ORIGINAL ARTICLE

Estrogen and Androgen Receptors as Comediators of Breast Cancer Cell Proliferation

Providing a New Therapeutic Tool

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Hypothesis: Dehydroepiandrosterone sulfate (DHEA-S) comediates breast cancer progression via estrogen receptors (ERs) and androgen receptors (ARs).

Design: Breast cancer cells that were ER positive–AR positive or ER negative–AR positive were pretreated with anastrozole, tamoxifen citrate, or bicalutamide, then stimulated with 228μM DHEA-S.

Setting: University Surgical Oncology Research Laboratory.

Main Outcome Measures: Receptor status was confirmed by reverse transcriptase polymerase chain reaction. Cellular activity was measured by a methylthiotetrazole proliferation assay in addition to ER nuclear translocation and mitogen-activated protein kinase activity by immunoassays.

Results: The use of DHEA-S induced growth of 43.4% in ER-positive–AR-positive cells but inhibited ER-negative–AR-positive cells by 22%. Tamoxifen reduced growth of ER-positive–AR-positive cells to 8.9%. Bicalutamide restored normal growth of ER-negative–AR-positive cells. The ER nuclear translocation rate of 51% was reduced to 11% with tamoxifen. The use of DHEA-S induced mitogen-activated protein kinase by 5.4-fold.

Conclusions: Stimulation with DHEA-S induced proliferation through the ER but inhibited cells via the AR. Therapeutic comediation of receptors may provide effective treatment for ER-negative–AR-positive breast cancers.

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THERAPEUTIC APPROACHES for the treatment of hormone-sensitive breast cancer have focused chiefly on suppressing estrogen production or the binding of estrogen to the estrogen receptor (ER). Antiestrogen therapy is used to prevent the cascade of events that transpire after the binding of estrogens to the ER. These include the activation of the mitogen-activated protein (MAP) kinase pathway,[1,2] nuclear translocation of the ER complex[3,4] and subsequent gene transcription,[5-7] and cellular proliferation.[8,9]

However, breast cancer cells express a variety of receptors in addition to the ER.[10-13] Androgen receptors (ARs) are in fact the most highly conserved receptors, having been detected in 66% to 80%[14,15] of breast carcinomas. Receptor binding studies of androgen precursors have demonstrated that many are capable of binding to the ER as well as the AR.[16-21] Included among these is dehydroepiandrosterone (DHEA), a steroid precursor of androgens and estrogens. Despite the low affinity of DHEA sulfate (DHEA-S) for the ER, DHEA-S has been shown to have estrogenlike effects at physiologic plasma concentrations in MCF-7 and T-47D breast cancer cells.[22] Although, in that study, this effect was likely due to conversion of DHEA-S to estrogens, DHEA can function as a direct activator of the ER.[23] This compound is able to compete with estradiol for the ER, induce the nuclear translocation of the ER, and activate the transcription of genes under the control of the estrogen response element.[18,24,25]

We previously demonstrated that serum levels of DHEA-S of 90 μg/dL or more (≥2.43 μmol/L) are a risk factor for disease progression in women with stage IV disease treated with third-generation aromatase inhibitors.[26] We subsequently reported that similar DHEA-S levels are a risk factor for disease progression in women treated with adjuvant tamoxifen citrate therapy.[27] Concomitant in vitro work demonstrated that DHEA-S used the ER to induce cell proliferation, an effect that was blocked by tamoxifen in a dose-depen-

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dent manner. These results demonstrated that DHEA-S had estrogenic effects on breast cancer.

Our in vitro studies showed that DHEA-S also affected cell growth of an ER- and progesterone (PR)–negative cell line. In contrast to the effect seen in ER-positive cells, growth was inhibited by DHEA-S. This demonstrated that DHEA-S also regulated cell proliferation via receptors other than the ER. We speculate that the other receptor may be the AR and that breast cancer cell proliferation is comediated by the ER and AR.

