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Androgen-induced human breast cancer cell proliferation is mediated by discrete mechanisms in estrogen receptor-α-positive and -negative breast cancer cells

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ABSTRACT

Androgens have important physiological effects in women. Not only are they the precursor hormones for estrogen biosynthesis in the ovaries and extragonadal tissues, but also act directly via androgen receptors (ARs) throughout the body. Studies of the role of androgens on breast cancer development are controversial and the mechanisms involved are not fully understood. In this report we demonstrate that a non-aromatizable androgen metabolite, dihydrotestosterone (DHT), stimulated cell proliferation *in vitro* of both estrogen receptor-α (ER-α)-positive MCF-7 cells and ER-α-negative MDA-MB-231 human breast cancer cells. A contribution of ER to the proliferative effect of DHT in MCF-7 cells was supported by actions of small interfering RNA (*siRNA*) ER-α transfection and of the specific inhibitor of ER, ICI 182,780 to block DHT-induced proliferation. A contribution of the possible conversion of DHT to androstane-3α, 17β-diol was not excluded in these MCF-7 cell studies. In MDA-MB-231 cells, a novel mechanism was implicated, in that anti-integrin αvβ3 or an Arg-Gly-Asp (RGD) peptide targeted at a small molecule binding domain of the integrin eliminated the DHT effect on cell proliferation. Anti-integrin αvβ3 did not affect DHT action on MCF-7 cells. A contribution from classical androgen receptor to the DHT effect in each cell line was excluded. A proliferative DHT signal is transduced in both ER-α-positive and ER-α-negative breast cancer cells, but by discrete mechanisms.

1. Introduction

The concept that androgen and estrogen exert opposing effects on the growth and development of human breast cancers has been widely advocated [1–3]. There are nonetheless recent epidemiologic observations to support the concept that androgen, in certain settings and like estrogen, can support breast cancer growth [4–7]. Such studies have relied upon radioimmunoassay to measure testosterone. This measurement approach is practical, but may not be sufficiently sensitive or specific to quantitate low levels of circulating testosterone in women [8]. Further, interpretation of a possible role of testosterone to breast cancer in women is complicated by a contribution of endogenous androgen to the estrogen pool [9,10]. The issue of whether testosterone, exogenous or endogenous, contributes to breast cancer growth remains unclear [7,11,12].

The relative roles of nuclear estrogen receptor (ER) and androgen receptor (AR) in breast cancer proliferation are controversial. Dehydroepiandrosterone sulfate (DHEAS) causes breast cancer cell proliferation via ER, but can inhibit proliferation through AR [13]. Yeh et al. have shown that AR plays an important role in breast cancer proliferation [14]. The nuclear AR is expressed in normal breast cells and up to 85% of breast tumors are AR-positive [12]. In addition, 25–82% of metastatic breast tumors that are ER- and progesterone receptor (PR)-negative still express a significant amount of AR [15,16].

In the present studies we show that dihydrotestosterone (DHT), a non-aromatizable androgen, enhances proliferation *in vitro* of ERα-positive and -negative human breast cancer cells. The proliferative DHT signal is transduced in ER-positive, AR-positive MCF-7 cells by an ERα-requiring and AR-independent mechanism and, remarkably, by a novel signaling pathway in ER-negative and AR-negative MDA-MB-231 cells that requires plasma membrane integrin αvβ3.

2. Materials and methods

2.1. Cell culture

The human breast cancer MCF-7 and MDA-MB-231 cells were purchased from ATCC (Rockville, MD). MCF-7 cells were main-

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tained in DMEM supplemented with 5% FBS and MDA-MB-231 cells were maintained in DMEM supplemented with 10% FBS in a 5% CO_2/95% air incubator at 37 °C. Prior to study, cells were placed in 0.25% thyroid hormone- and estrogen-stripped serum-supplemented medium for 2 days [17,18,20–22].

2.2. Reagents and antibodies

RGD and RGE peptide were obtained from Sigma Chemical Co. (St. Louis, MO). Polyclonal rabbit antibodies to integrin αvβ3 and αvβ5 and monoclonal mouse anti-PCNA were purchased from Santa Cruz (Santa Cruz, CA). Goat anti-rabbit IgG and rabbit anti-mouse IgG were purchased from Dako (Carpenteria, CA). Chemiluminescence reagent was from ECL (Amersham, Piscataway, NJ).

2.3. Cell fractionation

Fractionation of cells in a microfuge and preparation of nucleoproteins were by our previously reported methods [17–24]. Nuclear extracts were from a resuspension of crude nuclei in high salt buffer (420 mM NaCl and 20% glycerol) at 4 °C, with rocking for 1 h. The supernatants were collected after subsequent centrifugation at 4 °C and 13,000 rpm for 10 min.

