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Neonatal treatment of rats with diethylstilboestrol (DES) induces stromal-epithelial abnormalities of the vas deferens and cauda epididymis in adulthood following delayed basal cell development

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Abstract

This study investigated whether transient, neonatal (days 2–12) treatment of rats with the potent oestrogen, diethylstilboestrol (DES), altered the structure of the cauda epididymis/vas deferens in adulthood, and if the changes observed related to altered development of basal cells in early puberty. Neonatal treatment with 10 μg DES resulted in the following during adulthood: (a) coiling of the normally straight initial vas deferens, (b) gross epithelial abnormalities, (c) 4-fold widening of the periductal non-muscle layer, (d) infiltration of immune cells across the epithelium into the lumen, and (e) reduction/absence of sperm from the vas deferens lumen. Amongst affected animals >75% exhibited reduced epithelial immunoexpression of androgen receptor and aberrant oestrogen receptor-α immunoexpression and 63% exhibited multi-layering of basal cells coincident with increased epithelial cell proliferation. None of the aforementioned changes occurred in rats treated neonatally with 0.1 μg DES.

As basal cells play a key role in the development of epithelia such as that in the epididymis and vas deferens, we went on to investigate if neonatal DES treatment affected basal cell development. In controls, basal cells were first evident at day 10 (vas deferens) or day 18 (cauda). Rats treated with 10 μg, but not those treated with 0.1 μg, DES, showed ~90% reduction ($P < 0.001$) in basal cell numbers at day 15 and day 18. This decrease coincided with gross suppression of testosterone levels; co-treatment of rats with 10 μg DES + testosterone maintained basal cell numbers at control levels at day 18. However, suppression of testosterone production (GnRH antagonist treatment) or action (flutamide treatment) did not alter basal cell numbers. It is concluded that neonatal exposure to high oestrogen levels coincident with reduced testosterone action results in abnormal changes in the adult cauda/vas deferens that are preceded by delayed differentiation of basal cells. These findings imply a role for androgens and oestrogens in basal cell development and suggest that this may be pivotal in determining normal epithelial (and stromal) development of the cauda/vas deferens.

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Introduction

It is well established that oestrogens, as well as androgens, can affect development of the component parts of the male reproductive system. This information has come from two main sources. First, studies involving the administration of oestrogens in fetal/neonatal life to various mammals, including humans (Arai *et al.* 1983, Toppari *et al.* 1996). Second, studies of animals in which one or both oestrogen receptors (ERα, ERβ) have been inactivated using transgenesis in mice (Couse & Korach 1999). The precise physiological role(s) played by oestrogens in development of the male reproductive system is still unclear, but the prevailing impression gained from the aforementioned studies is that excessive exposure to oestrogens, and consequent disruption of the androgen–oestrogen balance, can cause permanent malformations (Arai *et al.* 1983, Toppari *et al.* 1996, Prins *et al.* 2001*b*, Rivas *et al.* 2002) and predispose to further aberrant changes in later life (Cunha 2001, Cunha *et al.* 2001, Prins *et al.* 2001*b*).

The mechanisms via which brief oestrogen exposure during development can lead to permanent structural and functional changes to the male reproductive tract are still unclear. Studies in rats treated neonatally with diethylstilboestrol (DES), have shown impaired development of the

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epithelium and relative overgrowth of stromal tissue in the epididymis (Williams *et al.* 2000, Atanassova *et al.* 2001), vas deferens (Atanassova *et al.* 2001), seminal vesicles (Williams *et al.* 2001), and prostate (Prins *et al.* 2001*b*, Williams *et al.* 2001), during or soon after the cessation of treatment. These gross structural changes are associated with reduced expression of the androgen receptor (AR; Prins 1992, Prins & Birch 1995, Williams *et al.* 2000, 2001, McKinnell *et al.* 2001), and with induction of abnormal expression of ERα (Atanassova *et al.* 2001, Prins *et al.* 2001*b*, Williams *et al.* 2001). These changes in receptor expression appear to involve a degree of 'reprogramming', at least in the prostate, as they are still evident in adulthood despite the restriction of oestrogen treatment to the neonatal period (Prins 1992, Prins & Birch 1995, Prins *et al.* 2001*b*, Risbridger *et al.* 2001*a*). The remarkable uniformity of oestrogen effects throughout the developing male reproductive tract suggests that there may be a common underlying mechanism that accounts for such changes. It is well-established that communication between the stromal and epithelial compartments of the developing reproductive tract are central to its normal development, and that stromal tissue from one region of the reproductive tract can be used to 'programme' development of epithelium from another region (Higgins *et al.* 1989, Donjacour & Cunha 1991, Hayashi *et al.* 1993, Aboseif *et al.* 1999). Moreover, experiments using recombination of epithelial and stromal prostatic tissue from ERα-knockout and ERβ-knockout mice have shown that ERα-mediated effects of DES on both the epithelium and mesenchyme are necessary for DES-induction of abnormal epithelial changes in adulthood (Prins *et al.* 2001*a*, Risbridger *et al.* 2001*a*).

One suggested mechanism for the DES-induced abnormal changes in adulthood in the prostate is altered development/programming of epithelial basal (p63-positive) cells, as over-proliferation of such cells is a common finding in adulthood following oestrogen treatment neonatally or in adulthood (Prins *et al.* 2001*a*, Risbridger *et al.* 2001*a*,*b*). It has been suggested that the basal cells exert a modifying influence on the overlying epithelial cells, which may in turn be a trigger for abnormal proliferation of epithelial cells during aging (Prins *et al.* 2001*b*, Risbridger *et al.* 2001*a*). Evidence for a key role of basal cells in development of the female reproductive tract has recently been reported (Kurita *et al.* 2004*b*). This study showed that neonatal DES treatment of female mice suppressed development of p63-positive (basal) cells in epithelium from the distal müllerian duct, and this resulted in development of columnar (uterine), instead of squamous (cervico–vaginal), epithelium in these regions; persistence of this change into adulthood probably explains how developmental DES exposure can induce adenosis (Kurita *et al.* 2004*b*). Whether oestrogen-induced changes to basal cell development occurs in male reproductive tract tissues that derive from the Wolffian duct, with comparable consequences for epithelial development to that in the müllerian duct, is unknown.

The aim of the present studies was therefore to establish if transient neonatal DES treatment of male rats resulted in gross structural and/or cellular changes to the epithelium and stroma of Wolffian-duct derived tissues (distal cauda epididymis and proximal vas deferens) in adulthood, that might be relevant to normal function and fertility. Having established that major structural and cellular changes were evident in such animals, we then sought for evidence of involvement of altered basal cell development earlier in life as a possible cause of such changes.

Materials and Methods

Animals, treatments, sample collection and processing

Wistar rats, bred in our own animal house, were maintained under standard conditions and were maintained on a soy-free diet (rat and mouse soya-free breeding diet; SDS, Dundee, Scotland). On the day after birth, rats from several litters were cross-fostered into all-male litters of 8–12 pups and these animals were then allocated to one of two to three different treatments ($n = 4/5$ per group) which were then treated by subcutaneous injection as follows:

(a) 10 μg or 0.1 μg DES (Sigma Chemical Co, Poole, Dorset, UK) in 20 μl corn oil on days 2, 4, 6, 8, 10 and 12; these doses were chosen on the basis of their differential ability to suppress testosterone levels and AR expression and to induce reproductive tract abnormalities neonatally, including effects on the epididymis and vas deferens (Atanassova *et al.* 2001, Williams *et al.* 2001, Rivas *et al.* 2002).

