PAPER

Dehydroepiandrosterone Sulfate Causes Proliferation of Estrogen Receptor–Positive Breast Cancer Cells Despite Treatment With Fulvestrant

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Hypothesis: Dehydroepiandrosterone sulfate (DHEA-S) causes a proliferation of estrogen receptor (ER)–positive breast cancer cells, even with tamoxifen citrate blockade. The ER antagonist ICI 182 780 (fulvestrant) will more effectively stop the proliferative effect of DHEA-S on breast cancer cells.

Design: Examination of in vitro breast cancer cell growth in the presence of fulvestrant and DHEA-S.

Setting: Surgical oncology research laboratory.

Interventions: The ER-positive and ER-negative breast cancer cells were pretreated with fulvestrant and stimulated with 900 µg/dL (22.8 µmol/L) of DHEA-S.

Main Outcome Measures: Assays using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, thiazolyl blue, were performed on the third, fifth, and seventh days poststimulation and permitted the calculation of growth percent change.

Results: The ER-positive and progesterone receptor–positive cells demonstrated universal proliferation of 107% by day 7 when treated with fulvestrant, regardless of the dose. The ER-negative and progesterone receptor–negative cells demonstrated growth inhibition.

Conclusions: The DHEA-S circumvented fulvestrant inhibition and caused ER-positive breast cancer cell growth.

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BREAST CANCER remains a major epidemiological concern, with estimates that the disease will have affected 5 million women in the last decade.[1] Although endocrine therapy has been clinically available for the past century and has proven beneficial for many patients, disease progression still occurs.

There are 3 mechanisms by which antiestrogen therapy for hormone-sensitive breast cancer may be implemented. The source of the estrogens may be ablated, the estrogen receptor (ER) may be blocked with antagonists, or the conversion of estrogens from precursors may be blocked with aromatase inhibitors.

Currently, most patients receive tamoxifen citrate, an antagonist that competitively inhibits the binding of estrogen to its receptor. Whereas tamoxifen achieves antitumor effects via antagonism of the ER, the drug also retains partial to full agonist properties depending on the target organ and species.[2-4] Although beneficial to bone density and serum lipid profiles, ER agonistic properties are thought to be responsible for the increase in endometrial cancers reported among tamoxifen users.[2,5] Of concern is the fact that this agonistic effect may be largely responsible for tamoxifen resistance and subsequent disease progression.[6]

Fulvestrant (ICI 182 780) is a pure ER antagonist.[7,8] It has been shown in in vitro studies to down-regulate the ER[3] and has recently been approved as a second-line hormonal therapy.[9] In vitro studies have also demonstrated that some breast cancer cell lines resistant to tamoxifen retain their sensitivity to fulvestrant, indicating that the drug has a role in the treatment of cancers that progress during adjuvant tamoxifen therapy.[10] Because it lacks estrogen agonist properties, adverse effect profiles appear limited with no increased risk of endometrial thickening or thrombogenicity. However, the beneficial effects found in the lipid levels and bone density patterns of tamoxifen users are not seen with fulvestrant therapy.

Current therapeutic approaches have focused chiefly on estrogens and their ef-

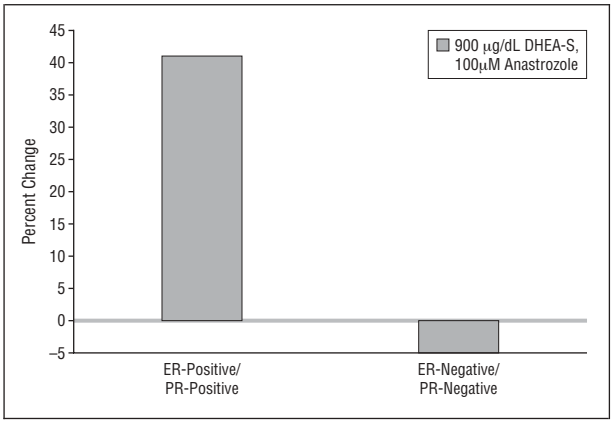

Figure 1. Percent change of cell growth of estrogen receptor (ER)– and progesterone receptor (PR)–positive (T-47D) and ER- and PR-negative (HCC1937) cells when blocked with anastrozole and stimulated with 900 µg/dL of dehydroepiandrosterone sulfate (DHEA-S). The ER- and PR-positive cells demonstrated significant proliferation vs ER- and PR-negative cells, which were inhibited by treatment. To convert DHEA-S value to micromoles per liter, multiply by 0.027.

