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ORIGINAL PAPER

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The role of estrogen receptor, androgen receptor and growth factors in diethylstilbestrol-induced programming of prostate differentiation

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Abstract Recently, others and we have demonstrated that prenatal exposure to an extremely low dose of diethylstilbestrol (DES) and other estrogenic compounds produces a significant effect on mouse prostate development in vivo and in vitro in the presence and absence of androgen. In this study, we investigated the mechanism by which DES produces this effect and determined the role of its estrogenic activity on the growth and branching, induced by DES in the 17-day-old fetal prostate in culture. Additionally, we investigated whether the androgen receptor (AR) plays a role and whether any of the growth factors, namely, EGF and IGF-1 which are known to modulate the estrogen receptor (ER) and androgen receptor (AR)-dependent process, mediate the DES-induced effects. Using the organ culture bioassay of prostate development, we demonstrate that DES enhanced the growth and branching of the prostate at both 0.1 and 0.5 pg/ml dosages, thus, confirming a previous report of ours. An anti-estrogen, ICI164,387 blocked both of the effect of DES, suggesting that both of these two effects are ER dependent. Anti-androgen, flutamide also blocked both branching and prostatic growth induced by DES, while cyproterone acetate blocked only the branching effect, suggesting a role for AR in the DES-induced effects. Depletion of EGF by anti-EGF antibody blocked the DES-induced effects and this was reversed following EGF replacement in the organ culture system. Anti-IGF-1 antibody, on the other hand, only blocked the branching effect, but produced no effect on the prostatic growth, induced by DES. Estrogenic chemicals, bisphenol A and DES enhanced EGF-mRNA level of the cultured prostates. Taken together, it appears that DES-induced prostatic enlargement involves enhancement of ER-dependent EGF and IGF-1 synthesis, mediating prostatic enlargement and androgen action.

Key words Epidermal growth factor · Insulin-like growth factor · Diethylstilbestrol · Prostate · Estrogen receptor · Sexual differentiation

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Introduction

Recently, it has been reported from our laboratory and others that prenatal exposure to estrogenic chemicals has profound effect on prostate growth and function [12, 27, 28, 30]. Diethylstilbestrol when used at extremely low dosage enhanced prostate growth and androgen binding activity permanently [12, 30]. The growth promoting effect of DES was also observed in organ culture experiments [12], suggesting that DES affect prostate development directly.

The mechanism by which DES modulates prostate development is not known and the experiments, described here, were designed to investigate this. Since DES is a potent estrogenic compound [17], we investigated whether its estrogenic activity plays a role in inducing malformation. Additionally, if estrogenic activity plays a role, it was also our interest to determine whether this estrogenic activity interacts with the androgen receptor (AR)-dependent pathway of prostate development [7] and how these two pathways are interlinked. It has been shown that some of the growth factors, such as, epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) mediate estrogen effects in the adult uterus [1, 16]. EGF was shown to enhance estrogen receptor (ER) mediated transcriptional activity, thus mimicking estrogen-like effects [16]. EGF and IGF-1 were also reported to enhance androgen receptor (AR)-mediated transcriptional activity [6]. Recently, we showed that EGF enhances AR-mediated transcriptional activity in the primary mesenchymal cells of the fetal reproductive tract [13] and that EGF plays

roles in fetal reproductive functions [14, 15]. Additionally, receptors for these growth factors were detected in the developing male reproductive tract [29]. Thus, it is possible that these two growth factors may link the ER- and AR-mediated pathways during the DES-induced malformation. In this study we therefore investigated the role of ER, AR and the growth factors by determining (1) whether DES-induced prostatic enlargement is an ER-mediated effect, (2) whether AR also plays a role in DES-induced prostatic enlargement and (3) whether EGF or IGF-1 mediates the DES-induced malformations. The results shown here suggest a role for EGF and IGF-1 mediating the AR/ER-dependent DES-induced prostatic enlargement.

Materials and methods

Animals

The CD-1 timed pregnant mice were purchased from the Charles River breeding laboratory (Wilmington, Mass.). The day of breeding was counted as day zero of gestation. The pregnant mice were killed by cervical dislocation at 17th day of gestation and fetuses were collected to use in the organ culture experiments. All these animal procedures were approved by the NIH guideline of animal care.

