

Measurement of Androgen Sensitivity in the Human Prostate in In Vitro Three-Dimensional Histoculture

Jack Geller, Lida R. Sionit, Kenneth Connors, and Robert M. Hoffman

Department of Medicine, University of California, San Diego (J.G.); Mercy Hospital and Medical Center (J.G., L.R.S.); AntiCancer, Inc., San Diego (K.C., R.M.H.); Laboratory of Cancer Biology, Department of Pediatrics, University of California, San Diego, School of Medicine (R.M.H.)

We have adopted an in vitro three-dimensional histoculture technique for assay of androgen sensitivity in explants of human benign prostatic tissue. The assay is based on the uptake of ^3H -thymidine/ μg protein in explants of prostate incubated in parallel with dihydrotestosterone (DHT) and hydroxyflutamide (HF) controls. The ratio of ^3H -thymidine/ μg protein in DHT treated samples per ^3H -thymidine/ μg protein in HF treated samples provides an index of androgen sensitivity. The DHT/HF index measured in 24 BPH specimens averaged 3.6. To determine the specificity of the HF effect, we measured the DHT/HF index in a single prostate at different concentrations of HF in the presence of fixed concentrations of DHT (2×10^{-8} M) and noted a dose-response relationship. In addition we noted no effects of HF on ^3H -thymidine incorporation over a range of 2×10^{-4} M compared to 2×10^{-7} M, except at the highest concentration. Of surprise was the finding of an average DHT/HF index in 5 different nonprostate tissues, including breast, uterus, colon, kidney, and thyroid, that was similar to the index found in prostates. We plan to adapt this androgen sensitivity assay to measure the DHT/HF index in biopsy-size samples of prostate, since such an assay could then be utilized to determine androgen sensitivity in individual patients with prostate cancer. © 1992 Wiley-Liss, Inc.

Key words: hydroxyflutamide, dihydrotestosterone, 5α -reductase, ^3H -thymidine

INTRODUCTION

Although the majority of prostate cancers are androgen dependent, the assessment of androgen sensitivity in individual cases of metastatic prostate cancer would provide a logical basis for the selection of appropriate treatment as well as prognosis. If the tumor is judged androgen sensitive, androgen withdrawal would appear to be the indicated therapy; if not androgen sensitive, the tumor should be treated with chemotherapy.

Recently we became interested in adapting an in vitro three-dimensional histoculture technique on collagen sponge gels [1], which is currently used for predicting chemotherapeutic response in various solid cancer tissue, to the measurement of androgen sensitivity in prostate tissue from patients with benign prostatic hyperplasia

Received for publication August 10, 1992; accepted September 2, 1992.

Address reprint requests to Jack Geller, M.D., Department of Medical Education, Mercy Hospital and Medical Center, 4077 Fifth Avenue, San Diego, CA 92103-2180.

(BPH). The ultimate goal is to develop such an assay for use in prostate cancer. The basis for developing a test for androgen sensitivity is the measurement of ^3H -thymidine incorporation into tissue when dihydrotestosterone (DHT) is added to the culture media and compared to parallel incubations and uptake of ^3H -thymidine when DHT activity is blocked. The in vitro three-dimensional sponge gel culture system has been well established [1] and has the following advantages:

1. It allows the growth of both normal and tumor tissues in 3 dimensions and the preservation of native tissue architecture.
2. It allows simultaneous testing of multiple agents.
3. It requires relatively small amounts of tissue [1].
4. In vitro results in histoculture parallel clinical responses.

This paper is a preliminary report on the use of the collagen sponge gel technique to establish androgen sensitivity in BPH prostates and normal nonprostate tissues using the ratio of ^3H -thymidine incorporation in DHT treated compared to hydroxyflutamide (HF) treated tissue explants. We call this ratio the *DHT/HF index*. HF is an anti-androgen whose mechanism of action is to block androgen receptor binding to DHT and thereby to block effectively androgen-mediated action.