2.4. Transfection of siRNA

Small interfering RNAs (*siRNAs*) of AR and ERα and scrambled RNA (scRNA) were purchased from Santa Cruz (Santa Cruz, CA). Breast cancer cells were seeded onto 6-well tissue culture plates and grown to 60–80% confluence in the absence of antibiotic for 24 h before transfection. Immediately prior to transfection, the culture medium was removed and the cells were washed once with PBS, then transfected with either *scRNA* or *siRNA* (0.2 μg/well), using Oligofectamine (2 μg/well) in Opti-MEM I medium according to the manufacturer's instructions (Ambion, Austin, TX). After transfection, cultures were incubated at 37 °C for 4 h and then placed in fresh culture medium. Cells were studied after an additional 24 h.

2.5. Thymidine incorporation

Cells were seeded in 24-well trays and fed with 10% hormone-stripped FBS-supplemented medium for 2 days, then treated with 0.25% hormone-stripped medium prior to starting experiments. Aliquots of cells were incubated with reagents as indicated including 1 μCi [^{3}H]-thymidine (final concentration, 13 nM) for 24 h, then washed twice with cold PBS. TCA (5%, 1 ml) was added and the plate was held at 4 °C for 30 min. The precipitate was washed twice with cold ethanol; 2% SDS (1 ml) was added to each well and the TCA-precipitable radioactivity was quantitated in a liquid scintillation counter.

2.6. Immunoblotting

The blotting techniques have been standardized in our laboratory [17–24]. In brief, nucleoproteins were separated on discontinuous SDS-PAGE and then transferred by electroblotting to nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 5% milk in Tris-buffered saline containing 0.1% Tween, the membranes were incubated with various antibodies overnight. Secondary antibodies were either goat anti-rabbit IgG (1:1000) or rabbit anti-mouse IgG (1:1000), depending upon the origin of the primary antibody. Immunoreactive proteins were detected by chemiluminescence and immunoblots were quantitated densitometrically with a Storm 860 phosphorimager; this was followed by analysis with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.7. Studies of breast cancer cell proliferation in non-perfusion bellows cell system.

To study effect of DHT on cell proliferation in human breast cancer MCF-7 and MDA-MB cell lines, several experiments were carried out using *in vitro* bellows cell culture system developed in our laboratory. MCF-7 and MDA-MB-231 cells were seeded in non-perfusion bellows bottles (1×10^7 cells/bottle) and brief agitation was scheduled at 2-min intervals overnight to permit cells to attach to polyethylene flakes. After overnight incubation, 5 flakes were harvested and trypsinized to determine the average number of cells attached to flakes. It was found that about 20% of seeded cells (2×10^6 cells) attach to flakes. Media were changed as 0.25% hormone-stripped FBS containing medium for 2 days. Cells were then refed with 1% hormone-stripped FBS-containing medium and agents were added to medium to obtain final concentrations as indicated. Ten flakes from each treatment bottle were harvested daily and trypsinized and the cells were collected for counting. Experiments were continued as indicated and reagents and medium were refreshed daily.

2.8. Quantitation of results and statistical analysis

Immunoblot densities were measured with a Storm 860 phosphorimager followed by analysis with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Student's *t*-test with $p < 0.05$ as the threshold for significance was used to evaluate the significance of the hormone and inhibitor effects.

3. Results

3.1. DHT induces cell proliferation in breast cancer cell lines

Human ER-positive breast cancer MCF-7 cells were treated with a non-aromatic androgen, DHT (10^{-10} to 10^{-6} M) daily for 5 days. [^{3}H]-Thymidine incorporation was applied 24 h prior to cell harvest. DHT increased [^{3}H]-thymidine incorporation (Fig. 1A, upper panel). [^{3}H]-Thymidine incorporation in 10^{-9}, 10^{-8} and 10^{-7} M DHT-treated cells was significantly increased ($p = 0.04$, $p = 0.005$ and $p = 0.03$, respectively), compared to untreated controls. Parallel experiments were conducted and cells were harvested and proliferating cell nuclear antigen (PCNA) expression was determined. DHT increased PCNA expression in MCF-7 cells ($p = 0.015$, $p = 0.01$ and $p = 0.044$ in 10^{-9}, 10^{-8} and 10^{-7} M DHT-treated cells as compared to untreated control) (Fig. 1A, middle panel). In order to confirm that DHT increased cell proliferation, an *in vitro* non-perfusion bellows cell culture system was used. Several concentrations of DHT (10^{-10} to 10^{-6} M) were added daily with refreshed medium. Results shown in Fig. 1A (lower panel) indicated that there was 25–65% increase of total cell numbers in 10^{-10} to 10^{-7} M DHT-treated cells, compared to untreated control in 3-day incubations. There was 2-fold increase of cell numbers in 10^{-8} M DHT-treated cells as compared to untreated control after 5 day incubation ($p = 0.008757$). We also examined the effect of DHT on ER-negative human breast cancer MDA-MB-231 cells that were similarly treated and found a similar proliferative response to 10^{-8} M DHT (Fig. 1B). Total cell numbers were increased 32–93% with exposure to 10^{-10} to 10^{-7} M DHT, compared to untreated controls in 3-day incubation. Other studies have also shown DHT-induced proliferation in human breast cancer cells [25].