In the present study, we explore the mechanisms by which DHEA-S may affect cellular proliferation via comediation of the ER and AR. We hypothesize that the cellular response will depend on the receptor status of the cell and the hormones available for binding. This knowledge will permit optimization of hormone therapy for hormone-sensitive breast cancer and, more important, open avenues for hormone therapy for ER- and PR-negative, but AR-positive, tumors.

METHODS

Breast cancer cells lines, T-47D (ER positive–PR positive) and HCC1937 (ER negative–PR negative) and Rat1 fibroblasts (to serve as a negative control) were grown in type-specific hormone-depleted media. After pretreatment with 100µM anastrozole (a gift from AstraZeneca Pharmaceuticals, Wilmington, Del), cells were stimulated with 22.8µM or 228µM DHEA-S (Sigma-Aldrich Co, St Louis, Mo). Cells were treated with anastrozole to prevent the conversion of DHEA-S to estrogens. Control cells were grown and treated as above except without the addition of DHEA-S. Cell proliferation was determined with a methylthiotetrazole assay (Sigma-Aldrich Co) performed on post-stimulation days 3, 5, and 7. This assay, which counts only living cells, is a rapid colorimetric assay composed of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide[28] that was read on a microplate reader (Dynatech MR580; Dynatech Laboratories Inc, Chantilly, Va) at a wavelength of 570 nm.

To determine whether DHEA-S induced cellular proliferation through the ER, experiments were designed as just described, except that equal numbers of cell cultures were also treated with the antiestrogen tamoxifen. Cellular responses that occurred after DHEA-S treatment, but were abrogated by tamoxifen treatment, were considered indicative of ER involvement. Similarly, AR involvement was investigated as described, except that cell cultures were treated with 10µM bicalutamide, an antiandrogen (a gift of AstraZeneca Pharmaceuticals), to demonstrate DHEA-S and AR interaction.

For cell lines used in this study, AR gene expression was established by reverse transcriptase polymerase chain reaction. Total RNA was isolated by phenol-chloroform extraction (TRIzol; Invitrogen Corp, Carlsbad, Calif). The AR gene was amplified according to the manufacturer's protocol with the use of gene-specific primers (ResGen; Invitrogen Corp), yielding a 137–base pair DNA fragment. Amplification products were separated by 2% agarose gel electrophoresis (GIBCO; Invitrogen Corp) and detected by ethidium bromide staining.

The presence and cellular localization of ER and AR protein was documented by immunohistochemistry. T-47D cells were fixed with paraformaldehyde after they were cultured in 228µM DHEA-S, with or without 10nM tamoxifen citrate. Antibodies of the ER and AR (Molecular Probes Inc, Eugene, Ore) and fluorescence microscopy were used to localize the receptors to different regions of breast cancer cells. Nuclear staining was defined as the predominance of ER staining being in the nucleus rather than the cytoplasm. Cytoplasmic staining was defined as the predominance of ER staining being contained in the cytoplasm. Experiments were done in triplicate, and approximately 500 cells were examined for each of the various treatment conditions. The percentage of cells with nuclear vs cytoplasmic staining was calculated for each treatment condition.

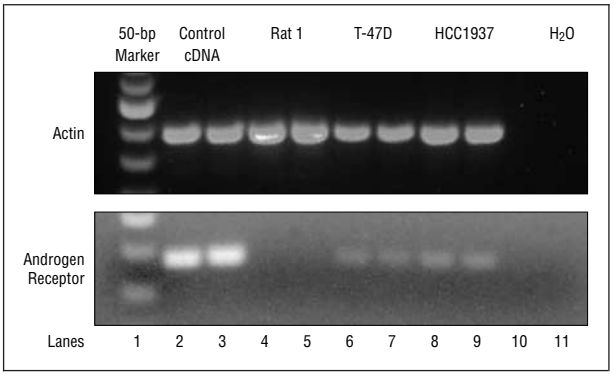

Figure 1. Androgen receptor gene expression in cell lines used in this study. Lane 1, 50–base pair (bp) ladder; lanes 2 and 3, positive control complementary DNA (cDNA); lanes 4 and 5, rat 1 cDNA; lanes 6 and 7, T-47D cDNA; lanes 8 and 9: HCC1937 cDNA; lanes 10 and 11, water control (H_2O). Total RNA was isolated by phenol-chloroform extraction. Reverse transcriptase polymerase chain reaction amplification of the androgen receptor gene yielded a 137-bp DNA fragment. Amplification of the actin gene served as a positive control. Products were separated by 2% agarose gel electrophoresis and detected by ethidium bromide staining.