fects on breast cancer growth, but the role that estrogen precursors play in cellular proliferation has not been fully explored. We previously demonstrated that high levels of dehydroepiandrosterone sulfate (DHEA-S), a steroid precursor of 75% to 100% of the estrogens in women, is a risk factor for disease progression in women with stage IV breast cancer treated with third-generation aromatase inhibitors.[11] We reported that ER-positive cell lines proliferated when exposed to DHEA-S in vitro. The conversion of DHEA-S into estrogens was prevented with the use of anastrozole, demonstrating the specificity of this compound. We subsequently reported that high DHEA-S levels are a risk factor for disease progression in women treated with adjuvant tamoxifen therapy. Concomitant in vitro studies demonstrated that DHEA-S used the ER and induced cell proliferation even in the presence of tamoxifen. We speculated that because tamoxifen is a competitive inhibitor of the ER, sufficient quantities of DHEA-S were able to overcome its inhibitory blockade.[12]

On the basis of these studies and suppositions, we hypothesized that blockade of the ER by the pure antagonist fulvestrant could ameliorate DHEA-S–induced proliferation of hormone-sensitive breast cancer cells. Therefore, in vitro studies were undertaken in which cells were cultured with high levels of DHEA-S. Fulvestrant was used as the ER blocker to further delineate the ability of DHEA-S to induce cellular proliferation.

METHODS

We obtained ER-positive and progesterone receptor(PR)–positive (T-47D) and ER-negative and PR-negative (HCC1937) cell lines from ATCC (Rockville, Md). Cells were plated onto 96-well plates in triplicate at a concentration of 1×10^4 cells per well. They were then grown in type-specific hormone-depleted media composed of phenol red–free RPMI (Gibco/Life Technologies, East Syracuse, NY), 5% dextran charcoal-treated fetal bovine serum (HyClone, Logan, Utah), 1% sodium pyruvate, and 0.5% gentamicin sulfate (Gibco BRL, Rockville), either with (T-47D) or without (HCC1937) 1% insulin (Gibco BRL), at 37°C with 5% carbon dioxide.

DHEA-S STIMULATION

After 5 days of incubation in hormone-depleted media and following 4 hours of pretreatment with 100µM anastrozole (AstraZeneca, Wilmington, Del), cells were stimulated with 900 µg/dL (22.8 umol/L) of DHEA-S (Sigma, St Louis, Mo). Cells with only anastrozole and the vehicle were maintained in tandem. Parallel cultures were pretreated with 100µM anastrozole and fulvestrant (ICI 182 780; AstraZeneca) at concentrations ranging from 10µM to 0.0001nM prior to stimulation with 900 µg/dL (22.8 µmol/L) of DHEA-S. Wells containing the vehicle, anastrozole, and fulvestrant but no DHEA-S were again maintained as controls.

Cell proliferation was determined using an assay (Sigma) performed on poststimulation days 1, 3, and 5. This assay, which counts only living cells, is a rapid colorimetric assay composed of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, thiazolyl blue.[13] A microplate reader (MRX; Dynatech Technologies, Chantilly, Va) recorded optical density readings of each plate at a wavelength of 570 nm. Growth or inhibition was reported as growth percent change of the DHEA-S–treated cells vs those grown in the vehicle, anastrozole, and fulvestrant. Statistical significance of differences in growth between various cell cultures was determined using a 2-tailed *t* test.