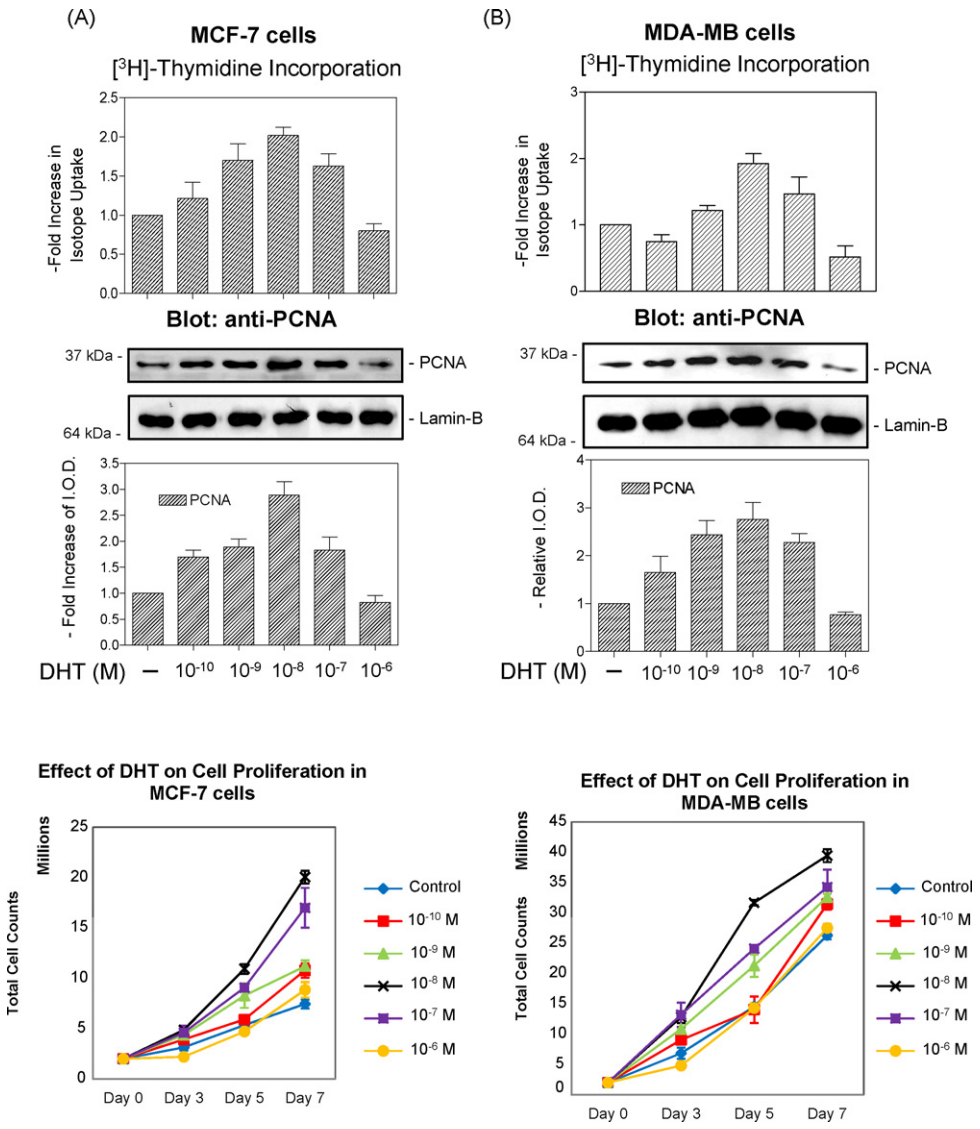

Fig. 1. Dihydrotestosterone (DHT) induces proliferation in ER-positive and ER-negative breast cancer cell lines. (A) *Upper panel*: MCF-7 cells grown in 0.25% stripped serum containing DMEM for 2 days were treated with different concentrations of DHT (10^{-10} to 10^{-6} M) daily for 5 days. Cell cultures were exposed to 1 μCi [^{3}H]-thymidine 24 h prior to harvest for thymidine incorporation assay. Radioactivity was quantitated in a liquid scintillation counter. *Middle panel*: Cells were treated with same condition and harvested for Western blotting analysis as described in Section 2. *Lower panel*: Cells were treated with 0.25% stripped serum containing medium for 2 days and refed with 1% stripped serum containing medium. Cells were treated with different concentrations of DHT and with refreshed medium daily. Cells were harvested and counted as described in Section 2 at date indicated. (B) *Upper panel*: MDA-MB-231 cells grown in 0.25% stripped serum containing DMEM for 2 days were treated with different concentrations of DHT (10^{-10} to 10^{-6} M) daily for 5 days. Cell cultures were treated with 1 μCi [^{3}H]-thymidine 24 h prior to harvest for thymidine incorporation assay. Radioactivity was quantitated in a liquid scintillation counter. *Middle panel*: Cells were treated with same condition and harvested for Western blotting analysis as described in the section of Section 2. *Lower panel*: Cells were treated with 0.25% stripped serum-containing medium for 2 days and refed with 1% stripped serum containing medium. Cells were treated with different concentrations of DHT and with refreshed medium daily. Cells were harvested and counted as described in Section 2 at date indicated. All graphs presented show mean ± SEM of change from normalized control values.