Activity of MAP kinase in T-47D cells stimulated with DHEA-S was determined as follows. Cells were grown as already described in depleted media and then collected by enzyme disruption after DHEA-S stimulation of 0, 5, and 12 minutes. Cells from parallel untreated control cultures were also collected. Collected proteins were size-fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted and transferred to polyvinylidene fluoride membranes. Membranes were incubated in Phospho-p44/42 MAP Kinase antibodies (Cell Signaling Technology Inc, Beverly, Mass) and detected by chemiluminescence using an imaging device (Lumi-Imager) and its associated software (LumiAnalyst; Roche Diagnostics Corp, Indianapolis, Ind).

RESULTS

The ER and AR gene expression was demonstrated by reverse transcriptase polymerase chain reaction (**Figure 1**) and by immunohistochemistry (results not shown) in the T-47D cells. Only AR gene expression was demonstrated in the HCC1937 cells. No gene expression of the ER or AR was observed in Rat1 fibroblasts.

Cultures of T-47D cells exposed to DHEA-S exhibited increased growth of 43.4% compared with control cultures on day 5. Cultures exposed to DHEA-S but pretreated with tamoxifen showed 8.9% growth compared with cultures receiving DHEA-S, but no tamoxifen, a reduction of 34.5% (**Figure 2**).

Cultures of HCC1937 cells exposed to DHEA-S had cell growth inhibited by 22.0% compared with control cultures on day 5. This inhibitory effect of DHEA-S on HCC1937 cells was completely blocked with the addition of bicalutamide by day 5 (**Figure 3**). Rat 1 fibroblast cells were unaffected by DHEA-S treatment.

Five minutes after the addition of DHEA-S to T-47D cell cultures, there was a 3.4-fold increase in phosphorylated MAP kinase proteins. There was a 5.4-fold in-

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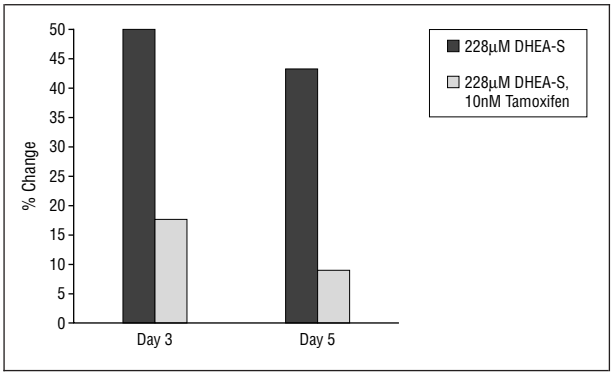

Figure 2. Percentage change in cell growth of estrogen receptor– and progesterone receptor–positive (T-47D) cells. Cultures were blocked with anastrozole and stimulated with 22.8µM dehydroepiandrosterone sulfate (DHEA-S), then measured at days 3 and 5 after stimulation. In parallel, cultures were stimulated and blockaded as described, but in addition were pretreated with 10nM tamoxifen citrate.