Organ Culture

Fetal prostate was collected by micro-dissection from the urogenital sinus, at the onset of prostate development; i.e. 17th day of gestation as described elsewhere [7]. The organ culture assay of prostate development was carried out as described previously by Lipschultz et al. [21] and Lasnitzki et al. [19]. In brief, stainless steel grids were placed inside the 60 mm organ culture Falcon Petri dishes (Miles Plastics, Oxnard, Calif.). A strip of 1% Difco Agar (3 mm thick, 45 mm long, and 10 mm wide) was placed on the grid. The fetal prostates (two per culture) were arranged on the surface of agar and dye-free culture medium [Dulbecco's modified eagle medium (DMEM) and DMEM-F-12 1:1]. No serum was used, but insulin and transferrin were added, each at 10 ug/ml into the medium. DES (Sigma Chemical Company, St Louis, Mo.), anti-EGF, IGF-1 antibodies (Upstate Biotechnology, N.Y.), EGF (Sigma Chemical Company, St Louis, Mo.), IGF-1 (Collaborative Research Products, Bedford, Mass.) ICI 164,387 (Ligand Pharmaceutical Inc, San Diego, Calif.), cyproterone acetate (Schering AG, Berlin, West Germany) and flutamide were added in the culture medium as described in the text. The medium was changed every other day. To mimic the in vivo condition of testicular testosterone, drugs were tested in the presence of a physiological level of testosterone in some experiments. The culture dishes were then placed in a humidified 5% CO_2 incubator and incubated at 37 °C with various agents. The culture was stopped after 6 days. On day 7, the cultures were terminated and the organs were fixed in Bouin's solution for 4 h; they were then transferred into 70% alcohol and finally transferred into 20% sucrose-PBS buffer and allowed to equilibrate for 20 h each time. The fixed tissue was then used for histological sectioning as described below.

Histology and assessment of differentiation

The histology was performed as described earlier [19, 21]. In brief, the fixed tissue specimens were placed in Tissue-Tek OCT (Miles, Elkhart, Ind.) and frozen at –70 °C. Six-micrometer sections were cut transversely from the fixed specimen using an AO histostat microtome and stained with hematoxylin and eosin. All serial sections of the specimen were examined and branching appearance and the size of the specimen were monitored. The size of the specimen was determined by a calibrated micrometer disc attached to the microscope. Usually, the mid-section of a specimen demonstrates the full status of the development; i.e., branching appearance and size of the specimen. The branching was quantified by determining the no of tips per square millimeter.

EGF mRNA determination

The concentration of EGF mRNA was determined using an internally controlled quantitative reverse transcription polymerase chain reaction (RT-PCR) assay developed in our laboratory [22].Total RNA was isolated from the prostate using RNAzol and the extract was treated with DNAse to remove any contaminating DNA. An aliquot of the RNA preparation (0.1 ug) was mixed with 2 ng of in vitro synthesized EGF with a deletion in the target sequence. The deletion EGF RNA has been prepared in this laboratory [22]. The mixture was co-reverse transcribed using MMTV reverse transcriptase and co-amplified using direct incorporation of 32P dCTP. The primer pairs for EGF are: (nt 3228–3401) 5′CCT GAC TCT ACC GCA CCC TCT 3′ (upstream) and 5′AT CCC CAG AAT AGC CAA TAA CAC 3′(downstream). The amplification reaction was started by heating at 72 °C for 5 min and this was followed by repetitive cycles of 60 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C. Thirty cycles were carried out as this gave optimal amplification and the amplification was completed with an additional extension at 72 °C for 10 min. An aliquot of the PCR product was separated on a 10% nondenaturing PAGE. The amplified products was detected by staining with ethidium bromide. Subsequently, the gel was used for autoradiography and the intensity of the amplified bands was quantified by densitometric analysis. The expected product for the EGF gene is 173 bp size and its mutant product is 100 bp size.

A standard curve using different amounts of EGF mutant RNA was generated to determine the RNA present in the unknown samples. The signal intensity for EGF was normalized against the signals of the amplimer generated from the deletion EGF RNA and the results were used in determining the amount of RNA from the standard curves.

Three kinds of negative controls were used (1) no reverse transcriptase was added in the reverse transcription reaction to detect possible EGF DNA contamination in RNA sample, (2) no RNA was added in reverse transcription and (3) cDNA product was omitted in the amplification reaction. The experiment was considered useful only if no band was observed in all these three negative controls.