3.2. Discrete mechanisms for DHT-induced cell proliferation in ER-positive and ER-negative breast cancer cells

Having established that DHT can cause proliferation of human breast cancer cells, we next studied mechanisms by which DHT may act on breast cancer cells *in vitro*. First, we determined the contributions of ER and AR to DHT-induced breast cancer cell proliferation. MCF-7 cells were treated with 10^{-9} M DHT in the presence or absence of an androgen inhibitor, flutamide, for 3 days. No inhibition of MCF-7 cell proliferation occurred in the presence of DHT and flutamide (Fig. 2A, upper panel). Cell count in the non-perfusion bellows bottle system also showed that DHT increased cell proliferation ($p = 0.028$) and that flutamide did not affect DHT-induced cell proliferation ($p = 0.71$, cell numbers in cultures treated with DHT in the presence or absence of 1 nM flutamide) (Fig. 2A, lower panel). On the other hand, an ER inhibitor, ICI 182,780, blocked the DHT effect on cell proliferation in MCF-7 cells (Fig. 2B, upper panel). Studies of cell number also indicated that ICI blocked DHT-induced cell proliferation ($p = 0.028$, cell numbers in cultures treated with DHT in the presence or absence of 1 nM ICI) (Fig. 2B, lower panel).

Results shown in Fig. 3A indicate that *ERα-siRNA*, but not *AR-siRNA*, blocked DHT-induced MCF-7 cell proliferation (Fig. 3A, upper panel), as anticipated from results of the pharmacological inhibitor studies. A monoclonal antibody to ERα also inhibited DHT-induced proliferation in MCF-7 cells (Fig. 3A, lower panel). This interesting result is consistent with studies from several laboratories that contend functional ERα can reside in the plasma membrane (see Section 4). Taken together, these results indicate that the prolifer-