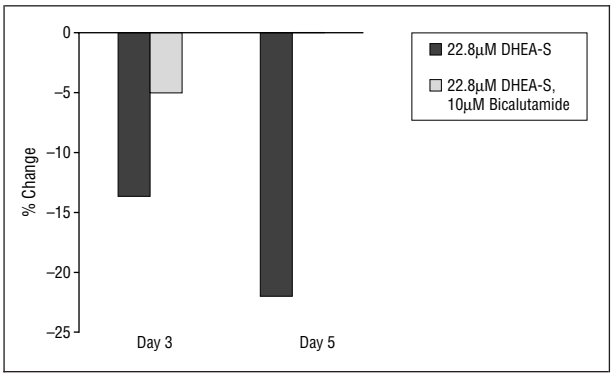

Figure 3. Percentage change in cell growth of estrogen receptor– and progesterone receptor–negative (HCC1937) cells when cultures were blocked with anastrozole and 10µM bicalutamide, before stimulation with 22.8µM dehydroepiandrosterone sulfate (DHEA-S).

crease in phosphorylated MAP kinase in T-47D cells at 12 minutes after stimulation by DHEA-S (**Figure 4**).

Use of DHEA-S induced the nuclear translocation of the ER. Twelve minutes after the addition of DHEA-S, virtually all ER was present in the cytoplasm of cells, with only 1.1% of cells showing positive nuclear staining for the ER. At 30 minutes, 51.2% of cells showed positive nuclear staining for the ER. At 60 minutes, only 6.4% of cells continued to exhibit positive nuclear staining for the ER, with the majority of cells again showing presence of the ER in the cytoplasm (**Figure 5** and **Figure 6**).

At the peak nuclear translocation time of 30 minutes, only 11.1% of cells pretreated with tamoxifen and then stimulated with DHEA-S exhibited positive nuclear staining of the ER. This was a 40.1% reduction in nuclear staining compared with cells stimulated with DHEA-S alone (Figure 6).

COMMENT

The hormonal response of breast cancer is determined by the receptor status as well as the ligands available for binding to those receptors. Traditionally, the chief factors considered in the hormone treatment of breast cancer have been the ER and PR status and estrogen levels.

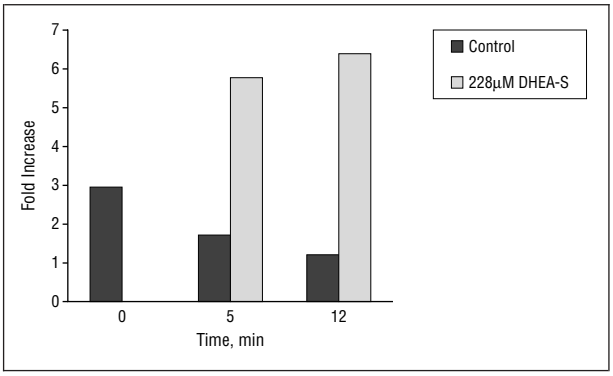

Figure 4. Mitogen-activated protein kinase activity in T-47D cells stimulated with dehydroepiandrosterone sulfate (DHEA-S). Cells were grown in depleted media and then collected by enzyme disruption after DHEA-S stimulation of 0, 5, and 12 minutes. Cells from parallel untreated control cultures were also collected. See "Methods" section for details.

However, the findings of this study indicate that the factors considered must also include the AR status and the levels of hormone precursors, such as DHEA-S.

The hormone precursor DHEA-S is capable of inducing growth of T-47D breast cancer cells. In contrast, DHEA-S inhibits growth of HCC1937 cells. These opposite responses can be attributed to the different receptor statuses of the 2 cell lines. The T-47D cell line is ER and PR positive.[29] Conversely, the HCC1937 cells are ER and PR negative.[30] We demonstrated that both cell lines are also AR positive. This difference in receptor status resulted in completely different responses to the same hormone.

Within the T-47D cell line, the stimulatory effect of DHEA-S was mediated through the ER. This is supported by the fact that the effect can be blocked by pretreatment with tamoxifen. Further support is provided by the fact that no stimulatory effect was seen in the HCC1937 cells, which lack any ER expression. Estrogen-induced stimulation of ER-positive breast cancer cells has been documented to be associated with increased activity of the MAP kinase signaling pathway and shuttling of the ER to the cell nucleus. Similarly, we observed an increase in activated MAP kinase as well as shuttling of the ER to the cell nucleus after addition of DHEA-S to T-47D cell cultures. All of these results indicate that DHEA-S–induced growth is mediated by signaling pathways similar to those used by estrogens.