Results

Effect of ER antagonist on prostate growth induced by DES

We have shown previously that prenatal exposure to low dosage of DES causes enlargement of prostatic growth [12]. Similar results were obtained when DES was tested with the organ culture model of prostate development [12]. In this study, we examined whether the estrogenic activity of DES [17] plays a role in affecting prostate development. Thus, we tested whether an antiestrogen, ICI168,387 [1] at 1 ug/ml blocks DES-induced prostatic enlargement. Figure 1 shows that DES caused enlargement of the prostate and branching at low dosages, 0.5 and 0.1 pg/ml, as expected. ICI (1 ug/ml) blocked both of the effects of DES, suggesting that these effects are ER-dependent. ICI produced no effect on control

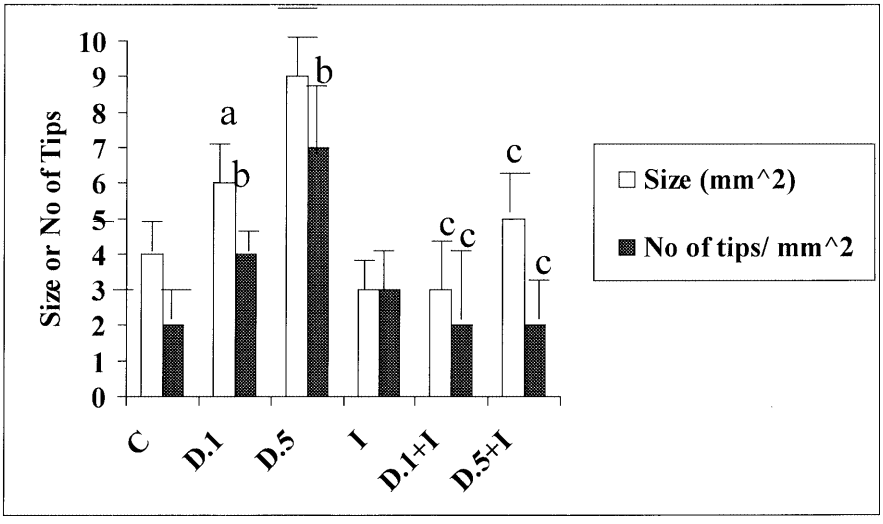

Fig. 1 Effect of DES on the growth and branching of the 17-day old fetal prostate in organ culture. *C* represents the control (8 specimens); *D.1* (4 specimens and *D.5* (8 specimens) represent the specimens exposed to 0.1 and 0.5 pg/ml DES respectively. *I* represents ICI164,387 (5 specimens,1 ug/ml). The experiment was performed four times. The data represent mean ± SD; *a* $P < 0.05$ compared to control growth; *b* $P < 0.05$ compared to control branching; *c* $P < 0.05$ compared to DES-treated controls. The results were analyzed by an ANOVA test. The experimental details are included in this paper. The symbol mm^2 represents mm^2

prostate growth or branching. The representative specimens from the treatment of ICI with or without DES are shown in the Fig. 2.

Effect of EGF antibodies on prostate enlargement by DES

Since estrogenic activity of DES plays a role in mediating DES-induced malformation, next, we determined whether any of the growth factors, EGF or IGF-1 which mediate-estrogenic activity in the adult uterus [16], plays a role in mediating DES-induced malformation. Thus, we determined the effect of anti-EGF antibody (neutralizing antibody) on DES-induced prostatic development. As shown in Fig. 3, anti-EGF antibody (50 and 10 ug/ml) blocked both prostatic enlargement and branching, induced by DES. EGF replacement (100 ng/ml) reversed the effect of anti-EGF antibody (Fig. 3), suggesting a specific role of EGF in prostatic enlargement. Anti-EGF antibody produced no effect on prostatic growth or branching of the control samples (Fig. 3). Figure 2 demonstrates a representative effect of anti-EGF antibody on DES-induced prostatic enlargement. Thus, EGF appears to play a role in DES-induced prostatic enlargement.