(b) 10 mg/kg of a long-acting GnRH antagonist (GnRHa, Teverelix; Ardana, Edinburgh, Scotland) in 20 μl corn oil on days 2 and 5 alone. This treatment regimen has been shown previously to suppress testosterone levels and testis weight at days 18–25 to a similar extent to that induced by treatment with 10 μg DES (Atanassova *et al.* 1999, Williams *et al.* 2001, Rivas *et al.* 2002).

(c) 10 μg DES plus 200 μg mixed testosterone esters (TE, Sustanon; Organon Labs, Cambridge, UK) in 20 μl corn oil on days 2, 4, 6, 8, 10 and 12; the dose of TE used has been shown previously to prevent most DES-induced abnormal changes to the developing male reproductive tract when co-administered with DES (McKinnell *et al.* 2001, Rivas *et al.* 2003).

(d) 50 mg/kg of the androgen receptor antagonist, flutamide (Sigma) in 20 μl corn oil on days 2, 4, 6, 8, 10 and 12. This dose was chosen based on studies by Imperato-McGinley *et al.* (1992) who showed that it caused major reproductive tract abnormalities in male offspring when administered to pregnant rats; we have shown that administration of this dose neonatally retards normal development of the

reproductive tract in rats (Atanassova *et al.* 2001, Rivas *et al.* 2002).

(e) Subcutaneous injection of 20 μl corn oil alone (control).

Rats from each of the treatment groups described above were killed on day 18 while some animals in groups (a) and (e) were also sampled on days 10, 15 or in adulthood (~day 90); for most treatment groups, 2 or 3 independent experiments were conducted and the data presented is amalgamated from these experiments. Animals were anaesthetized with flurothane, blood collected into a heparinised syringe by cardiac puncture and the animals then killed by cervical dislocation. Plasma was separated by centrifugation and stored at −20 °C until used for testosterone assay as described below. The right testis was dissected out and weighed whilst the left and right epididymides with the vas deferens still attached were fixed for ~5 h in Bouins. After fixation, tissue was transferred into 70% ethanol before being processed for 17.5 h in an automated Leica TP1050 processor (Leica Microsystems (UK) Ltd. Milton Keynes, UK) and embedded in paraffin wax. Sections of 5 μm thickness were cut and floated onto slides coated with 2% 3-aminopropyltriethoxy-silane (Sigma) and dried at 50 °C overnight before being used for the studies described below.

Immunohistochemistry

Antibodies used for immunohistochemistry, their dilutions and sources are listed in Table 1. Unless otherwise stated, all incubations were performed at room temperature for 30 min. Sections were deparaffinised in Histoclear (National Diagnostics, Hull, UK), rehydrated in graded ethanols and washed in water. For some antibodies, sections were subjected to a temperature-induced antigen retrieval step (Norton *et al.* 1994) in either 0.05 M Glycine, pH 3.5 and 0.01% EDTA (for CD45 and ERβ) or 0.01 M citrate buffer, pH 6.0 (for AR, ERα, CKHMW, p63 and Ki67). Sections were pressure-cooked for 5 min at full pressure, left to stand undisturbed for 20 min, then cooled under running tap water. At this stage and after all subsequent steps, sections were washed twice (5 min each) in Tris–buffered saline (TBS; 0.05 M Tris–HCl, pH 7.4, 0.85% NaCl). Endogenous peroxidase activity was blocked by immersing sections in 3% (v/v) H_2O_2 in methanol (both from BDH Laboratory Supplies, Poole, Dorset, UK). To block non-specific binding sites, sections were incubated with an appropriate normal serum diluted 1:5 in TBS containing 5% bovine serum albumin (BSA; Sigma). Goat serum was used for CD45, for AR swine serum was used and for all other antibodies rabbit serum was used (all from Scottish Antibody Production Unit, Carluke, Scotland). Primary antibodies were added to the sections (Table 1) and incubated overnight at 4 °C in a humidified chamber. For CD45 only, sections were then incubated with mouse EnVision-HRP system (Dako, High Wycombe, UK). For all other antibodies, a biotinylated secondary antibody was used, namely a 1:500 dilution in blocking mixture of swine anti-rabbit IgG (Dako) for AR, rabbit anti-sheep IgG (Vector Laboratories, Peterborough, UK) for Neutrophil Elastase (NE) and ERβ, or rabbit anti-mouse IgG (Dako) for the remainder, followed by incubation with avidin-biotin conjugated to horseradish peroxidase (ABC-HRP; Dako) diluted in 0.05 M Tris–HCl, pH 7.4, according to the manufacturer's instructions. Immunostaining was developed using 3,3′-diaminobenzidine (Liquid DABplus; Dako), according to the manufacturer's instructions, until staining in controls was well-developed, when the reaction was stopped by immersing all sections in distilled water. All sections were then lightly counterstained with haematoxylin, dehydrated in graded ethanols, cleared in xylene and mounted using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK).

To allow comparative evaluation of the immunostaining, sections of tissues from control and treated animals were processed in parallel on at least three occasions to ensure reproducibility of results; on each occasion tissue sections from four to six animals in each treatment group were run. Within each run, the intensity of immunostaining for each antigen was scored on an arbitrary scale ranging from negative (−) through weakly positive (+) to intensely positive (++++), and this was then used to compile a table of results. For all of the present immunohistochemical studies, appropriate negative controls (replacement of the primary antibody by appropriate normal serum or in the case of AR, preabsorption with the immunising peptide) were included in every run and were used to ensure that any staining observed was specific. In no instance did any of

Table 1 Antibodies used for immunohistochemistry.

Antibody	Type	Source	Dilution
α-Smooth muscle actin (SMA)	mouse mAb	Sigma, Poole, UK	1:5000
Pan-cytokeratin (PCK)	mouse mAb	Sigma	1:50
CD45 (leukocyte common antigen)	mouse mAb	BD Pharmingen, Oxford, UK	1:50
Neutrophil elastase (NE)	sheep IgG	ICN Pharmaceuticals, Basingstoke, UK	1:500
Androgen receptor (AR)	rabbit IgG	Santa Cruz Biotechnology, Santa Cruz, USA	1:200
Oestrogen receptor-α (ERα)	mouse mAb	Novocastra, Newcastle-upon-Type, UK	1:20
Oestrogen receptor-β (ERβ)	sheep IgG	Gift from Dr P Saunders (Saunders *et al.* 2000)	1:1000
Cytokeratin high molecular weight	mouse mAb	DAKO, High Wycombe, UK	1:100
p63	mouse mAb	Santa Cruz Biotechnology	1:500
Ki67	mouse mAb	Novocastra	1:50

the antibodies used present problems with non-specific staining using the specified protocols, which were carefully optimised prior to their use in experiments. More detailed validation of the specificity of several of the antibodies used in the present studies (AR, ERα, ERβ, Ki67) has been reported previously (Atanassova *et al.* 2001, McKinnell *et al.* 2001, Williams *et al.* 2000, 2001).

