and free testosterone, previously observed in men undergoing the intervention, may account for part of the reduced LNCaP growth, inasmuch as adding these hormones to subject serum increased serum-stimulated cell growth in vitro. Other serum hormones, growth factors, or binding proteins may
play a role in the observed reduction in LNCaP cell growth using the diet-and-exercise subject serum, inasmuch as the addition of all three hormones accounted for only 50% of the growth reduction observed in the cell culture assay. We recognize the limitations in extrapolating the results of in vitro data to the in vivo situation. However, our results with LNCaP cells in vitro reflect the epidemiological data which suggest that a low-fat diet and/or exercise reduces the risk for clinical prostate cancer. Clinical studies are ongoing to see if our in vitro findings apply to patients with prostate cancer on “watchful waiting.”

Acknowledgments and Notes

This publication was made possible by funds received from the National Cancer Institute (Grant CA-42710) Cancer Research Fund, under Interagency Agreement #97-12013 (University of California Contract 98-00924V) with the Department of Health Services, Cancer Research Program. Mention of trade name, proprietary product, or specific equipment does not constitute a guaranty or warranty by the Department of Health Services, nor does it imply approval to the exclusion of other products. This does not constitute a guaranty or warranty by the Department of Health Services, Cancer Research Program. Address correspondence to R. J. Barnard, 3219 Life Science Bldg., 621 Charles Young Dr. So., Los Angeles, CA 90095. Phone: (310) 825-3794. FAX: (310) 206-9184. E-mail: jbarnard@physci.ucla.edu.

Submitted 3 May 2001; accepted in final form 4 October 2001.

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