Effect of IGF-1 antibodies on prostate enlargement by DES

Next, we investigated the role of the other growth factor, IGF-1 which was also shown to mediate the estrogen effect in the adult uterus [1, 23]. As shown in Fig. 4, a neutralizing IGF-1 antibody (10 ug/ml) blocked prostate branching induced by DES. However, prostate size remained unaffected by the antibody (Fig. 2, Fig. 4). Again IGF-1 (50 ng/ml) replacement reversed the effect of anti-IGF-1 antibody (Fig. 4). Anti-IGF-1 antibody had no effect on the control specimen (Fig. 4). Figure 2 demonstrates the typical effect of IGF-1 antibody on prostate differentiation. Thus, EGF and IGF-1 both played a role in prostatic enlargement.

Effect of DES on EGF mRNA synthesis

To further determine the role of EGF in DES-induced prostatic effects, we examined whether DES enhances EGF-mRNA synthesis in the developing prostate. Thus, 18-day fetal prostates (4 per culture) were cultured with and without DES (0.5 pg/ml) and bisphenol A (50 pg/ml). Bisphenol A, like DES, was shown to enhance prostatic branching and size [12]. After 6 days of culture, the tissues were snap-frozen and used in determining EGF mRNA synthesis using the RT-PCR procedure. The results as shown in Fig. 5 indicate enhanced EGF synthesis by both DES and bisphenol A. Thus, enhanced EGF synthesis by DES and bisphenol A may play a role in prostate differentiation induced by the chemicals.

Effect of anti-androgens on DES-induced prostatic enlargement

The above results indicate that ER plays a role in mediating DES-induced prostatic enlargement. However, prostatic development is an androgen dependent process [7] and it is not known whether AR also plays a role in this process or whether this is an AR-independent effect. To determine this, we investigated the effect of anti-androgens, namely, cyproterone acetate and flutamide [14, 24]. The results as shown in Fig. 2, and Fig. 6, indicate that flutamide inhibited both prostatic enlargement and branching induced by DES. Cyproterone acetate, on the other hand, inhibited prostatic branching but had no effect on the control of prostate growth (data not shown). Thus, AR also plays a role in mediating the DES-effects.

Discussion

Many synthetic chemicals can bind to the estrogen receptor and thereby affect specific gene expression and cellular function [18]. The persistence of these compounds and their metabolites in the environment pose risks for both human and animal populations. The mechanism by