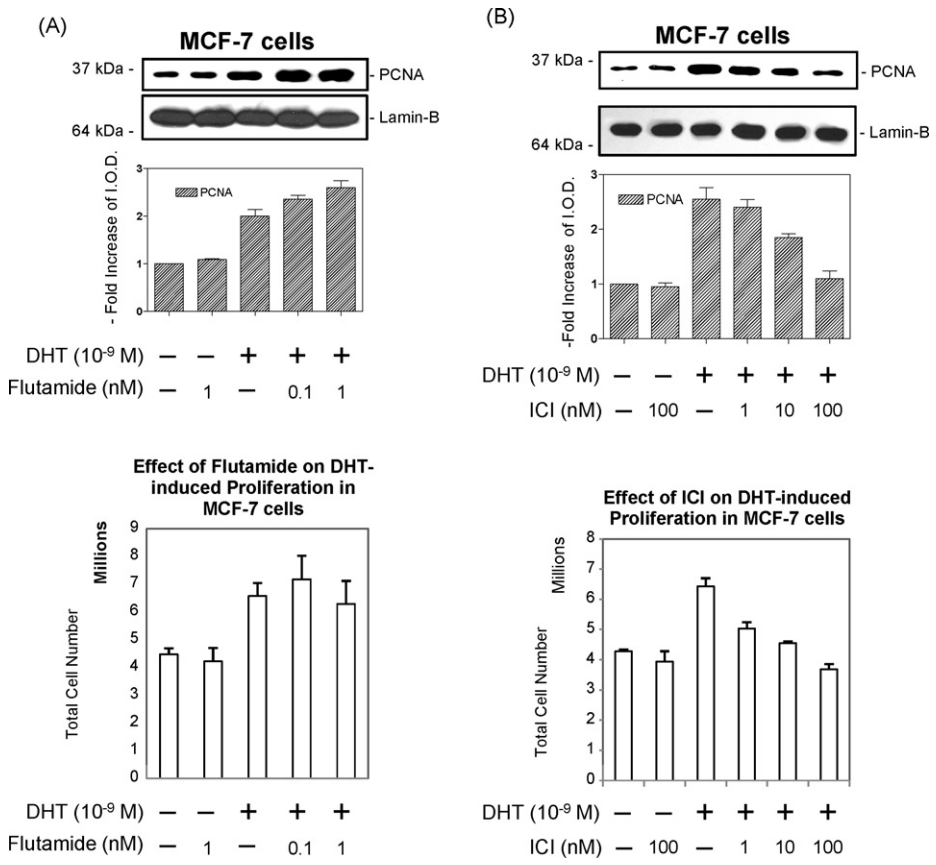

Fig. 2. Inhibitor of androgen receptor did not block DHT-induced cell proliferation in MCF-7 cells. (A) MCF-7 cells were exposed to 10^{-9} M DHT daily in the presence or absence of flutamide for 3 days. Flutamide did not inhibit DHT-induced PCNA expression (upper panel) and cell proliferation (lower panel). MDA-MB-231 cells are ER-negative and AR-negative, therefore we did not test for an effect of the inhibitor on DHT-induced proliferation in MDA-MB-231 cells. (B) MCF-7 cells were exposed to 10^{-9} M DHT daily in the presence or absence of ICI 182,780 (ICI) for 3 days. ICI treatment blocked DHT-induced PCNA expression (*upper panel*) and cell proliferation (*lower panel*).

ative effect of DHT in MCF-7 cells is ERα-dependent and does not require AR. Similar studies were also conducted in MDA-MB-231 cells, an ER-negative and AR-negative breast cancer cell line [26], and results indicated that neither AR *siRNA* nor ER *siRNA* blocked DHT-induced PCNA expression in MDA-MB-231 cells (Fig. 3B).

We have shown elsewhere that thyroid hormone via plasma membrane integrin αvβ3 induces cell proliferation in glioma cells *in vitro* [20] and that the hormone also induces MCF-7 cell proliferation [21], apparently via the iodothyronine receptor on the integrin [19,20]. We examined here where DHT-induced cell proliferation is initiated in MDA-MB-231 cells. We showed surprisingly that DHT-induced MDA-MB-231 cell proliferation is blocked by an Arg-Gly-Asp (RGD) peptide that can block actions of small molecules on the integrin [19,20,22–24], but not by a control RGE peptide (Fig. 4A, upper panel). The inhibitory effect of RGD on DHT-induced MDA-MB cell proliferation was also examined by using non-perfusion bellows bottle cell culture system. RGD peptide (500 nM), but not control RGE peptide, inhibited 10^{-9} M DHT-induced MDA-MB-231 cell proliferation (RGD, $p = 0.0092$, decrease in cell number in cultures treated with DHT in the presence or absence of RGD, vs. RGE, $p = 0.868057$) (Fig. 4A, middle panel). Treatment of MDA-MB cells with αvβ3 antibody blocked DHT stimulation of MDA-MB-231 cell proliferation. A control antibody, anti-αvβ5, was ineffective as an inhibitor of DHT (Fig. 4A, lower panel). Thus, in ER-negative MDA-MB-231 cells, the proliferative action of DHT is initiated at a cell surface integrin. On the other hand, we found that anti-αvβ3 had no effect on the action of DHT on ER-positive MCF-7 cells (Fig. 4B), nor did RGD peptide (Fig. 4B). Thus, a contribution of the integrin to DHT action in MCF-7 cells was excluded.

4. Discussion

The reported epidemiologic association of circulating androgen levels with increased breast cancer risk [1–8] is difficult to interpret because of the hormone measurement methodology used, as pointed out above (Introduction). Women with breast cancer do tend to have higher levels of androgenic DHEAS and androstenedione [27–30] than unaffected controls. DHEAS may be a moderate estrogen agonist [31,32] and adrenal androstenedione may be converted to estrone in postmenopausal women [33]. Interpretation of a possible contribution of DHEA to breast cancer biology is made difficult by experimental data showing discrete DHEA effects depending upon the abundance of AR and ER in the tumor cell line [34]. Against this background, it is apparent that additional information is needed about the possible molecular mechanisms by which androgens may act on breast cancer cells. In the present studies, we investigated the molecular basis by which DHT induced cell proliferation in human breast cancer MCF-7 cells that are ER-positive and AR-positive (Fig. 1A), as well as in MDA-MB-231 cells that are ER-negative and AR-negative. A novel cell surface receptor was implicated in the action of DHT in MDA-MB-231 cells.

