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Expression of c-*fos* and c-*jun* protooncogenes in the uteri of immature mice neonatally exposed to diethylstilbestrol

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Summary. We studied the cell-type-specific and temporal expression of c-*fos* and c-*jun* protooncogenes after 17ß-estradiol (E_2) stimulation in the uteri of immature 3-week-old mice neonatally exposed to diethylstilbestrol (DES), DES-mice, and the ontogenic expression of these genes in the uteri of DES-mice using immunohistochemistry and *in situ* hybridization. A single E_2 injection induced the transient and rapid expression of c-*fos* mRNA and c-Fos protein in the endometrial epithelium and endothelial cells of the blood vessels in both 3-week-old vehicle-treated controls and DES-mice; a peak of mRNA expression was 2 hours after E_2 injection and that of protein expression was 2 to 3 hours after the injection. The expression of c-*fos* mRNA and protein after E_2 stimulation was lower in the DES-mice than in the control animals. There were no significant differences in the c-*jun* expression patterns in both experimental groups before and after the E_2 injection. The E_2 injection transiently down-regulated the c-*jun* expression in the epithelium and up-regulated it in the stroma and myometrium. The uterine epithelium of DES-mice showed much stronger c-Jun immunostaining on days 4 and 10, compared with those of controls. Neonatal DES treatment reduced c-Jun immuoreactivity in the uterine epithelium on days 4 and 10, and increased the reaction in the stroma on day 4. These results suggested that the neonatal DES treatment induces permanent changes in the c-*fos* expression pattern independent of the postpuberal secretion of ovarian steroids. The changes in the expression of c-*fos* and c-*jun* protooncogenes, particularly during postnatal development, are likely to play important roles in the production of uterine abnormalities in the DES-mice.

Key words: c-Fos, c-Jun, Diethylstilbestrol, Uterus, Immature mouse

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Introduction

Estrogen treatment of prenatal or neonatal female mice is associated with long-term effects, including preneoplastic and neoplastic changes in the vaginal, cervical and uterine epithelium (Bern et al., 1975; McLachlan et al., 1975; Takasugi, 1976; Forsberg, 1977). Estrogen stimulates cell proliferation and differentiation in the female reproductive organs through its nuclear receptors, known as estrogen receptor (ER). Recent studies have demonstrated that mice, rats and humans have two types of ER: ERα (classical type) and ERß (recently cloned type). ERα is the major type of ER in the female genital tract of rodents (Couse et al., 1977; Kuiper et al., 1997).

Hormone-occupied ERs regulate the transcription of target genes by binding to their estrogen-responsive elements (EREs). Furthermore, liganded ERs have also been shown to modulate the transcriptional activity of activator protein-1 (AP-1) transcription factors, dimers of Fos and Jun family proteins, that bind to AP-1 enhancer elements (Gaub et al., 1990; Paech et al., 1997). Recent studies have indicated that the expression of c-*fos* and c-*jun* protooncogenes is directly controlled by estrogen in the uterus of rodents (Loose-Mitchell et al., 1988; Weisz and Bresciani, 1988; Kirkland et al., 1992; Bigsby and Li, 1994; Nephew et al., 1995; Yamashita et al., 1996). In the uterus of ovariectomized adult rodents, estrogen transiently activated c-*fos* expression in the epithelial cells and stimulated c-*jun* expression in stromal and myometrial cells but suppressed c-*jun* expression in epithelial cells (Bigsby and Li, 1994; Nephew et al., 1995; Yamashita et al., 1996). The expression patterns of c-Fos and c-Jun proteins should play an important role in the morphogenesis and epithelial characteristics of the uterus in normal and neonatally estrogen-exposed mice since the long-term expression of c-Fos proteins has been shown to cause a loss of epithelial polarity irreversibly and to trigger epithelial-fibroblastoid cell conversion (Reichmann et al., 1992), while an excess

expression of c-Jun proteins induces a loss of polarity in mammary epithelial cells (Fialka et al., 1996).