Double immunostaining for α-smooth muscle actin and cytokeratins

To delineate the structural changes induced by DES treatment, both smooth muscle and epithelial tissues were labelled immunohistochemically on the same sections. Following antigen retrieval in 0.01 M citrate buffer, pH 6.0, sections were immunostained for α-smooth muscle actin as described above. A biotinylated rabbit anti-mouse IgG secondary antibody (Dako) was used. After development of immunostaining with DAB as described above, some sections were again incubated with blocking mixture followed by application of mouse anti-pan cytokeratin antibody overnight at 4 °C. Sections were then incubated in rabbit anti-mouse IgG (Dako) at 1:60 dilution in blocking mixture, followed by application of mouse alkaline phosphatase anti-alkaline phosphatase (APAAP; Dako) diluted 1:100 in blocking mixture. Slides were washed in TBS and then in 100 mM Tris buffer, pH 9.5, containing 100 mM NaCl and 50 mM MgCl, followed by the addition of 337.5 μg/mL 4-Nitro blue tetrazolium chloride (Boehringer GmbH, Mannheim, Germany), 175 μg/mL 5-Bromo-4 chloro-3-indolylphosphate (Boehringer) and 0.001% levamisole (Sigma) in 10 ml Tris–MgCl buffer until immunostaining was optimal in control sections. Slides were very lightly counterstained in haematoxylin before being dehydrated rapidly in absolute ethanol, cleared in xylene, and mounted using Pertex mounting medium.

Immunostained sections were examined and photographed using an Olympus Provis microscope (Olympus Optical, Honduras Street, London, UK) fitted with a Kodak DCS330 camera (Eastman Kodak, Rochester, NY, USA). Captured images were stored on a G4 computer (Apple MacIntosh) and compiled using Photoshop 7.0.

Measurement of the width of the periductal muscle-free layer in the adult vas deferens and quantification of basal cell numbers on days 15 and 18

Tissue sections from adult rats treated neonatally with vehicle or with 10 or 0.1 μg DES were double immunostained for pan-cytokeratin (blue) and smooth muscle actin (brown), as described above, to clearly demarcate epithelial and periductal muscle tissues respectively (see Fig. 1). Using a ×20 objective, symmetrical cross-sectional profiles of the initial vas deferens were identified and images captured. Using NIH Image Proplus (Media Cybernetics, Silver Spring, MA, USA) the width of the periductal actin-negative layer, from the base of the epithelium to the edge of the muscle (actin-immunopositive) layer, was measured at 50 μm intervals along the edge of the vas deferens using the line tool; a total of 50 measurements were made for each animal and a mean value then computed for the width of the layer in each animal.

To quantify basal cells, tissue sections immunostained for p63 (to stain basal cell nuclei), as described above, were used. Sections from rats aged 15 and 18 days that had been subjected to various neonatal treatments were examined using a ×40 objective and symmetrical cross-sectional profiles of the initial vas deferens were identified and images captured. The numbers of p63-immunopositive cell nuclei were then counted along both sides of the vas deferens lumen within a measured length of the epithelium (length measured using the line tool in NIH Image with ×10 objective), and the number of basal cells per mm epithelium then calculated. All suitable cross sections were evaluated in this way (i.e. there was no selection) and the total length of epithelium that was evaluated per animal ranged from ~1.2–4 mm.

Measurement of plasma testosterone levels

Plasma levels of testosterone were measured using an enzyme-linked immunosorbent assay adapted from an earlier RIA method (Corker & Davidson 1981), as described previously (Atanassova *et al.* 1999, Rivas *et al.* 2002). The limit of detection was 8 pg/ml and the intra-assay coefficient of variation was 10.4%.

Statistics

Comparison of the width of the adult periductal layer of the vas deferens and of plasma testosterone levels and basal cell numbers at days 15 and 18 in the various treatment groups, was made using analysis of variance (ANOVA); data for testosterone levels were logarithmically transformed prior to analysis to obtain a normal distribution and to equalize group variances. Where significant differences between groups were indicated, sub-group comparisons also utilised ANOVA but used the variance from the experiment as a whole (for that parameter) as the measure of error. Comparison of the frequency of morphological changes in the adult vas deferens of control and DES-treated animals used Fisher's exact test.

Results

Phenotypic characterization of the epididymis and vas deferens in adult animals

Analysis of gross morphology and other parameters (below) revealed that some animals treated neonatally with 10 μg DES exhibited more severe changes than did other animals. To take account of this variation and to establish possible inter-relationships between phenotypic changes, each investigated parameter/phenotypic change was evaluated systematically in each animal in each treatment group, and wherever possible was quantified or