MATERIALS AND METHODS

Histoculture

Prostate tissue obtained at surgery was immediately placed in ice-cold MEM media. The tissue was cut into small 1.0 mm^3 pieces and then thoroughly mixed. These tissue minces were then planted onto previously hydrated flexible sponge gels derived from the extracellular matrix of pigskin on plates, each of which contained 6 wells. For each tissue studied, 2 plates were treated with DHT alone (treated plates), while 2 plates were used as controls, representing the absence of an androgen effect. A DHT concentration of $2 \times 10^{-8}\text{ M}$ was chosen as a near-maximal stimulus for growth stimulation, since organ culture studies by Shao et al. [2] in the rat prostate indicated a similar concentration to be optimal as well as physiologic. In our initial studies of 8 different prostates, ethanol alone was added to the control plates. These trials gave erratic results and suggested that the controls may have contained endogenous androgen. Therefore, in the subsequent 5 studies of prostate tissue, DHT ($2 \times 10^{-8}\text{ M}$) plus HF ($2 \times 10^{-5}\text{ M}$) was added to control plates to block androgen. It has been shown by Kennealey and Furr [3] that HF in this concentration effectively blocks DHT binding to the androgen receptor. Subsequently, all experiments were done using HF alone, $2 \times 10^{-5}\text{ M}$, for the control plates.

Since there was no statistical difference between the ^3H -thymidine in control plates treated with DHT plus HF in the doses indicated above compared to controls treated with HF alone, these 2 groups of studies were ultimately pooled as noted in Figure 2 and were referred to as the HF controls. For each prostate study, approximately 800–1000 mg of total tissue was utilized for the 4 plates. To each sponge in each well, 2.0 ml of media was added. The media was not changed during the 5-day incubation. The media was made up in batches as follows: To 500 ml of MEM with Earle salts, the following was added: 1.0 ml fetal bovine serum (final concentration = 0.2 percent) from which all steroid had been removed by charcoal-dextran adsorption; 5.0 ml of gentamicin sulfate (10 mg/ml) to give a final concentration of 0.1

mg/ml; 5.0 ml of nonessential amino acids, $100 \times$ (NEAA); 2.5 ml of insulin (10 U/ml) to give a final concentration of $50 \mu\text{U/ml}$; 2.5 ml of spermine ($200 \mu\text{g/ml}$) to give a final concentration of $1 \mu\text{g/ml}$; 2.5 ml of spermadine ($200 \mu\text{g/ml}$) to give a final concentration of $1 \mu\text{g/ml}$; 2.5 ml of vitamin A (0.028 mg/ml) to give a final concentration of $0.14 \mu\text{g/ml}$.

Following the initial 24 hours after planting of the explants, DHT in $5 \mu\text{l}$ ethanol was added daily for 4 days to all wells of the 2 treated plates to give a final concentration of $2 \times 10^{-8} \text{ M}$ in each well. HF in $5 \mu\text{l}$ ethanol or HF plus DHT in $5 \mu\text{l}$ ethanol, or in the early experiments $5 \mu\text{l}$ ethanol alone, were added daily for 4 days to all wells in the other 2 control plates. On the morning of the fourth day following tissue planting, $8 \mu\text{C}$ of ^3H -thymidine was also added to each well and the experiment was stopped 24 hours later. After stopping the experiment, all plates were frozen and kept at -70°C until assay. Ethanol was $<0.1\%$ in all wells. Normal nonprostatic human tissues consisting of colon, kidney, uterus, breast, skin, muscles, and thyroid that were obtained at surgery were studied in an analogous fashion, with 2 plates having DHT $2 \times 10^{-8} \text{ M}$ added daily, while the control contained HF $2 \times 10^{-5} \text{ M}$.

Processing Tissue for ^3H -Thymidine Uptake and Protein Assay

Tissue explants from each plate were pooled and washed until an aliquot of 1/20 of the saline wash was less than 100 cpm. The tissue was then placed in a test tube in a beaker of ice and homogenized in .05 M Tris buffer, pH 7.8, using a Techmar homogenizer operating at 3,000 RPM for 10-second bursts times 6. The homogenate was then centrifuged in a refrigerated centrifuge at 3,000 RPM, and the supernatant was removed for protein assay. The precipitate, which represented DNA, was digested in 0.3 ml hyamine at $70-80^\circ\text{C}$ for 2 hours. The solution was counted for ^3H -thymidine CPM in a LS 100 Beckman liquid scintillation counter with an efficiency of 59% for tritium.