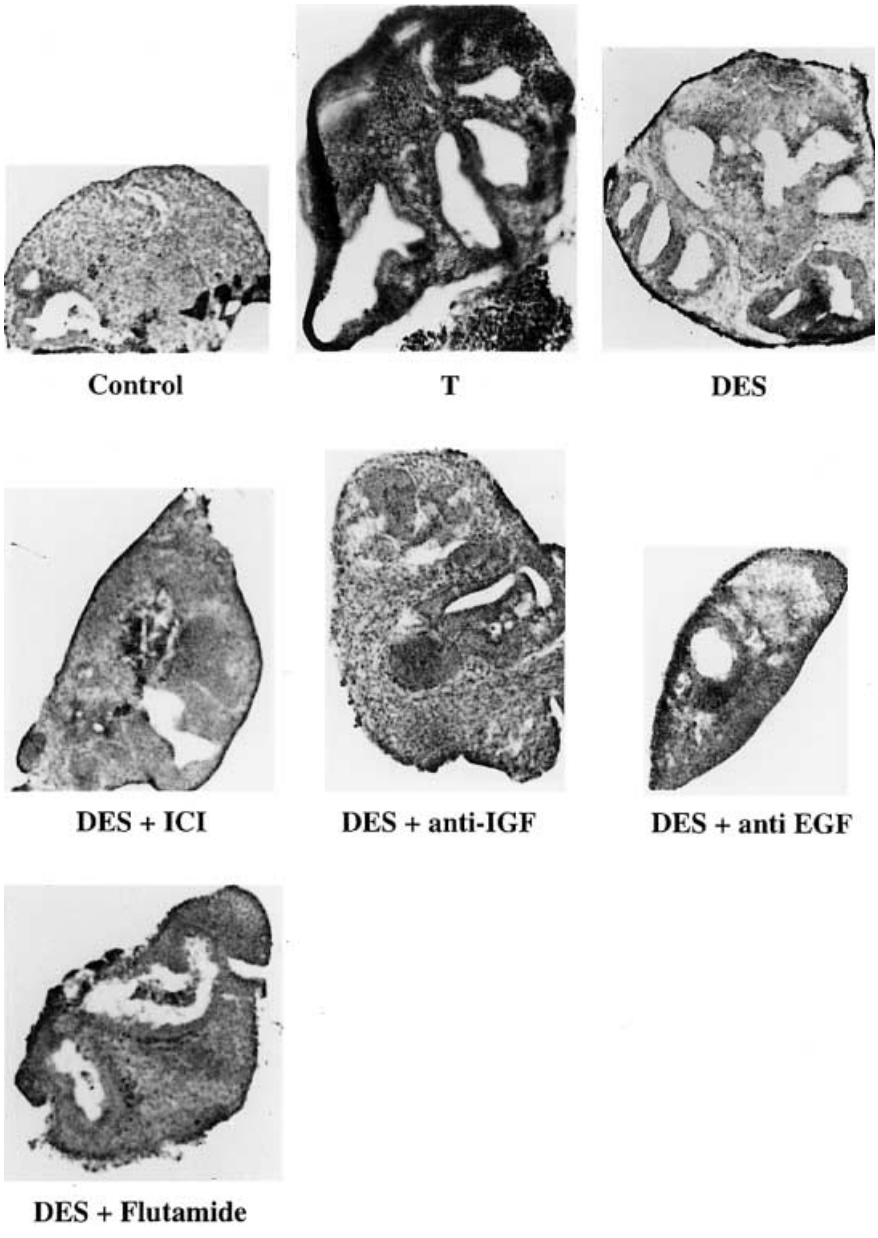

Fig. 2 Photo-microscopic appearance of the histologic sections of representative specimens from different experiments. *T* represents testosterone treatment at 10 ng/ml; *DES* represents DES treatment at 0.5 pg/ml; *Anti-IGF* represents treatment with 10 ug anti-IGF-1 antibody; *Anti-EGF* antibody represents treatment with 50 ug/ml of EGF antibody. *DES* plus *ICI* represents treatment with DES (0.5 pg/ml) plus ICI (1 ug/ml); DES plus Flut represents treatment with DES plus flutamide (10 ug/ml)

which these chemicals induce male reproductive malformations is not known. Although DES is a potent estrogenic compound [17], the effects were observed with extremely low doses of this compound. Therefore, it seems unlikely that estrogenic activity alone produced the adverse effects during fetal development. Some suspected endocrine disruptors have been shown to interact not only with the estrogen receptor (ER) but also with the androgen receptor (AR) or to interfere with steroid hormone synthesis or metabolism [18, 20]. Our observation that both anti-estrogen and anti-androgen inhibited DES-induced prostatic development suggests that both AR- and ER-mediated pathways are involved in inducing this malformation. It may be possible that these two pathways interact at some point to induce this permanent effect. One possibility for such an effect could be through some growth factor production. It has been reported that estrogenic chemicals enhance EGF and IGF-1 synthesis in the adult uterus [8, 9] and that EGF and IGF-1 enhance AR-mediated transcription activity [26]. Our findings that anti-EGF-1 and anti-IGF-1 blocked DES-induced prostatic development and that DES enhances EGF synthesis suggest that these two growth factors may link the AR-mediated and ER-mediated pathways during DES-induced prostatic malformation.

The results described here using ICI suggest a role for ER in mediating the DES effect. However, there are two ER subtypes [25] in the prostate and the phytoestrogens were shown to have a higher binding affinity for the ER beta protein [2]. At this time, it is not known whether ER alpha or ER beta or both is involved in inducing malformation. The estrogenic potency is another issue. The estrogenic potency is dependent on a number of factors, such as differential effects on the transactivation