ENZYME IMMUNOASSAY FOR ESTROGENS

The T-47D cells were plated and cultured and subjected to pretreatment, blockade, and DHEA-S stimulation, as mentioned previously. Cells were lysed with RIPA buffer (150nM sodium chloride, 50nM Tris-hydrochloride, 1% Nonidet P-40 (Roche, Basel, Switzerland), 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate, and 1 tablet of protease inhibitor (Complete Mini; Roche). After the lysates were pelleted, the resultant cell suspensions were collected and purified using a YM-10 micron filter (Millipore, Bedford, Mass). Estradiol enzyme immunoassays (ALPCO Diagnostics, Windham, NH) were performed and read using a microplate reader (MRX; Dynatech Technologies) at 450 nm.

RESULTS

ENZYME IMMUNOASSAY FOR INTRACELLULAR ESTROGEN LEVELS

Immunoassays performed on T-47D cells demonstrated that negligible levels of estradiol were detected in all cells treated with DHEA-S and anastrozole. This indicates that, when present, anastrozole provides effective blockade of the conversion of DHEA-S into estrogens.

EFFECTS OF DHEA-S STIMULATION

When ER-positive T-47D cells were stimulated with 900 µg/dL (22.8 umol/L) of DHEA-S while blocking the DHEA-S conversion to estrogens with anastrozole, cell growth was 41% higher than that achieved with cells exposed only to the vehicle and anastrozole. The ER-negative HCC1937 cell lines failed to exhibit growth in identical conditions (growth, –5%) (**Figure 1**).

Growth of T-47D cells was inhibited by fulvestrant treatment at all concentrations, ranging from 0.0001nM to 10µM. However, no concentration of fulvestrant within this range prevented cell proliferation when ER-

positive T-47D cells were exposed to 900 µg/dL (22.8 umol/L) of DHEA-S and anastrozole. Cell cultures containing fulvestrant concentrations of 0.0001nM to 0.001nM exhibited a mean growth increase 26% higher than controls. Cell cultures with concentrations of 0.1nM to 10nM exhibited an average cell proliferation of 32%, whereas those with fulvestrant concentrations between 0.1µM and 10µM resulted in an average cell proliferation of 39% (**Figure 2**). The ER-negative HCC1937 cell line was unaffected by exposure to fulvestrant blockade. In identical experimental conditions, HCC1937 cells demonstrated no proliferation. Growth inhibition was similar to that seen in cultures treated only with DHEA-S and anastrozole (–5%).

That DHEA-S induces the proliferation of T-47D cells even in the presence of fulvestrant is graphically depicted in **Figure 3**. With all cultures undergoing fulvestrant blockade, there was no significant difference in growth on day 3 between cells stimulated with DHEA-S and those that were not ($P=.73$). In the presence of DHEA-S, there was a 34% increase in cell growth on day 5 ($P=.04$) and a 107% increase on day 7 ($P=.02$) compared with controls on the same days.

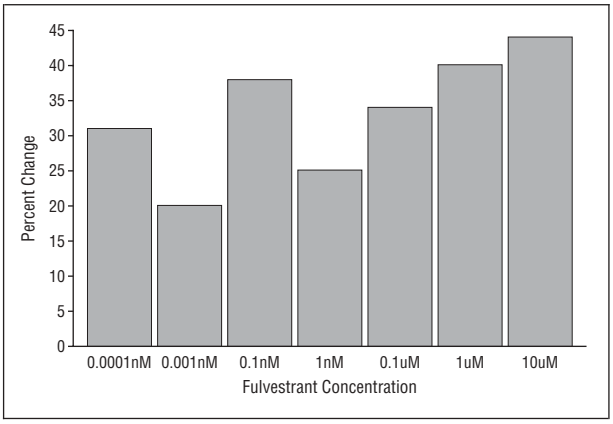

Figure 2. Percent change of cell growth of estrogen receptor– and progesterone receptor–positive (T-47D) cells when blocked with anastrozole and various concentrations of fulvestrant prior to stimulation with 900 µg/dL of dehydroepiandrosterone sulfate (DHEA-S). No concentration of fulvestrant prevented stimulation of cell growth by DHEA-S. To convert DHEA-S to micromoles per liter, multiply by 0.027.