The supernatant was assayed for protein by the method of de Boer [4]. Androgen sensitivity was expressed as the ratio of ^3H -thymidine/ μg of protein in the DHT incubation/ ^3H -thymidine/ μg protein in the HF incubation since HF at $2 \times 10^{-5} \text{ M}$ effectively blocks DHT receptor binding [3]. Such blockade interrupts the pathway of androgen mediated action and prevents any biological androgen response. This DHT/HF ratio, therefore, appears to describe the androgen sensitivity of the tissue tested. Studies of the nonprostate tissue were performed in a similar manner and were expressed as a similar ratio.

Studies of Specificity of the HF Effect

We performed several different experiments to demonstrate the specificity of the HF effect. In one such experiment, we measured the effect on ^3H -thymidine incorporation, when the concentration of HF was varied from $2 \times 10^{-5} \text{ M}$ in one histoculture plate to $2 \times 10^{-7} \text{ M}$ in another, while keeping the concentration of DHT at $2 \times 10^{-8} \text{ M}$ in both plates.

In addition, in another experiment, we measured the effect on ^3H -thymidine incorporation when we varied the concentration of HF in histoculture from $2 \times 10^{-4} \text{ M}$ to $2 \times 10^{-7} \text{ M}$, looking for any nonspecific concentration-dependent effect of this anti-androgen.

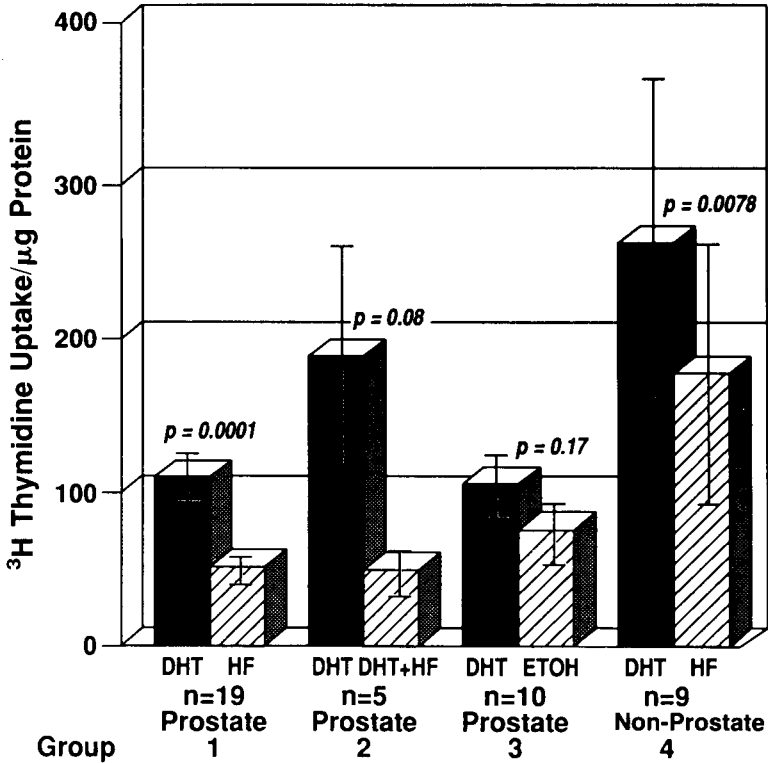


Fig. 1. ³H-thymidine uptake per microgram of protein for 2×10^{-8} M DHT (solid bars) stimulated prostate and nonprostate incubations and the various parallel control incubations (slashed bars) with either HF, HF plus DHT, or ethanol. The standard error of the mean (\pm S.E.M.) is indicated for each group by a vertical line above and below the top of each bar. Group 1 represents incubations in which flutamide, 2×10^{-5} M, was used for control incubations. Note the statistically significant increase in the ³H-thymidine uptake per microgram of protein in the DHT compared to the flutamide group. Group 2 represents incubations showing a trend toward a significant increase in the ³H-thymidine uptake in the DHT treated incubations compared to parallel controls treated with DHT, 2×10^{-8} M, plus flutamide, 2×10^{-5} M. In Group 3, ³H-thymidine uptake per microgram of protein was compared in 10 prostates incubated in parallel with DHT and ethanol controls. Group 4 shows the mean value for ³H-thymidine uptake per microgram of protein in nonprostatic tissues incubated with DHT compared to flutamide controls. T-test comparisons of DHT with the control tissue are shown above each set of bars for each of the 4 groups.