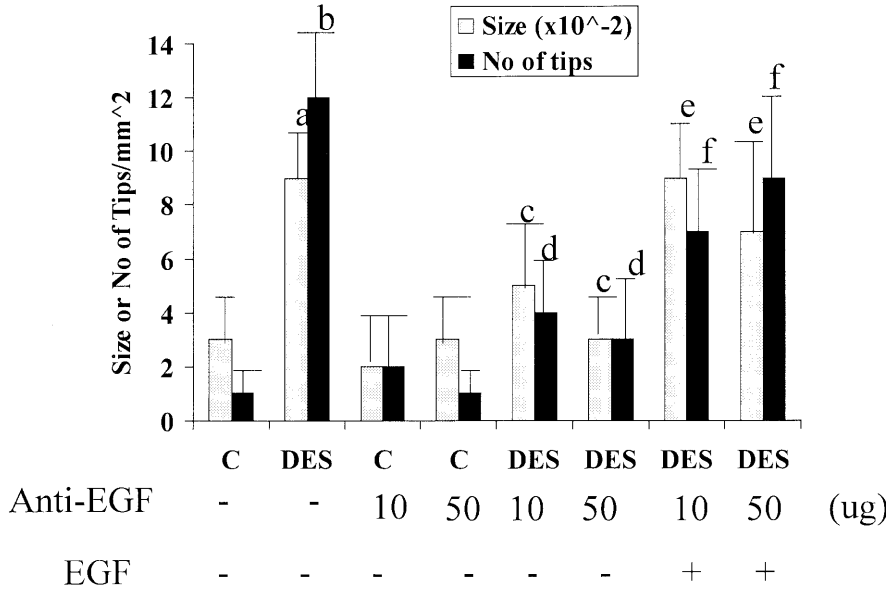

Fig. 3 Effect of anti-EGF antibody on the DES-induced prostatic development in vitro. *C* represents the control (4 specimens). DES represents the specimens exposed to 0.5 pg/ml of DES (7 specimens). The effect of anti-EGF antibody was challenged with EGF (100 ng/ml, 6 specimens). The experiment was repeated four times and the results were analyzed by an ANOVA test. The data represent mean ± SD; *a* and *b* $P < 0.05$ compared to the control; *c* and *d* $P < 0.05$ compared to DES-treated controls; *e* and *f*, $P < 0.05$ compared to DES plus anti-EGF antibody. The symbol mm^2 represents mm^2

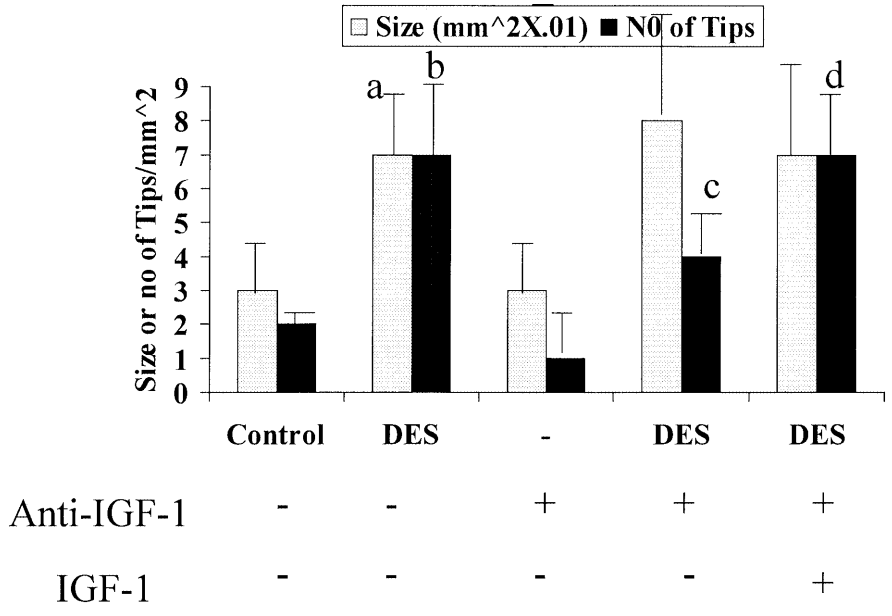

Fig. 4 Effect of anti-IGF-1 antibody on the development of prostate in vitro. *DES* represents the specimens exposed to 0.5 pg/ml of DES (6 specimens). Anti-IGF-1 antibody was added at 10 ug/ml (4 specimens) and this was challenged with 50 ng IGF-1/ml (4 specimens). The experiment was repeated three times and the results were analyzed by an ANOVA test. The data represents mean ± SD; *a*, *b* $P < 0.05$ compared to the control; *c* $P < 0.05$ compared to DES-induced branching; *d* $P < 0.05$ compared to branching induced by DES and anti-IGF-1. The symbol mm^2 represents mm^2

functionalities of the receptor, the particular coactivator recruited and cell- and target-gene promoter context [4]. The apparently lower transactivational activity of ER beta compared with ER alpha has also been reported in transient transfection experiments using different cell lines [5]. In contrast, in human endometrial cells, the transcription activity of ER beta was higher than that of ER alpha [31]. The reason for these differences in transcription activity of ER subtype is at the moment unknown, but it might reflect differential expression of transcription coactivators or differential stability of the receptor protein [4]. At the present it is not known whether ER alpha or ER beta or both are involved in inducing the malformation. Additionally, it remained to be determined whether any of the co-activators for these receptors are induced by DES treatment.