When transfected with AR, breast cancer cells that lack ER, AR and progesterone receptor respond to treatment with DHEAS and an aromatase inhibitor (AI) [35]. This suggests that AR is involved in the inhibition of breast cancer cell proliferation by DHEAS. Studies by Tilley's group indicate that reduced levels of AR or impaired function of AR contribute to the failure of breast carcinoma cells to respond to progestin medroxyprogesterone acetate (MPA) [26] which has been used as a second-line hormonal therapy for metastatic breast cancer. They also further point out that syn-

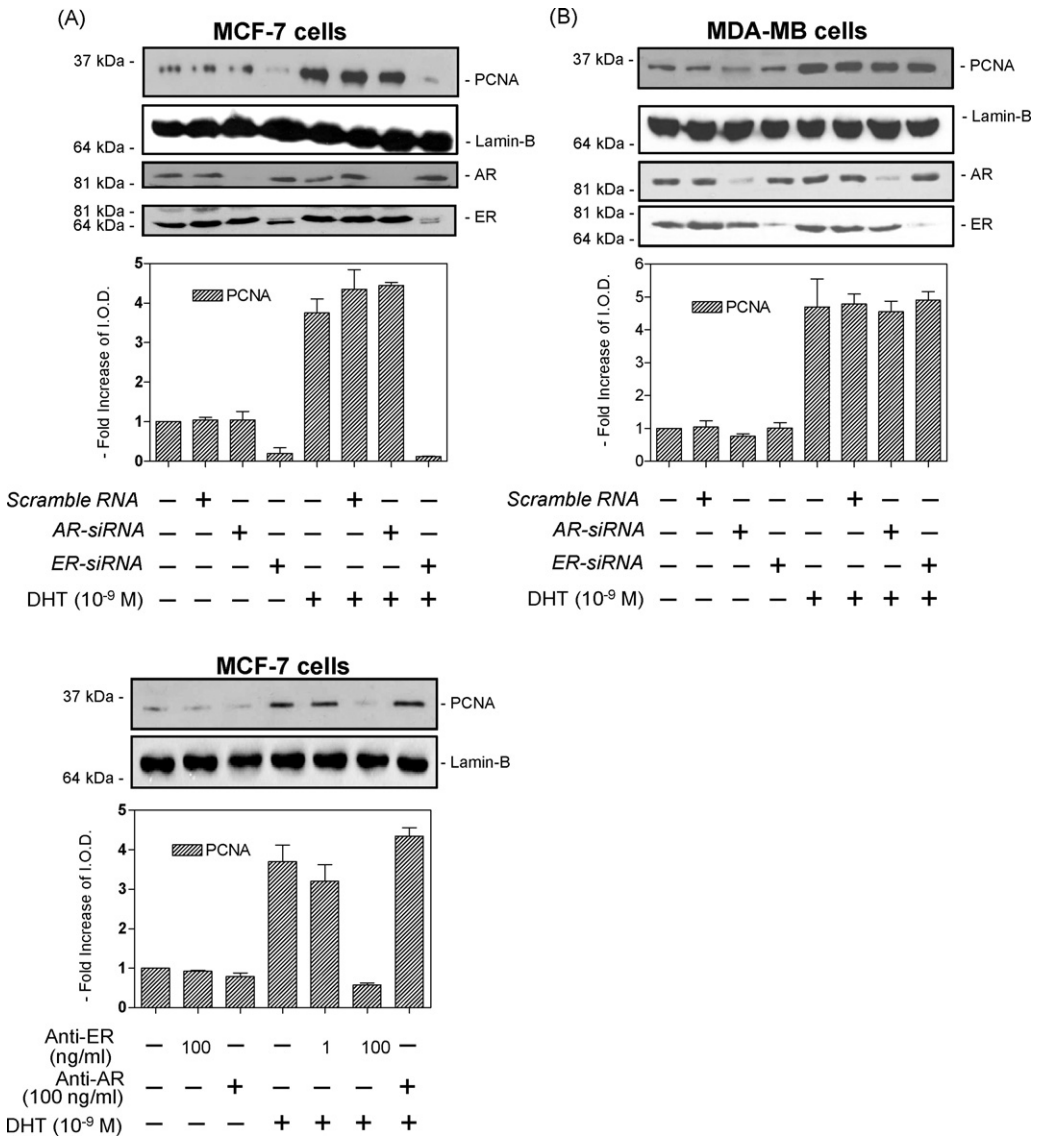

Fig. 3. DHT-induced cell proliferation occurs via ER in ERα-positive breast cancer cells. (A) *Upper panel*: MCF-7 cells were transfected with *siRNA*s of AR or ERα or with scrambled RNA. Cells were treated 10^{-9} M DHT daily for 3 days. DHT-induced PCNA was decreased in cells transfected with *ERα siRNA*, but not *AR siRNA* or scrambled RNA. *Lower panel*: MCF-7 cells were treated either ERα antibody or AR antibody prior to daily 10^{-9} M DHT treatment for 3 days. Anti-ERα blocked DHT-induced PCNA expression, but AR antibody was ineffective. (B) MDA-MB-231 cells were transfected with siRNAs of AR or ERα or with scrambled RNA. Cells were treated 10^{-9} M DHT daily for 3 days. DHT-induced PCNA was not affected in cells transfected with *ERα siRNA*, *AR siRNA* or scrambled RNA.

thetic progestins disrupt androgen receptor signaling may increase risk of developing breast cancer [36]. The conclusion has been postulated to be due to abrogation of the inhibitory effect of AR on ER signaling [36]. In the current studies, treatment of MCF-7 cells with DHT induced cell proliferation which was blocked by an inhibitor of ER, ICI 182,780 (Fig. 2B), by ERα *siRNA* trasfection (Fig. 3A, upper panel) and by monoclonal antibody to ERα (Fig. 3A, lower panel). DHT-induced cell proliferation was not affected by the AR inhibitor, flutamide (Fig. 2A), AR *siRNA* transfection or AR antibody (Fig. 3A). These observations are consistent with an action by DHT via ER to stimulate proliferation of ER-positive, AR-positive breast cancer MCF-7 cells. In what cellular compartment the ER exists in MCF-7 cells in order for the DHT effect to occur is not yet clear, since more than one estrogen receptor may exist on the cell surface [37,38]. Interaction of DHT with plasma membrane integrin αvβ3 may play an important role in DHT-mediated MDA-MB-231 cell growth in the absence of ERα, whereas the role of the integrin in MCF-7 cells may be secondary. Our results in MCF-7 cells do not exclude the possibility that DHT is converted to estrogenic androstane 3α, 17β-diol. It should be noted that Toth-Fejel et al showed that DHEA-S induced proliferation through ER, but the agent inhibited cell proliferation via AR [13]. This suggests a therapeutic strategy could be based on DHEA-S for ER-negative, AR-positive breast cancers [13].

More remarkably, DHT was shown here to stimulate MDA-MB-231 cell proliferation via a mechanism involving plasma membrane integrin αvβ3 (Fig. 4A) and an initiation site apparently located at the RGD recognition domain of the integrin that is critical to the binding by the integrin of extracellular matrix (ECM) proteins [39,40]. Several small non-peptide molecules have recently been reported by us to bind to integrin αvβ3 at the RGD recognition site and to induce changes in cell behavior. Among these small molecules are thyroid hormone [19,20,23,24] and estrogen-like stilbenes [22,23]. Acting on the integrin, thyroid hormone induces ERα phosphorylation and breast cancer cell proliferation in MCF-7 cells [21]. The thyroid hormone-induced effect can be blocked by RGD peptide in glioma cells [20,24] and in thyroid cancer cells [23].

The present observations suggest that testosterone may be capable of stimulating human breast cancer cell proliferation under