In contrast, DHEA-S had an inhibitory effect on the growth of HCC1937 cells. Growth inhibition could not have been mediated through the ER or PR in this cell line, leaving the AR as a possible protein through which the growth inhibition was mediated. The fact that the AR antagonist bicalutamide reversed the inhibition of cellular proliferation strengthens the argument that the binding of DHEA-S to the AR is responsible for the inhibition of HCC1937 cell growth.

The addition of the DHEA-S to T-47D cell cultures resulted in stimulation of growth despite the expression of AR. Apparently, when both ER and AR are present, the estrogenic effect of DHEA-S predominates. This may be due to the relative availabilities of the ER and the AR, or it may be due to differences in their binding affinities for DHEA-S. Perhaps when the ER is blocked by tamox-

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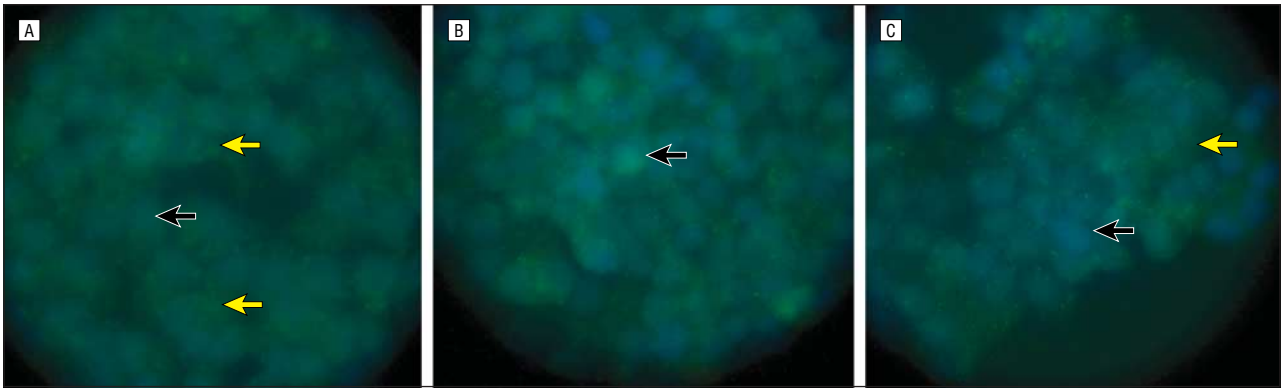

Figure 5. Presence and cellular localization of estrogen receptor (ER) protein documented by immunohistochemistry. T-47D cells were fixed with paraformaldehyde after they were cultured in 228μM dehydroepiandrosterone sulfate (DHEA-S). Antibodies for the ER and fluorescence microscopy were used to localize the receptors to different regions of breast cancer cells. A, Nuclear translocation of the ER at 12 minutes. Nuclei are blue and ER protein is green. Black arrow points to nucleus containing no ER. Yellow arrows point to ER in the cytoplasm. B, Nuclear translocation of ER at 30 minutes. Black arrow points to ER staining in the nucleus. C, Nuclear translocation of ER at 60 minutes. Black arrow points to nucleus with no ER staining. Yellow arrow points to ER staining in the cytoplasm.

ifen, there is increased binding of DHEA-S to the AR. It is conceivable that a portion of the inhibitory effect of tamoxifen in our study is attributable to the binding of DHEA-S to the AR when the ER is blocked.

The concept of comediation of breast cancer cells by multiple receptors and hormone precursors requires a comprehensive approach to the hormone treatment of breast cancer. It also opens new treatment options for some patients with breast cancers that are ER and PR negative, a group in which hormone therapy has not been used.

Patients within this group who are demonstrated to have AR-positive tumors are candidates for potential therapies that exploit the AR as a pathway that inhibits cellular proliferation.