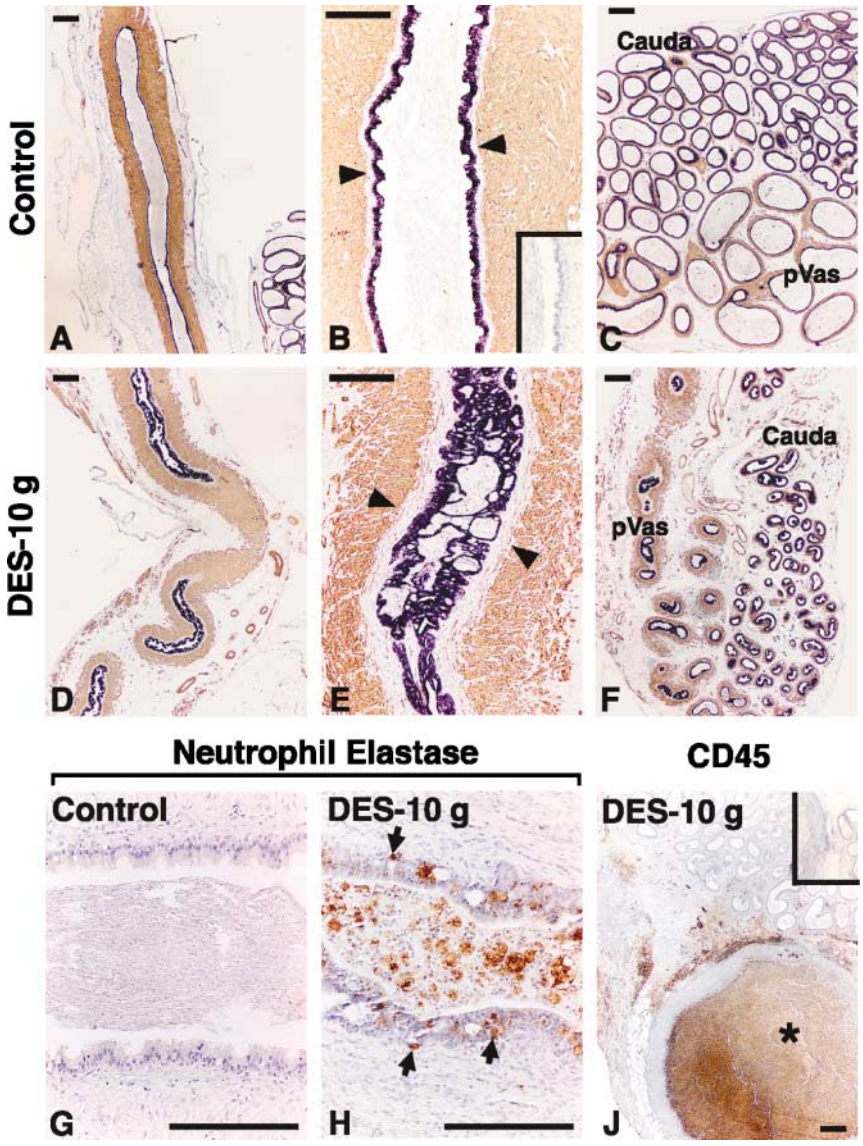


Figure 1 Representative gross morphology of the cauda epididymis and the initial part of the vas deferens in adult rats treated neonatally with vehicle (control) or 10 μg DES. Panels A–F illustrate sections double immunostained for pan-cytokeratin (blue) or for smooth muscle actin (brown) to demonstrate the relative morphology of the epithelium and periductal muscle layer, respectively, in controls (A–C) and in DES-treated animals (D–F). Note the relative under-development of the duct and epithelium of the cauda and proximal vas deferens (pVas deferens) in DES-treated animals (F) compared with controls (C) and the abnormal coiling of the initial part of the vas deferens (D) in the same animals (compare panels A and D). In the latter areas, note also the abnormal layering and branching of the epithelium, the widening of the periductal actin-free layer (arrowheads) and the break-up of the smooth muscle layer in DES-treated animals (E) compared with controls (B). Panels G–J illustrate the evidence for inflammatory changes in the initial part of the vas deferens in DES-treated animals (H, J) compared with controls (G) in sections immunostained for neutrophil elastase (G, H) or for CD45 (J). Note the invasion of neutrophils across the epithelium into the vas deferens lumen (arrows) in DES-treated animals (H), in several instances leading to formation of a large granuloma (asterisk, J). Insets in panels B and J show negative controls. Scale bars represent 500 μm.

semi-quantified (e.g. immunohistochemistry score); in some instances, it was simply recorded whether or not a particular phenotypic change was detectable (e.g. coiling of the initial vas deferens). A summary of the results and their statistical analysis is presented in Table 2.

Gross morphology of the adult vas deferens and cauda epididymis – DES induction of an inflammatory response

In controls, the vas deferens, as it emerged from the cauda epididymal 'bulb', was slightly coiled and then became a conspicuously straight duct (Fig. 1A and B). Similar morphology was evident in rats treated neonatally with 0.1 μg DES (Table 2) or with a GnRHa (not shown). However, in most rats treated neonatally with 10 μg DES the vas deferens exhibited conspicuous coiling of the normally straight initial region, so that it appeared snake-like (Fig. 1D; Table 2). To discern the underlying cause for this change, double-immunostaining of the vas deferens with antibodies to SMA and PCK was undertaken to demarcate stromal smooth muscle and epithelial tissue respectively (Fig. 1A–F). This demonstrated that the normal simple epithelium of the vas deferens evident in controls, with its conspicuous variations in height, was distorted in rats treated neonatally with 10 μg DES, such that the epithelium was relatively unvarying in height and multi-layered/branched (Fig. 1E), and was often so tortuously arranged that a simple duct-like profile was difficult to discern. Cyst-like pockets filled with liquid were frequently formed within the expanded epithelium (Fig. 1E). Another obvious change in rats treated with 10 μg DES was a 4-fold widening of the immediate periductal stromal cell layer (Table 2) which was immunonegative for SMA (comparison of Fig. 1B and E); this layer is probably composed mainly of undifferentiated fibroblast-like cells (see Atanassova *et al.*

Table 2 Summary of the main abnormal changes observed in the adult vas deferens of rats following their neonatal treatment with either corn oil (control) or diethylstilboestrol (DES).

	Neonatal treatment[a]		
Phenotype/parameter	Corn oil ($n = 8$)	10 μg DES ($n = 14$)	0.1 μg DES[g] ($n = 8$)
Coiling of proximal vas deferens	0[b]	11*	0
Width of periductal non-muscle layer (μm)[c]	15.4 ± 1.6	68.1* ± 7.8	12.1 ± 1.2
Abnormal epithelium[d]	0	12*	0
Numerous (+++) sperm in vas lumen	8	2*	8
Immune cell infiltration[e]	0	11*	0
Intense (++++) epithelial AR immunoexpression	8	6 $p = 0.018$	8
Occurrence of epithelial ERα immunoexpression	0	8 $p = 0.018$	0
Basal cells multi-layered[f]	0	5 $p = 0.115$	0
High frequency (+++) of Ki67-immunopositive cells	0	7 $p = 0.023$	0

[a]Treatments administered on alternate days between day 2 and day 12; [b]number indicates how many animals in this group exhibited the phenotype/parameter listed on the left; [c]see Fig. 1F for illustration; [d]branching and/or cystic in regions (see Fig. 1F for illustration); [e]based on immunostaining for neutrophils and CD45-immunopositive cells in stroma, epithelium and/or duct; see Fig. 1H for illustration; [f]based on immunoexpression of cytokeratin-HMW and p63; see Fig. 2 for illustration; [g]none of the parameters in this group differed from controls. *$p < 0.001$.

2001). In association with this change there was peripheral displacement of smooth muscle and the normally homogenous and continuous muscle layer was interspersed with SMA-immunonegative fibroblastic stroma (Fig. 1E). The latter two changes probably explain the abnormal coiling of the vas deferens in rats treated with 10 μg DES. In addition to these morphological abnormalities in the vas deferens, the rats also exhibited conspicuous structural changes to the cauda epididymis, similar to those described earlier (Atanassova *et al.* 2001), the main features being under-development of the epithelium relative to the stroma (comparison of Fig. 1C and F).

Another major change in rats treated neonatally with 10 μg DES was a reduction in numbers of sperm in the lumen of the vas deferens. In controls, the lumen always contained copious numbers of sperm (Fig. 1G) whereas most animals treated neonatally with with this dose had no sperm present or markedly reduced numbers of sperm (Table 2; see also Fig. 2). In the latter animals, the lumen exhibited cellular debris that included epithelial cells and leukocytes (CD45 positive cells), indicative of an inflammatory response (Table 2). Inflammation was characterized by the identification of neutrophils (immunopositive for NE), which were identified in the stroma as well as in the lumen and which appeared to migrate from the stroma through the epithelium with final accumulation in the lumen of the vas deferens (Fig. 1H; see also Fig. 2). In instances of very severe inflammation (4 of 14 animals), huge granulomas were found to occupy most of the caudal space (Fig. 1J).

Comparative evaluation of the above morphological abnormalities in individual animals (Table 2) demonstrated that those which exhibited an abnormal vas deferens epithelium also exhibited coiling of the vas deferens, a widened periductal layer, leukocyte infiltration and a lack of, or decrease in numbers of, sperm in the vas deferens lumen, suggesting that these changes are interrelated. None of these abnormalities were observed in adult rats that had been treated neonatally with a 100-fold lower dose (0.1 μg) of DES (Table 2) or in which neonatal suppression of testosterone levels had been induced by administration of a GnRHa (not shown).