RESULTS

Paired T-Tests Comparing the Effect of Androgen (DHT to Controls HF or Ethanol) on ³H-Thymidine Uptake per Microgram Protein in Explants of Human BPH in Culture

DHT, 2×10^{-8} M, added daily to prostate explants, increased ³H-thymidine/ μ g protein an average of 2.2 times in comparison to HF controls where HF was added to the same tissue explants as shown for Group 1 (Fig. 1). A paired t-test showed a significant stimulation of thymidine uptake ($P = 0.0001$) in the DHT treated samples for 19 prostates studied.

A trend toward similar results was noted in 5 other prostate incubations (Group

2. Fig. 1) in which DHT, 2×10^{-8} M alone, was added to the explant cultures and ^3H -thymidine uptake was compared to parallel tissue explants to which DHT 2×10^{-8} M plus HF 2×10^{-5} M had been added as a control. A paired t-test gave a value of $P = 0.08$. By contrast, when 10 different prostate explants were incubated with 2×10^{-8} DHT and ^3H -thymidine/ μg protein compared to values obtained in parallel incubations with ethanol controls alone, a paired t-test gave a value of $P = 0.17$ (see Fig. 1, Group 3). Protein concentrations in all prostate supernatants following homogenization ranged from 225 $\mu\text{g}/\text{ml}$ to 2657 $\mu\text{g}/\text{ml}$.

Similar studies of 9 nonprostatic tissues are shown as Group 4 in Figure 1 (1 breast, 1 muscle, 1 skin, 2 uteri, 2 kidneys, 1 thyroid, and 1 colon) gave a significant increase of ^3H -thymidine/ μg protein in the DHT incubations as compared to the HF treated control tissue ($P = 0.0078$). Protein concentrations in these 9 tissues ranged from 82 $\mu\text{g}/\text{ml}$ in breast skin to 4055 $\mu\text{g}/\text{ml}$ in uterine tissue.

Comparison of the DHT/HF Ratios in Prostate and Nonprostate Tissues

The ratio of ^3H -thymidine/ μg protein in DHT incubations/ ^3H -thymidine/ μg protein in control incubations (HF, HF + DHT or ethanol) provides a DHT/HF index that is an expression of androgen sensitivity of the tissue, as described in Materials and Methods. In Figure 2 we show this index for parallel incubations of 10 prostates treated with DHT 2×10^{-8} M vs. ETOH alone. The ratio averaged 1.77 ± 1.11 SD. In 4 of these 10 studies, the DHT/HF was less than one. In comparison, the DHT/HF index for DHT vs. HF controls in 24 other prostates averaged 3.60 (S.D. ± 3.8), and in only 2 samples was the ratio less than 1. The unpaired P value comparing the DHT/HF ratio in these 2 groups is significant, with $P = 0.0346$. This suggests that incubation of prostate tissue without any added HF may contain endogenous androgen, thus resulting in a relatively low DHT/ethanol ratio.

No significant difference was noted in the DHT/HF index in unpaired t-tests for nonprostate tissues in comparison to prostate tissue ($P = 0.47$). The higher mean value was due to 1 uterine tissue in which the ratio was 41.9. If this value is excluded, the DHT/HF ratio for nonprostatic tissues is decreased to 3.3 ± 0.45 SEM. Individual values for nonprostate tissues are shown in Table I.

Specificity of HF Effect

Evidence for the probable specificity of HF in blocking androgen action is shown in Figure 3. This figure shows that when DHT (2×10^{-8} M) alone was added in ethanol to incubations of prostate explants and compared to incubations of combined DHT (2×10^{-8} M) plus HF (2×10^{-5} M), there was significant inhibition noted in the ^3H -thymidine uptake per microgram protein in the latter, as shown in Figure 3. When the HF concentration was reduced to 2×10^{-7} M in combination with DHT (2×10^{-8} M), there was a dramatic reversal of the inhibitory effect of HF. In addition, histocultures of aliquots of a single tissue with HF concentrations ranging from 2×10^{-4} down to 2×10^{-7} M (Fig. 4) showed no difference in ^3H -thymidine incorporation, except at the highest concentration, which was obviously toxic. The absence of any noticeable differences in ^3H -thymidine incorporation over the range of 2×10^{-5} M to 2×10^{-7} M of HF (ranges used in our studies) supports the specificity of the HF effect as an androgen receptor blocker (Fig. 4).