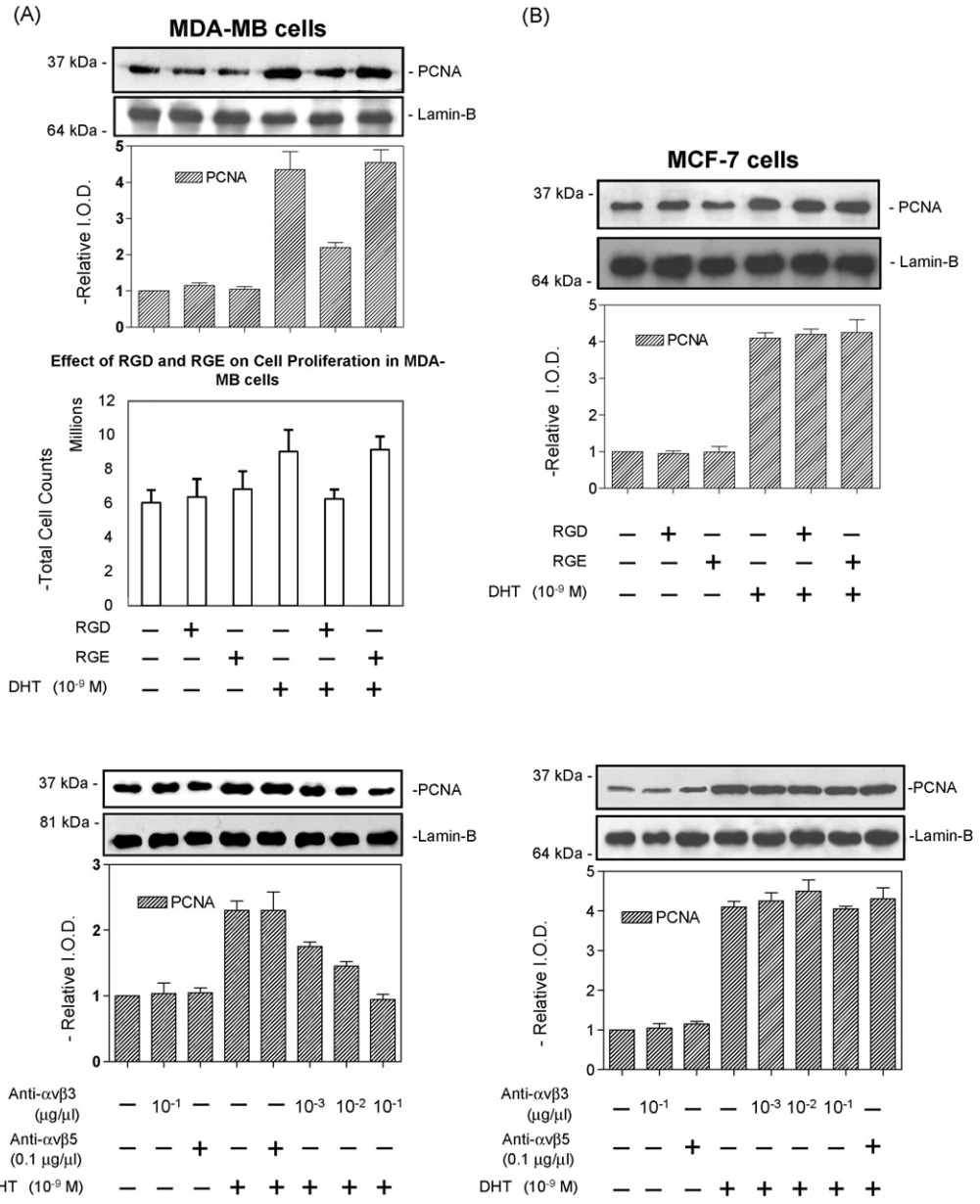

Fig. 4. Integrin αvβ3 is implicated in DHT-induced cell proliferation in ER-negative but not in ERα-positive human breast cancer cells. (A) *Upper panel*: MDA-MB-231 cells were treated with 500 nM RGD or RGE peptide prior to 10^{-9} M DHT treatment for 3 days. RGD peptide, but not RGE peptide, blocked DHT-induced PCNA expression in MDA-MB-231 cells. *Middle panel*: MDA-MB-231 cells were treated with 500 nM RGD or RGE peptide prior to daily 10^{-9} M DHT treatment for 3 days. RGD peptide, but not RGE peptide, blocked DHT-induced cell proliferation in MDA-MB-231 cells. *Lower panel*: MDA-MB-231 cells were incubated with anti-integrin αvβ3 or anti-αvβ5 for 24 h prior to DHT (10^{-9} M) treatment for 3 days. Integrin αvβ3 antibody inhibited DHT-induced PCNA expression in a concentration-dependent manner, whereas anti-αvβ5 did not affect DHT-induced PCNA generation. (B) *Upper panel*: MCF-7 cells were treated with 500 nM RGD or RGE peptide prior to daily 10^{-9} M DHT treatment for 3 days. Neither RGD peptide nor control RGE peptide blocked DHT-induced PCNA expression in MCF-7 cells. *Lower panel*: MCF-7 cells were incubated with anti-integrin αvβ3 or αvβ5 antibody for 24 h prior to 10^{-9} M DHT treatment for 3 days. Neither anti-integrin αvβ3 nor anti-integrin αvβ5 antibody inhibited DHT-induced PCNA expression.

certain clinical conditions, for example, in postmenopausal women where an increase in circulating androgen levels occurs relative to estrogen levels. Enhancement of breast cancer cell proliferation by androgen need not be AR-dependent, as our studies show. That there is redundancy in the molecular mechanism of androgen action in breast carcinoma cells was clearly shown here. That a cell surface protein, integrin αvβ3, mediated the androgen response in ER-negative cells is another novel observation, but this structural membrane protein played no role in the androgen response observed in ER-positive cells. The putative androgen receptor on the integrin is a potential drug target in the setting of ER-negative breast cancer that has recurred in the absence of endogenous estrogen. The use of non-aromatizable DHT in the current studies excluded the possibility of conversion of the androgen into estrogen.

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