Immunoexpression of AR, ERα and ERβ in the cauda epididymis and vas deferens in adulthood

In controls, AR was immunoexpressed intensely in epithelial cells and in sporadic stromal cells whereas no cells exhibited detectable immunoexpression of ERα (Fig. 2A and C). A similar pattern was found in rats treated neonatally with 0.1 μg DES (Table 2). In rats treated neonatally with 10 μg DES, there was variation between animals in the degree of disruption of the control pattern of AR immunoexpression (Table 2). In animals that exhibited an abnormal epithelium plus coiling of the vas deferens (Table 2), the changes were most marked and involved a pronounced reduction in intensity of AR immunoexpression in epithelial, but not in stromal, cells, coincident with induction of ERα immunoexpression in sporadic epithelial cells and in some periductal cells (Fig. 2B and D). In contrast, in the cauda epididymis, immunoexpression of AR and ERα remained unaffected by neonatal treatment with 10 μg DES (not shown). In animals treated neonatally with 10 μg DES, in which coiling of the adult vas deferens was not induced, AR immunoexpression was normal in intensity, and immunoexpression of ERα was not induced (not shown), suggesting that abnormal expression of AR and/or ERα are directly related to the occurrence of gross abnormalities. The intensity and distribution of immunoexpression of ERβ did not show any detectable treatment-induced change in the adult vas deferens (data not shown).

Basal cell distribution and cell proliferation (immunoexpression of Ki67) in the adult vas deferens

Basal cells were identified on the basis of immunoexpresion of cell-specific markers CK-HMW (cytoplasmic) and p63 (nuclear). In adult control rats, basal cells formed a thin, continuous, single-cell, layer along the base of the epithelium and beneath the principal cells of the vas deferens (Fig. 2E and G); a similar pattern was found in all animals treated neonatally with 0.1 μg DES and in most rats treated with 10 μg DES (Table 2). However, in approximately one third of rats treated neonatally with 10 μg DES, basal cells formed a multicellular layer in some regions of

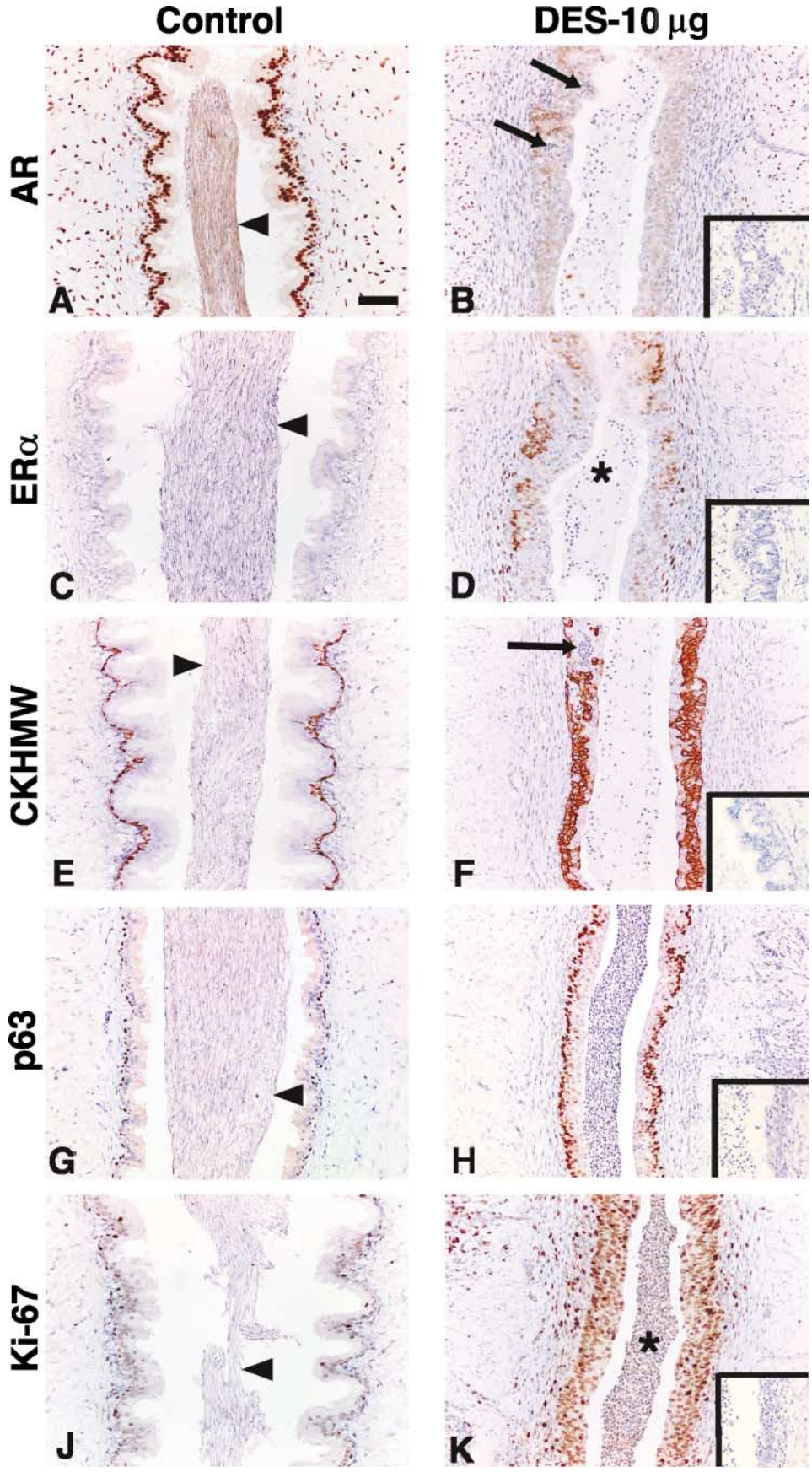


Figure 2 Representative changes in immunoexpression of the androgen receptor (AR; panels A, B) and oestrogen receptor-α (ERα; panels C, D) in relation to the distribution of basal cells (panels E–H) and cell proliferation (panels J, K) in sections from the initial part of the vas deferens from adult rats treated neonatally with vehicle (control) or 10 μg DES. Note the pronounced reduction in immunoexpression of AR in epithelial, but not stromal, cells in DES-treated rats (B) compared with controls (A) and the abnormal occurrence of sporadic ERα immunoexpression (compare panels C and D). Note also the abnormal multilayering of basal cells, identified by immunoexpression of cytokeratin high molecular weight (CKHMW; panels E, F) or p63 (panels G, H) in DES-treated rats compared with controls, and the association of this change with increased cell proliferation, based on immunoexpression of Ki67 (compare panels J and K). Finally, note that the large numbers of sperm present in the lumen of the control vas deferens (arrowheads) are largely replaced in DES-treated animals by immune cells (asterisks), which have probably infiltrated through the vas deferens epithelium (arrows). Insets in panels on the right show negative controls. Scale bar represents 100 μm.

the vas deferens that varied in cell number/depth independent of the overall height of the adjacent epithelium (Fig. 2F and H; Table 2); coincident with this change, there was a marked increase in the frequency of Ki67-immunopositive epithelial cells (Fig. 2J and K; Table 2), although the identity of these cells was not investigated. All adult animals that exhibited these basal cell abnormalities also exhibited disruption of the normal pattern of epithelial AR and ERα immunoexpression.