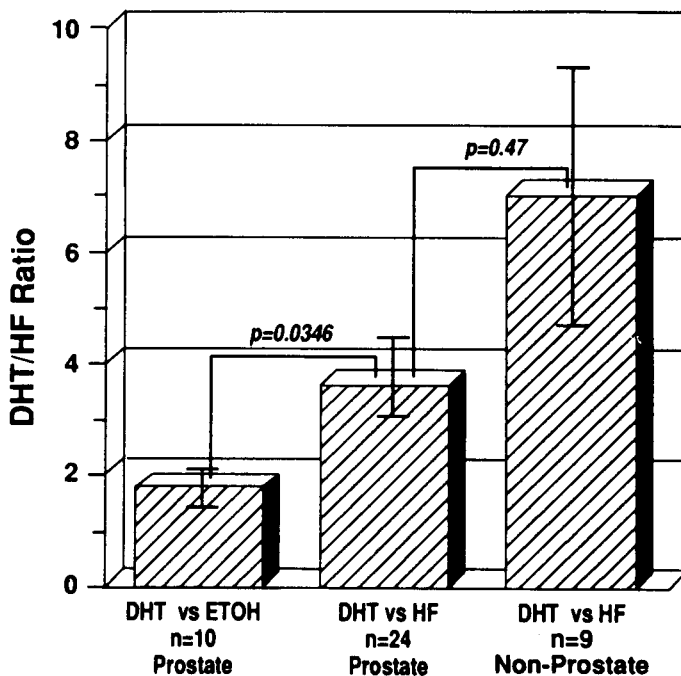


Fig. 2. The DHT/HF ratio is depicted for 3 different groups of tissues that were incubated according to the procedure described in Materials and Methods. The DHT/HF ratio is an expression of ^3H -thymidine per microgram of protein for the DHT incubations/ ^3H -thymidine/ μg protein for the HF controls. The standard error of the mean (S.E.M.) is indicated by the vertical line above and below the top of each bar. On the left is shown the ratio for BPH prostates that were incubated in parallel with DHT and ethanol. The ratio in this study was 1.77. The middle bar shows the DHT/HF ratio for explants incubated in parallel with DHT and HF. In 24 such samples the ratio was 3.6. This ratio was significantly greater than that noted in the DHT vs. ethanol group. On the far right is shown the DHT/HF ratio for explants of nonprostatic tissue, similar to those described in Figure 1. Note that there are only 9 samples in this group and, despite the fact that the mean value for the ratio was 6.98, this value is not significantly different from the DHT/HF ratio noted in the 24 BPH prostates.

TABLE I. DHT/HF Androgen Sensitivity Ratios for Nonprostate Tissues

Tissue	DHT/HF ratio
1. Thyroid	1.7
2. Kidney	1.03
3. Uterus, myometrium	2.3
4. Breast skin	1.18
5. Kidney	1.82
6. Uterus, myometrium	41.9
7. Colon	6.2
8. Muscle adjacent to breast	5.3
9. Breast tissue	1.4

DISCUSSION

We have attempted to develop a practical *in vitro* index to measure androgen sensitivity in prostate tissue, analogous to studies of chemosensitivity in malignant