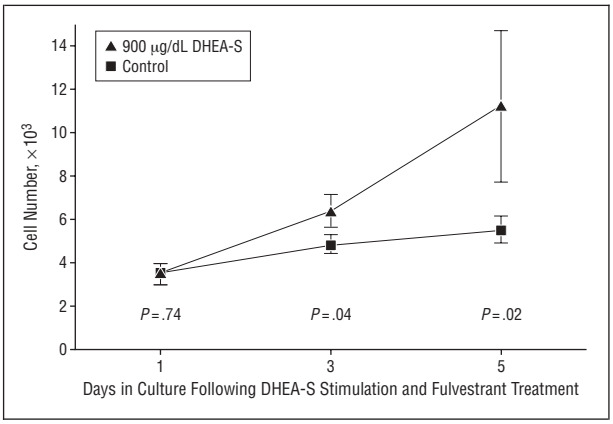

Figure 3. Comparison of cellular proliferation of estrogen receptor– and progesterone receptor–positive (T-47D) cultures blocked with fulvestrant in the absence and presence of 900 µg/dL of dehydroepiandrosterone sulfate (DHEA-S). Cells stimulated with this concentration of DHEA-S exhibited 34% increased growth on day 3 and 107% increased growth on day 5. To convert DHEA-S to micromoles per liter, multiply by 0.027.

COMMENT

Our previous studies demonstrated that DHEA-S stimulates the growth of ER-positive breast cancer cells[11] and that competitive tamoxifen blockade of the ER may be circumvented by sufficient quantities of DHEA-S.[12] In this study, the competitive blockade of the ER provided by fulvestrant failed to prevent DHEA-S–induced proliferation of ER-positive breast cancer cells.

Having shown in our studies that high levels of serum DHEA-S may function as an agonist ER ligand and that this may contribute to treatment failure during tamoxifen therapy, we hypothesized that fulvestrant, as a pure antagonist of the ER, would be more useful for blocking the effects of DHEA-S. This study demonstrated that DHEA-S induced cell proliferation even in the presence of a pure ER antagonist, fulvestrant. Contrary to our expectations, at no concentration of fulvestrant was growth inhibited. Because the immunoassay results demonstrated no conversion of estradiol from DHEA-S, it is likely that the DHEA-S was responsible for the proliferation of ER-positive cells.

Our results indicate that DHEA-S induces cellular proliferation in the presence of multiple agents and in a variety of settings. In environments where the estrogen concentration is low, such as after menopause or during third-generation aromatase inhibitor therapy, DHEA-S may function as a ligand for the ER and thereby induce cellular proliferation. Even in the presence of tamoxifen or fulvestrant, when levels of DHEA-S are high, it may be able to outcompete either agent for the ER, thereby functioning as a ligand and inducing proliferative cell-signaling pathways.

Extensive preclinical studies suggest that ER expression is down-regulated following fulvestrant treatment.[3,14-16] However, our results suggest that the inhibitory effect of fulvestrant is more likely due to blockade of the ER than down-regulation. If DHEA-S functions as an ER ligand, as our previous studies suggest, and if there were down-regulation of the ER by fulvestrant, some dose of fulvestrant should have been able to block the proliferative effects of DHEA-S. Because this was not observed with any dose of fulvestrant, it is more likely that this drug functions chiefly through competitive blockade of the ER. We are conducting ongoing studies to identify the changes in gene expression and cellular response to DHEA-S stimulation in the presence of tamoxifen or fulvestrant.

The results of this study have important clinical implications and warrant further investigation. Our previous studies indicated that serum DHEA-S levels higher than 90 µg/dL (2.28 µmol/L) are a risk factor for cancer progression among patients treated with third-generation aromatase inhibitors[11] and adjuvant tamoxifen therapy.[12] The findings of this study indicate that high serum DHEA-S levels are also a risk factor for disease progression in the presence of fulvestrant blockade. This study further supports the need to monitor serum sex steroid

levels, in particular DHEA-S, during endocrine therapy. Serially collecting these data will determine whether patients with low DHEA-S levels are more likely to respond to fulvestrant and thus will define a patient population for whom this drug is best indicated. In addition, actively lowering serum DHEA-S levels may increase the number of patients who will respond to fulvestrant treatment.