Ontogeny of basal cell development in the vas deferens (immunoexpression of CK-HMW and p63) and the effect of neonatal manipulation of androgen and oestrogen (DES) action

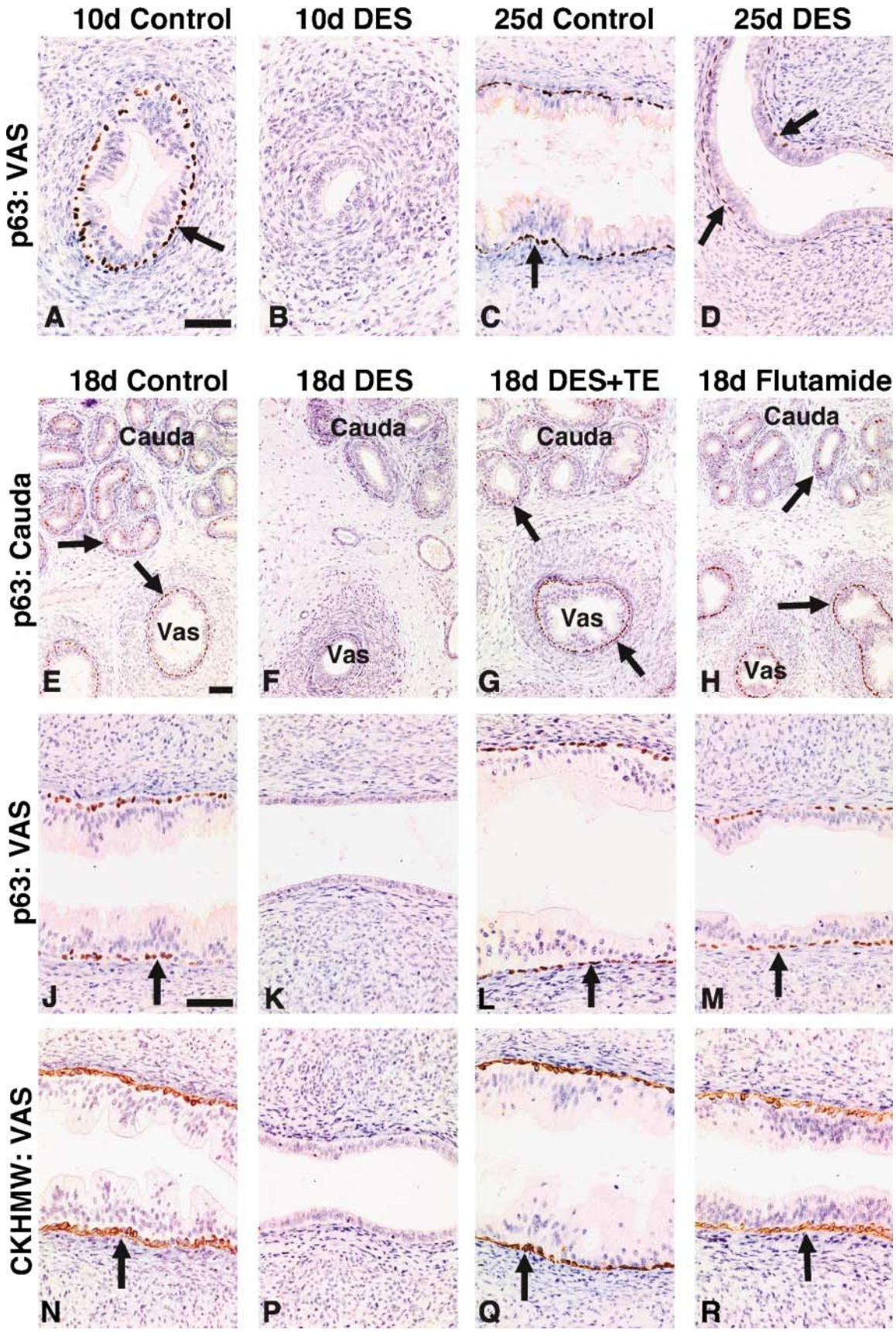
We next determined if altered basal cell development during neonatal life/early puberty might underlie the observed changes to the epithelium of the adult vas deferens in rats treated neonatally with 10 μg DES. In control rats, basal cells were first detectable at age 10 days in the region of the proximal vas deferens and onwards (Fig. 3A), and the relative numbers of immunopositive cells increased up to day 25, forming a single cell layer (Fig. 3E, C, J and N) which persisted into adulthood (Fig. 2); these findings are consistent with those reported by Hayashi *et al.* (2004). In addition to the continuous layer of basal cells in the proximal and distal vas deferens, CK-HMW/p63 immunopositive cells also appeared in the proximal cauda between days 10 and 18 but they did not form a continuous layer (Fig. 3E). Unexpectedly, neonatal treatment with 10 μg DES up to day 12 almost completely blocked the appearance of basal cells at 10 (Fig. 3B), 15 (Fig. 4) and 18 (Fig. 3F, K and P; Fig. 4) days of age in both the cauda and vas deferens (Fig. 4), and even at day 25 only sporadic basal cells were evident (Fig. 3D). Treatment with 10 μg DES also grossly suppressed testosterone levels (Fig. 4), as reported previously (Rivas *et al.* 2002, 2003). We tested if prevention of this decrease by co-administration of testosterone with 10 μg DES was able to restore normal numbers of basal cells at day 18, and this proved to be the case (Fig. 3G, L and Q; Fig. 4). However, neonatal treatment with GnRHa, which was equally as effective as 10 μg DES in lowering testosterone levels (Fig. 4), did not alter the normal development/numbers of basal cells in the cauda (not shown) and vas deferens (Fig. 4) and nor did neonatal treatment with the anti-androgen, flutamide (Fig. 3H, M and R; Fig. 4).

Discussion

The initial aim of this study was to investigate if neonatal treatment of rats with DES induced permanent changes in morphology and/or cellular composition of Wolffian duct-derived tissues (cauda epididymis, vas deferens) in adulthood. Our results show that transient neonatal treatment with a relatively high dose (10 μg) of DES, but not a 100-fold lower dose (0.1 μg), induces a wide spectrum of abnormal changes in most adult animals, including abnormal coiling of the vas deferens, gross abnormalities of the epithelium, abnormal stromal cell changes such as increase in thickness of the periductal fibroblast (muscle-free) layer and reduced epithelial immunoexpression of AR. Associated with these changes there were inflammatory changes and reduced amounts of sperm in the vas deferens lumen. Our findings provide new evidence that these changes may stem from reduction/delay in differentiation of basal cells during neonatal/early prepubertal life (10–25 days of age) coincident with reduced blood levels of testosterone. Our findings also demonstrate that deprivation of normal androgen stimulation during the neonatal period is essential, but not sufficient, for induction of the aforementioned changes; coincident over-exposure to high levels of oestrogens (as results from treatment with 10 μg dose of DES) at the time of androgen suppression is an absolute requirement for induction of delayed basal cell development and for induction of structural/cellular abnormalities in the cauda and vas deferens in adulthood. These observations further imply that (a) both androgens and oestrogens can affect basal cell development, and (b) that normal timing of basal cell development is of key importance in determining whether development of the cauda epididymis and vas deferens is normal or abnormal.