Prostatic development is an AR dependent process [7]. The blockade of DES-induced prostatic branching and growth by flutamide proves that point. Cyproterone acetate, however, was partially effective in blocking the DES-effects. Cyproterone acetate blocked prostatic branching but was unable to block the stromal growth induced by DES. This observation can be explained in two ways (1) cyproterone acetate is not a very specific anti-androgen and (2) stromal growth induced by DES is not an AR-mediated effect. There is support for the first possibility as cyproterone acetate was shown to have some progestational activity [3]. Further studies using prostatic cells transfected with and without AR would evaluate the role of AR in the DES-induced prostatic malformation more conclusively.

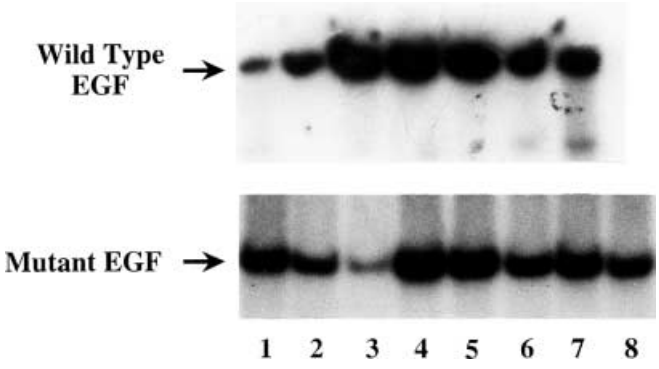

Fig. 5 RT-PCR amplification of EGF RNA in DES and bisphenol A treated 17 day old fetal prostates in culture. Polyacrylamide analysis of the PCR products: *Lane 1, 2*: control; *Lanes 3, 4, 5*: DES (0.5 pg/ml); *Lanes 6, 7*: Bisphenol A (50 pg/ml), *Lane 8*: mutant EGF product alone. The *upper band* represents the amplimer from the wild type EGF RNA and the *lower band* represents that from the mutant EGF RNA. The average results of the densitometric analysis of these products from different specimens are as follows: Control: 4.9; DES: 15.7; and Bisphenol A: 10.2

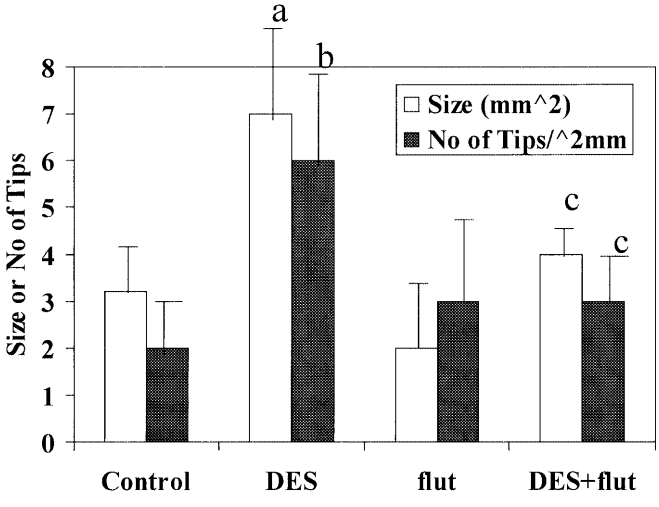

Fig. 6 Effect of flutamide on DES-induced prostate development. Flut represents flutamide (10 ug/ml; 4 specimens); DES represents 0.5 pg/ml DES (4 specimens). The experiment was repeated twice. The data represent mean ± SD; *a*, *b* $P < 0.05$ compared to the control; *c* $P < 0.05$ compared to the DES control. The symbol mm^2 represents mm^2

In summary, we have shown previously [12] that DES induces prostatic enlargement both in the presence and absence of testosterone in culture, thus replicating the in vivo effect of DES. Current findings confirm the DES effects in the absence of testosterone in culture and demonstrate that the DES-effects require the presence of ER, EGF, IGF-1 and AR. It may be possible that EGF/IGF synthesized by ER-dependent effect of DES mediate prostatic enlargement and enhanced AR functions. Further studies are needed to explore this speculation.

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