CONCLUSIONS

The compound DHEA-S is estrogenic in a low-estrogen environment; conversion to estrogens is not necessary for its activity. It appears to compete with both tamoxifen and the pure antagonist fulvestrant for binding to the ER. Given that DHEA-S can stimulate the growth of breast cancer cells in the presence of multiple therapeutic agents, it may be more prudent to control DHEA-S serum levels in patients with breast cancer.

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DISCUSSION

Roger E. Alberty, MD, Portland, Ore: When I was asked by Dr Pommier to comment on this paper, I thought, Why me, oh Lord? Then I was reminded of my days in the navy when I was qualifying for submarines. My division commander was interrogating me. I was explaining a very complicated hydraulic pump. I wasn't doing too well. He said, "Son, if you can't do any better than that, I will call in the chaplain. You can explain it to him. If he understands what you are saying, I know you know what you are talking about." So I come to you today as the chaplain. If I can understand this paper, anybody can.

The association of hormonal manipulation treating breast cancer goes back a long way and dates to a rather extraordinary operation in which Sir Percivall Pott resected bilateral herniated ovaries in a young woman and noticed a change in the progression of her breast development. Over 100 years ago there were papers published on oophorectomy for treating metastatic breast cancer. After World War II, glucocorticoids came on board and we were able to do adrenalectomies. For those of you in the audience who remember those days, some of the results were quite spectacular but unpredictable. This paper helps explain why some people respond and some don't.

This work makes me think of a stealth bomber. You don't see it coming, and it makes a big impact when it gets there. The literature is very recent and very scant. Dr Pommier's group is one of the few in the country that is investigating dehydroepiandrosterone. This work and other published works show that this hormone can block the effects of tamoxifen in vitro and clinically. As we see today, it works directly on estrogen receptor cells; it does not have to be converted to estrogen. I would like to emphasize that DHEA-S is the only hormone in their survey of a panel of hormones that was associated with progression of disease.

In this study, fulvestrant, which permanently rebinds estrogen receptors, was unable to block the effects of DHEA-S. Now this is an in vitro study. Evidently DHEA-S can block any estrogen blockade currently available. If we are therefore to control the DHEA-S, we are going to have to control the source. I have 2 questions: (1) Why in God's name did they study this hormone in the first place? (2) Will this lead to clinical work? Are we going to revisit Charles Huggins' works of the 1950s and 1960s when we are going back to either chemical or surgical adrenalectomy in the treatment of metastatic breast cancer?

This is truly an important paper, and it is extremely timely because there is hardly anything published on this outside of Pommier's group.

Electron Kebebew, MD, San Francisco, Calif: I find your results interesting. It has been demonstrated that there are dif-

ferent estrogen receptor subtypes (eg, α, β). In the cell lines you studied, have you characterized the estrogen receptor subtypes? Also, if it is not working through the estrogen receptor, what other alternative mechanism is it possibly working through? Lastly, did you ever consider using estrogen-positive but progesterone-negative receptor cell lines?

James E. Goodnight, Jr, MD, PhD, Sacramento, Calif: I have a brief question. The only way I know to block the dehydroepiandrosterone in vivo is to either take out the adrenal gland or block it with aminoglutethimide and then block the pituitary gland with hydrocortisone. Is there another method in vivo to block the hormone production?

Dr Pommier: We have been measuring the serum sex steroid levels of estrone, estradiol, testosterone, and DHEA-S in patients who have failed tamoxifen therapy for many years now. We treated such patients with a total endocrine ablation consisting of an oophorectomy, adrenalectomy, or total adrenal suppression with aminoglutethimide, which was the first-generation aromatase inhibitor. We verified that the estrogen and the DHEA sulfate levels went to zero to confirm that the endocrine ablation was in fact total. If patients still had high DHEA-S levels, then either they were not taking their aminoglutethimide or we had an incomplete adrenalectomy. Patients would generally respond to this therapy for a few years.