It is widely accepted that development of male reproductive tract tissues involves stromal–epithelial interactions and the action of androgens (Donjacour & Cunha 1991, Cunha *et al.* 1996). The latter are mediated by AR, expressed in epithelial and stromal cells. Our present finding, that reduced immunoexpression of AR in epithelial cells of the adult vas deferens coincided with the major cellular and structural abnormalities induced by neonatal treatment with 10 μg DES is therefore not surprising, and parallels similar findings in the adult prostate following neonatal oestrogen treatment (Prins & Birch 1995, Prins *et al.* 2001*a*,*b*). We have shown previously that even more pronounced loss of AR expression is found in the vas deferens and throughout the reproductive tract of DES-treated

Figure 3 Ontogeny of basal cells (arrows) in the distal cauda and proximal and initial vas deferens of rats treated neonatally with vehicle (controls), 10 μg DES, 10 μg DES + 200 μg testosterone (TE) or with the anti-androgen flutamide. Basal cells were identified by their immunoexpression of cytokeratin high molecular weight (CK-HMW; panels J-M) or p63 (all other panels). Basal cells were first detectable in the vas deferens of controls at day 10 (panel A), spreading to the cauda by day 18 (E). Treatment with 10 μg DES alone blocked the appearance of basal cells at day 10 (compare panels A and B) and 18 (compare panels E, J and N with F, K and P) until day 25 when some basal cells become apparent (C, D). Co-administration of TE to DES-treated animals resulted in normal appearance of basal cells at day 18 (G, L, Q) but treatment with flutamide did not prevent the appearance of basal cells at day 18 (H, M, R). Note also the general reduction in height of the epithelium of the vas deferens in animals treated with 10 μg DES alone and the loss of the natural variation in epithelial height seen in controls (e.g. compare panels J and K). Scale bars represent 100 μm.



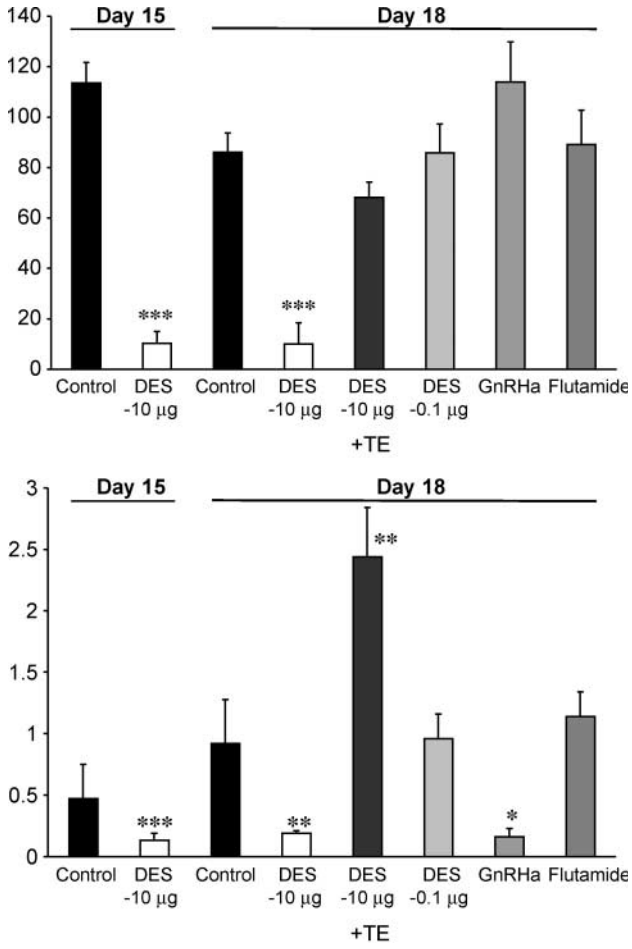


Figure 4 Effect of neonatal hormone treatment of rats on basal cell number in the epithelium of the vas deferens (top) in relation to plasma testosterone levels (bottom) at age 15 and 18 days. Note that although GnRHa reduces testosterone levels to a similar extent as treatment with 10 μg DES, it does not reduce basal cell numbers and nor does flutamide treatment. Values are means ± S.E.M. for 4–6 animals per group, except $n = 3$ for GnRHa group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, in comparison with respective control group.

animals in neonatal life and in early puberty, and this loss is causally linked to the abnormal structural changes that are seen at this time (McKinnell *et al.* 2001, Williams *et al.* 2001, Rivas deferens *et al.* 2003). Altered androgen action, both during development and in adulthood, is thus an integral feature of DES-induced reproductive tract abnormalities in the male. Recent findings indicate that the DES-induced reduction in AR expression occurs not through altered AR gene regulation but from increased proteosomic degradation of the AR protein (Woodham *et al.* 2003). Moreover, the remarkable similarity between the changes induced in the adult vas deferens by neonatal DES treatment (10 μg), as shown presently (widening of periductal fibroblast layer, loss of AR, induction of ERα, inflammatory changes), and those induced in the prostate by neonatal oestrogen treatment (Chang *et al.* 1999*a*,*b*, Prins *et al.* 2001*a*, Risbridger *et al.* 2001*a*,*b*, 2003), strongly supports the view that similar regulatory mechanisms operate to regulate normal growth and development in tissues that derive from the Wolffian duct as those that derive from the urogenital sinus. The present findings suggest that a key event in this process may be the timing of differentiation of basal (p63-immunopositive) cells.

The p63 protein is widely expressed in developing epithelia and up- or down-regulation of its expression by transgenesis results in abnormal development of stratified epithelial tissues in numerous organs (e.g. Daniely *et al.* 2004, Westfal & Pietenpol 2004), including in the reproductive tract (Ince *et al.* 2002, Kurita *et al.* 2004*a*). In some situations, over-expression of p63 may lead to oncogenic changes in the neighbouring epithelium (Westfal & Pietenpol 2004). Based on the rapidly growing literature for p63, it appears that its time-and cell-specific expression in epithelial basal cells is an essential prerequisite for normal development and function of the overlying epithelial cells, as for example in the prostate (Kurita *et al.* 2004*a*) and uterus/vagina (Kurita *et al.* 2004*b*). The reduction/delayed appearance of (p63-immunopositive) basal cells during puberty in the vas deferens and cauda of all 10 μg DES-treated rats, as shown in the present study, is thus a novel finding of potential importance. This delay is associated with under-development of the overlying epithelium (Atanassova *et al.* 2001, Rivas deferens *et al.* 2002) and reduced proliferation of epithelial cells (Atanassova *et al.* 2001) in early puberty (18–25 days of age) as well as subsequent occurrence of the various epithelial and stromal abnormalities in the adult cauda and vas deferens, that are presently reported. Though these associations do not prove definitively that the various abnormalities are a direct consequence of the abnormal timing of basal cell development in DES-treated animals, such a conclusion would fit with the other literature cited above. In this regard, it is also pertinent to address the origin of basal cells in the epididymis and vas deferens, which remains a matter for debate. Some evidence supports the view that basal cells may be modified immune cells that migrate into these tissues from outside (e.g. Holschbach and Cooper 2002). Our findings do not allow us to determine whether it is altered migration or delayed differentiation (i.e. switching on of p63 and CK-HMW) of basal cells *in situ* that accounts for the delay in appearance of p63-immunopositive cells in the epididymis and vas deferens after neonatal treatment with 10 μg DES.