To clinically exploit the inhibitory effects of DHEA-S on ER- and PR-negative but AR-positive breast cancer, conversion of DHEA-S into other hormones must be prevented. The DHEA-S is converted to estrogens and other hormones by the aromatase enzyme. In our study, we used the third-generation aromatase inhibitor anastrozole to prevent this conversion. Thus, any therapeutic compounds targeted to the AR should be administered together with a third-generation aromatase inhibitor.

The comediation of cellular response implies that it may be possible to potentiate hormonal therapy of ER-positive breast cancers when the AR is also present. The mainstay of therapy in these patients is to block the ER or the production of estrogen to inhibit cellular proliferation. However, the AR could be simultaneously manipulated to further down-regulate cell growth. The DHEA-S would not be a good candidate in this setting because it also has ER agonistic properties that may predominate over its inhibitory effects through the AR, as was seen in the present study. Rather, a new compound that is a pure AR agonist, a selective androgen receptor modulator, would need to be developed.

The results of this study indicate that the ER and AR have opposing actions on breast cancer cellular proliferation. The balance of these actions can be therapeutically manipulated to achieve desired clinical outcomes. Studies indicate that the AR is likely to be present in many breast cancers,[14,15] and may include those that are traditionally considered to not be hormone sensitive, such as the ER-negative–PR-negative HCC1937 cell line used in our study. This provides a new therapeutic approach. It opens the possibility of effective hormone therapy in the adjuvant and metastatic settings for patients with tumors that are ER negative–PR negative but

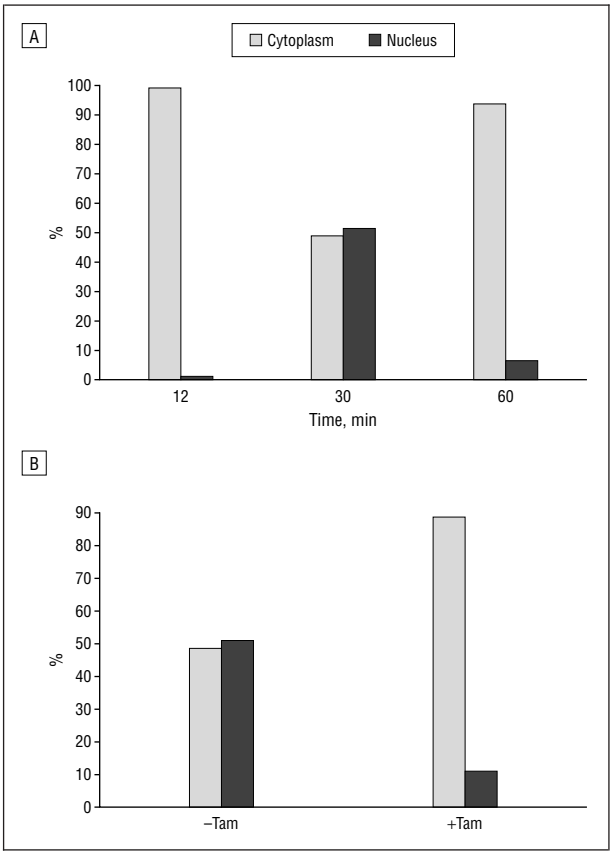

Figure 6. Percentage of cells showing nuclear or cellular localization of estrogen receptor protein, at various times after 228μM dehydroepiandrosterone sulfate stimulation (A), and with or without 10nM tamoxifen citrate blockade (–Tam or +Tam) (B). The influence of tamoxifen on receptor shuttling is shown at 30 minutes, the peak time of nuclear translocation of the estrogen receptors to the nucleus.

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AR positive. In addition, adding an AR agonist to conventional antiestrogen therapy could further hormone therapy for ER-positive–PR-positive and AR-positive tumors. Optimal hormonal therapy for breast cancer will be based on individual tumor receptor status, including AR status, and will require coordinated manipulation of all receptors.

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