When the third-generation aromatase inhibitors came out, we could substitute 4 daily doses of aminoglutethimide, 3 doses of hydrocortisone, and 1 dose of aldosterone with a single pill and avoid aminoglutethimide's side effects of lethargy and rash, so we switched to them. We continued to measure serum sex steroid levels in these patients, however. We were expecting the same response rates and durations that we had seen with aminoglutethimide. While some patients did respond to the new agents, some exhibited progressive disease after a short interval. Thinking that I was seeing the emergence of hormone-insensitive disease, I biopsied some of the new tumors and found that they were all still ER-positive. I then thought that perhaps the third-generation drugs did not inhibit conversion of DHEA into estrogens equally well in all patients, but the sex steroid panels showed that the estrogen levels were zero in all patients; the new drugs were quite effective at preventing conversion. As I plotted the data, I saw a clear division between the patients who were responding and those who were progressing. Those responding had low DHEA-S levels, generally less than 60 μg/dL. Those who were progressing had DHEA-S levels greater than 90 μg/dL, and the difference was highly statistically significant. The patients with low DHEA-S levels had response durations 3 times longer than those with high levels. When we lowered the DHEA-S levels in the patients who were progressing by giving them aminoglutethimide or an adrenalectomy, they all responded again. This made me wonder what DHEA-S itself could do to the estrogen receptor. Could it be estrogenic?

Dr Toth-Fejel and I then conducted laboratory experiments on the T-47D cells, stimulating them with DHEA-S but blocking the conversion of the DHEA into estrogens with anastrozole. The results were that the DHEA-S stimulated cellular proliferation as much as did estradiol. Our other manuscript presented at this meeting shows, by immunofluorescence staining, that the DHEA-S itself causes the estrogen receptor to translocate into the nucleus, an effect that is blocked by tamoxifen. It also activates the MAP [mitogen-activated protein] kinase pathway. These effects are the same as those seen with estradiol. The DHEA-S itself is estrogenic. The old paradigm on which third-generation aromatase inhibitor therapy is based is that if there is no conversion of DHEA-S, then there is no problem. Our new paradigm is that in a low-estrogen environment, no conversion is a big problem.

The only known ways to lower high DHEA-S levels are with aminoglutethimide, adrenalectomy, or high-dose steroids. Our other data, which are in press, indicate that it may be beneficial to lower high DHEA-S levels even in the adjuvant setting. Giving high-dose steroids (which causes Cushing syndrome) or doing adrenalectomies in the adjuvant setting are clearly going to be unacceptable. We have found a way to lower DHEA-S levels close to zero, with far fewer side effects. We use 2 rather than 4 doses of aminoglutethimide per day with full hydrocortisone replacement. The lethargy and rash associated with this drug are practically nonexistent with this regimen; all of our patients tolerate it very well, and their DHEA-S levels are close to if not zero. What we really need to develop is a drug that specifically blocks the production of DHEA-S and leaves synthesis of adrenal cortisol and aldosterone unaffected.

The T-47D cell line is ER-positive, PR-positive, and androgen receptor–positive. The HCC1937 cells are ER-negative, PR-negative, but androgen receptor–positive. That was shown in our other work presented at this meeting, along with the surprising finding that DHEA-S inhibited the growth of the HCC1937 cells. We think DHEA does this through the androgen receptor. Functionally, DHEA is neither estrogen nor androgen but can be converted into either. Apparently its structure resembles the structure of either one enough that it can bind to both the estrogen receptor when estrogen levels are low or to the androgen receptor when testosterone levels are low. It triggers different responses that are specific to the profiles of receptors.

We do not yet know whether DHEA binds to the progesterone receptor as well, but this may be another mechanism by which it functions, especially in the presence of fulvestrant and tamoxifen. We are beginning experiments with cells that are only PR-positive to further investigate this. Alternatively, there may in fact be a specific DHEA receptor. We intend to pursue that possibility.