Our findings also show that delayed development of basal cells only occurred in treatment groups in which testosterone levels/action were suppressed neonatally coincident with increased oestrogen (i.e. DES) action. Neonatal treatments that suppressed just androgen production (treatment with a GnRH antagonist) or action (treatment with flutamide) or which increased oestrogen (DES) action without suppressing testosterone levels (treatment with 10 μg DES), did not induce any delay in basal cell development and nor did these treatments result in observable abnormalities of the cauda/vas deferens in adulthood

(present study plus unpublished data by the authors of this paper). Furthermore, co-administration of testosterone to rats treated neonatally with 10 μg DES, was able to prevent the changes to basal cell development that this dose of DES induced when administered on its own, confirming that impaired androgen action is a prerequisite for DES-induction of delayed basal cell differentiation. These findings thus fit with our earlier conclusion that it is disruption of the normal androgen–oestrogen balance during neonatal life which mediates the DES-induced reproductive tract abnormalities in the male (McKinnell *et al.* 2001, Rivas *et al.* 2002, 2003), and parallel findings in the prostate strongly support such a view (Prins 1992, Prins & Birch 1995, 1997, Prins *et al.* 2001*b*).

Another change induced in the vas deferens and epididymis by neonatal treatment with high doses of DES that is manifest both during development (Atanassova *et al.* 2001, Williams *et al.* 2001) and in adulthood (present study) is aberrant expression of ERα in epithelial cells of the vas deferens. Similar up-regulation of ERα has been reported in the adult prostate of rats treated neonatally with oestrogens (Prins & Birch 1997, Prins *et al.* 2001*a*). In both the vas deferens (present studies) and adult prostate (Prins & Birch 1997, Prins *et al.* 2001*a*, Risbridger *et al.* 2001*b*), this aberrant expression of ERα is associated with epithelial abnormalities. In contrast, expression of ERβ in the vas deferens remained unchanged in both young (Atanassova *et al.* 2001) and adult rats (present study) after neonatal DES treatment, and similar findings have been reported for the prostate (Prins *et al.* 1998). Recombination studies using tissues from ER knockout mice (αERKO and βERKO) (Risbridger *et al.* 2001*a*), as well as *in vivo* experiments with neonatal treatment of these mutants (Prins *et al.* 2001*a*), have also shown that ERα, but not ERβ, mediates the acute and chronic pathological responses to developmental oestrogen treatment. Similar conclusions have been reached regarding mediation of adverse DES effects on the developing female reproductive system (Couse *et al.* 2001). The present findings on the vas deferens, as with earlier findings on the prostate, therefore show that down-regulation of AR and up-regulation of ERα coincides with epithelial hyperplasia and associated basal (p63-positive) cell multilayering. However, of potentially more significance is the relationship between delayed basal cell development at day 18, as observed presently, and the aberrant expression of ERα in epithelial cells of the vas deferens at the same age (Williams *et al.* 2001) in rats treated with 10 μg DES. A similar inhibitory effect of neonatal DES treatment on the appearance of p63-positive cells in müllerian duct-derived epithelium was reported recently in female mice and was shown to be mediated via ERα, and this finding and others allowed the authors to conclude that p63 played a key role in directing differentiation of müllerian duct-derived cells into squamous (vaginal) as opposed to columnar (uterine) epithelium (Kurita *et al.* 2004*b*). Similar evidence for a key role of basal cells in regulating differentiated functions of the overlying epithelial cells is emerging for the prostate (Kurita *et al.* 2004*a*). Taken together with our present findings, these results suggest that an abnormal profile of androgen-oestrogen action during the period when epithelial basal cells are differentiating (i.e. expressing p63) in epithelia of the reproductive ducts, may be a pivotal change that leads to permanent structural abnormalities in the corresponding epithelium in adulthood.

In the present study, nearly all of the adult rats treated neonatally with 10 μg DES exhibited abnormalities of the stromal tissue surrounding the duct of the vas deferens; these changes included dramatic widening of the periductal fibroblast (non-muscle) layer in both the cauda epididymis and vas deferens and disruption of the smooth muscle layer itself by interspersion with actin-negative (fibroblastic) cells; similar changes were found during early puberty in DES-treated rats (Atanassova *et al.* 2001). Changes in periductal stroma similar to those shown presently have also been reported in the prostate of adult mice treated neonatally with DES (Chang *et al.* 1999*a*,*b*, Prins *et al.* 2001*a*) as well as in adult hypogonadal mice implanted with oestradiol (Bianko *et al.* 2002). This permanent thickening of the periductal fibroblast, non-muscle layer may create a physical barrier that obstructs normal paracrine communications between stroma and epithelium (Chang *et al.* 1999*b*). It also seems likely that the DES-induced structural changes in the periductal stroma in the current study are responsible for the abnormal coiling of the vas deferens that was found, as these changes coincided in all but one animal. Whether these changes play any role in delayed basal cell development, as found in the present study, is unknown, but it has been suggested that changes in smooth muscle, with consequent changes in two-way signalling with the neighbouring epithelium, may be pivotal in regulating epithelial proliferation and malignant transformation in the prostate (Cunha *et al.* 1996, 2003).

Abnormal development of epithelial and stromal components of the vas deferens was associated in adulthood with an apparent reduction in the number, or lack, of sperm in the lumen of the vas deferens in 85% of animals treated with 10 μg DES in the present study, consistent with previous demonstration of reduction in testis size, sperm production and impaired fertility in such animals (Atanassova *et al.* 2000). This lack of sperm was accompanied by infiltration of neutrophils and CD45 positive cells into the lumen of the vas deferens. Based on the distribution of such cells, infiltration occurred from the stroma through the epithelium into the lumen. Our study provides the first evidence for leukocytic inflammation in the cauda epididymis and vas deferens in adult rats as a consequence of neonatal DES treatment, but similar findings have been reported in the adult prostate (Stoker *et al.* 1999, Prins *et al.* 2001*a*), and recent data indicates that prolactin may play a role in this change (Gilleran *et al.* 2003). In the adult rat there is an epithelial barrier at the

level of the tight junctions between adjacent principal cells in all regions of the epididymis (Robaire & Hermo 1988), and basal cells (which also express macrophage antigens; Seiler *et al.* 1999, 2000) are considered to play a role in minimizing interaction of sperm autoantigens with the immune system (Seiler *et al.* 1999, 2000). The present findings suggest that these mechanisms are disrupted in the vas deferens and cauda epididymis of adult rats after neonatal DES treatment, possibly because of the abnormal epithelial structure and/or the delayed appearance of basal cells (see Holschbach and Cooper 2002). Coexistence of immune cell infiltration and epithelial abnormalities/hyperplasia in the present study is similar to the positive correlation between inflammatory pathology and benign hyperplastic changes reported in the rat and human prostate (De Marzo *et al.* 1999, Putzi & De Marzo 2000, van Leenders *et al.* 2003).

Together with our previous studies showing that neonatal administration of high doses of DES caused permanent reprogramming of the hypothalamic–pituitary–testis axis (Atanassova *et al.* 1999), the current study of DES-induced changes in the vas deferens at puberty and in adulthood provide strong evidence that neonatal exposure to oestrogens (and associated suppression of androgen action) permanently alters the cellular composition of the male reproductive tract, resulting in aberrant growth, differentiation defects and reduced responsiveness to androgen. Delayed appearance of basal cells and/or expression of p63 during puberty may underlie these changes that are evident in adulthood. The present study also demonstrates several similarities between DES-induced changes in the epithelium and stroma of the vas deferens (derived from the Wolffian duct) and those reported in the prostate (derived from the urogenital sinus), reinforcing the view that common hormonal and cellular mechanisms may operate throughout the male reproductive tract to regulate its development and function.

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