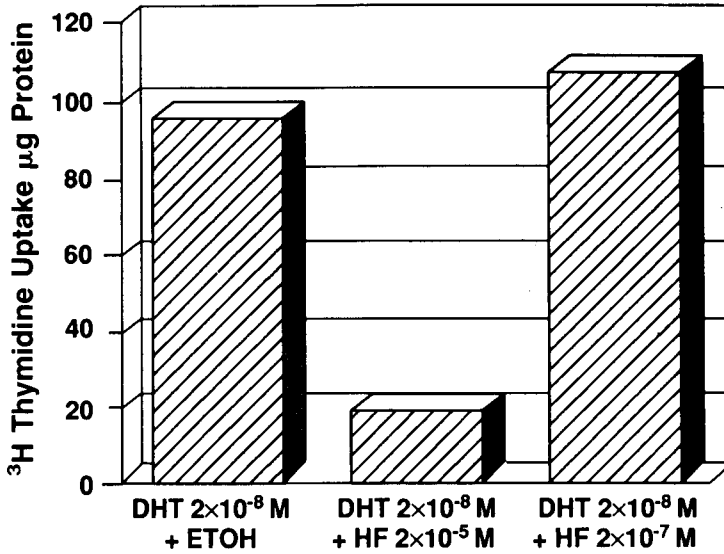


Fig. 3. ³H-thymidine uptake per microgram of protein in an experiment performed on a single tissue. The bar on the left indicates the uptake per microgram of protein for explants incubated with DHT 2×10^{-8} M plus ethanol. The center bar shows values for explants of the same tissue incubated with the same concentration of DHT and simultaneous HF, 2×10^{-5} M. On the right side of the figure the bar reflects the ³H-thymidine uptake per microgram of protein, when the same concentration of DHT was added to explants, but in which the HF concentration added simultaneously was decreased to 2×10^{-7} M. Note the dose-response relationship when the HF concentration was varied.

tumors done with in vitro assays. ³H-thymidine uptake appears to be an acceptable measure of cell growth and replication. There are exceptions to this when thymidine pool size or incomplete DNA synthesis may invalidate this procedure. This study establishes a simple technique for assaying androgen sensitivity by measuring a DHT/HF ratio for ³H-thymidine uptake per microgram of protein in benign prostatic tissue. This assay is based on a highly specific effect of a probe, HF, which blocks androgen receptor binding of DHT. No significant differences in DHT/HF ratios were noted when parallel incubations were performed with DHT vs. HF compared to DHT vs. HF plus DHT, indicating that the HF (2×10^{-5} M) concentration was more than adequate to block the majority of the receptor sites. No other direct effects of HF on DNA synthesis are known. Studies shown in Figure 3 of the dose-response relationship of HF to ³H-thymidine uptake support the specificity of the HF effect, as does the data shown in Figure 4. The present system of culture utilizes 200 mg of tissue per plate, but the system could possibly be miniaturized to handle biopsy-size specimens, since the ³H-thymidine counts average many thousand and the protein assay is very sensitive. To be useful in assessing androgen sensitivity in prostate cancer, a microassay using 20 mg of tissue would be necessary.

Prior attempts to develop predictors of androgen responsiveness with nuclear androgen receptor of tissue DHT assays did not prove to be practical. These include the studies of Trachtenberg and Walsh [5], who measured nuclear androgen receptor in prostate cancer cells to predict the response to androgen withdrawal. Although these studies did show that nuclear androgen receptor could be helpful in predicting

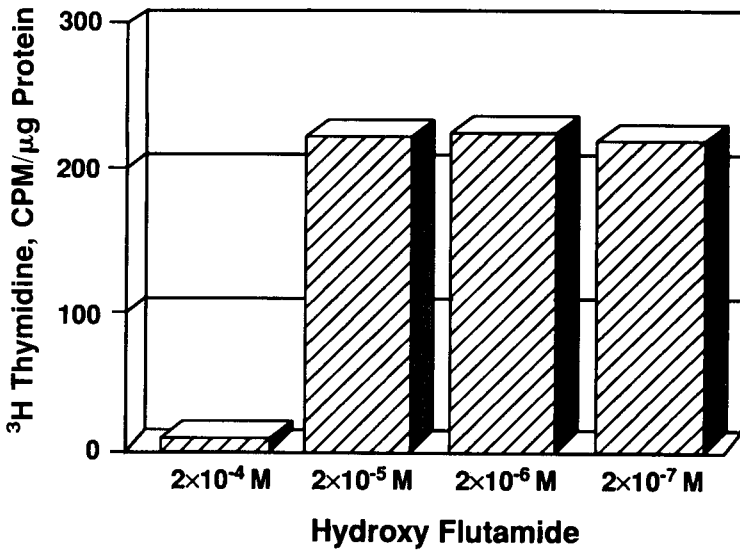


Fig. 4. ³H-thymidine incorporation per microgram of protein for parallel incubations of a single prostate exposed in 5-day histoculture to varying concentrations of hydroxyflutamide, ranging from 10^{-4} M shown on the far left down to 10^{-7} M. Note the dramatic inhibition of ³H-thymidine uptake at the highest concentration of HF and the absence of major differences at all other concentrations.

the response to androgen withdrawal therapy, the tissue requirements for good quantitative data to determine nuclear androgen receptor by Scatchard plot were greater than the biopsy material available in most cases of prostate cancer. The use of smaller amounts of tissue to do a single point assay as an alternative may not be reliable. Furthermore, the data shown by Trachtenberg and Walsh [5] did show an overlap of response to androgen withdrawal in patients who were above or below a critical level of nuclear androgen receptor defined by the authors.

Geller and his group [6] proposed measuring tissue DHT as a means of predicting the response to androgen withdrawal in metastatic prostate cancer. With this assay, minimum tissue requirements of 50 mg were also a practical consideration and usually exceeded the biopsy material available. Geller's studies, likewise, showed the usefulness of the tissue DHT assay, although overlap between good and poor prognostic groups was present.

Our present studies showed that blockade of presumed residual tissue androgen with HF was necessary to demonstrate the maximum androgen sensitivity. When controls consisting of ETOH alone were compared with DHT in parallel incubations, the DHT/HF ratio was significantly less than when HF was used in the control parallel incubations, implying that there was residual endogenous androgen in some of the incubations that required HF for blockade.

The surprise in our studies was the demonstration of androgen sensitivity, using the DHT/HF ratio in other normal tissues, including kidney, breast, muscle, skin, uterus, colon, and thyroid. All of these tissues except thyroid previously have been shown to have androgen receptors. Meggouh et al. [7] and Stebbings et al. [8] have identified androgen receptors in the human colon, including normal as well as cancer

of the colon. Androgen receptors were also identified in the human uterus by Tamaya et al. [9] and Muesler [10].

Abdelgadir [11] has shown that androgen receptors are present in the rat kidney, while Schleicher [12] has demonstrated the same in mice. Corrales-Hernandez [14] has demonstrated there are androgen receptors in the human kidney, and Corrales et al. [13], as well as Hoppen and Hammann [15], have identified androgen receptors in human breast tissue. Nevertheless, these tissues are not considered androgen target tissue. None of these tissues are known to contain 5α -reductase activity, except for breast tissue, and it is possible in vivo that only testosterone in significant amounts is found in these tissues. In our studies we used HF added to the incubations, which has a higher affinity for the receptor and may therefore produce a greater biologic effect. We are currently measuring the testosterone/HF ratios in incubations of both prostate and nonprostate explants to compare to the DHT/HF ratios in our studies.

We now plan to measure the DHT/HF ratio in prostate cancer. If this ratio gives values approaching 1 in patients with prostate cancer in relapse following androgen blockade, indicating growth that is independent of an androgen mediated mechanism, it would validate the usefulness of the DHT/HF ratio as a measure of androgen sensitivity and would provide a means of predicting androgen sensitivity in individual cases of prostate cancer.

CONCLUSIONS

In this study we have established that explants of prostate tissue in histoculture respond to androgen stimulation, as indicated by an increase in ^3H -thymidine uptake per microgram of protein in DHT treated as compared to hydroxyflutamide treated tissue samples. The CPM per microgram of protein for DHT treated incubates compared to ^3H -thymidine per microgram of protein in the hydroxyflutamide samples gives a ratio that serves as an index of androgen sensitivity. The specificity of the flutamide effect is supported by the uniformity of the effect of HF alone at concentrations of 2×10^{-5} to 2×10^{-7} and by the dose-response relationship between varying HF concentrations and fixed concentrations of DHT, using ^3H -thymidine uptake as the end point. The fact that the DHT/hydroxyflutamide ratio was similar in some nonprostate tissues was surprising to us but may be related to the fact that we used DHT in these incubations; in vivo these tissues are exposed to T, since many of them do not have 5α -reductase, although they do have androgen receptors.

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