We demonstrated that neonatal exposure to diethylstilbestrol (DES), a synthetic estrogen, elicited alterations in the expression patterns of c-*fos* and c-*jun* in the uteri of 12-week-old mice ovariectomized at the age of 10 weeks (Yamashita, 2001a; Yamashita et al., 2001). The c-*fos* mRNA expression before E_2 stimulation (at baseline) was about 2-fold higher and the c-*jun* mRNA level was slightly lower in the uteri of neonatally DES-exposed mice (DES-mice), compared with vehicle-treated control animals. However, the uteri of DES-mice had a lower sensitivity to c-*fos* and c-*jun* expression after E_2 administration than those of controls. Similar results concerning c-*fos* expression were reported by Kamiya et al. (1996) for the uteri of DES-mice.

The incidence of serious abnormalities, including adenocarcinoma, in the genital tract of female DES-mice was shown to increase in a time- and dose-related manner and to depend on postpubertally-secreted estrogens (Newbold et al., 1990). In this study, we examined the cell-type-specific and temporal expression of c-*fos* and c-*jun* protooncogenes after E_2 stimulation in the uteri of immature 3-week-old DES-mice which do not secrete endogenous estrogens, and the ontogenic expression of these genes in the uteri of DES-mice using immunohistochemistry and *in situ* hybridization to verify whether the altered expression of these genes is imprinted during neonatal development, including the period of DES-treatment, or is mainly induced by postpuberal endogenous estrogen exposure.

Materials and methods

Animals and tissue collection

Pregnant CD-1 mice were obtained from Clea Japan Inc. (Tokyo). Mice were housed at 21 to 22 °C with a 12-hour alternating light-dark cycle at Keio University Animal Facility, Tokyo, Japan. All animals were maintained and treated according to protocols approved by the Keio University Animal Care Committee. Mice were subcutaneously injected with 4 μg of DES (Sigma Chem., St. Louis, MO) dissolved in 0.02 ml of sesame oil or with the vehicle alone for 5 days, starting within 24 hours after birth. At the age of 3 weeks, the animals received an intraperitoneal injection of 0.1 ml of 17ß-estradiol (E_2, 20 μg/kg.bw; Sigma Chem.) and were sacrificed at 0 (without treatment, baseline), 1, 2, or 3 hours after the injection; six or seven animals of each experimental group were killed. In addition, six or seven animals were killed on day 4 (after 3 injections), and days 10 and 15, respectively, without receiving the E_2 injection.

Immunohistochemistry

The immunohistochemistry of c-Fos and c-Jun has been previously reported (Yamashita et al., 1996). Small pieces of the uterine horn were mounted in OCT compound and frozen in dry ice-cooled acetone. Frozen sections (6 μm thick) were fixed with Zamboni's fixatives for 30 minutes at room temperature. After treatment with 0.2% glycine in phosphate-buffered saline (PBS) and successively with 10% normal goat serum and 1% bovine serum (BSA) in PBS, the sections were incubated with anti-c-Fos rabbit antibody (Oncogene Sciences Inc., MA) or anti-c-Jun rabbit antibody (Oncogene Sciences), each at a 50-fold dilution, overnight at 4 °C. The sections were then treated with horseradish peroxidase-linked $F(ab')_2$ fragments (Amersham Life Sciences, UK), 100-fold dilution, for 2 hours at room temperature. For the control, antibodies absorbed with the peptides used as immunogens were employed (Yamashita et al., 1996). For the controls, normal rabbit IgG or antibodies pre-absorbed with the peptide antigens were employed as the primary antibodies; S-14-C or T-15-D (Oncogene Sciences) were used for the absorption of anti-c-Fos or anti-c-Jun antibodies (Yamashita et al., 1996). The peroxidase enzyme activity was examined using a 3,3'-diaminobenzidine (DAB) solution containing nickel and cobalt ions (Adams, 1981).

In situ hybridization

Digoxigenin (Dig)-labeled RNA antisense and sense probes were prepared from the cDNA fragments of c-*fos* (195 bp) and c-*jun* (198 bp), which were inserted at the Hinc II site of pSPT18 (Boehringer Mannheim, Germany), (Yamashita et al., 1996, 2001). The tissue blocks for the *in situ* hybridization were rapidly frozen in isopentane quenched with liquid nitrogen and subsequently freeze-substitution-fixed and embedded in paraffin, as previously described (Yamashita et al., 1996). After deparaffinization, the sections were treated with 0.2N HCl and protease K (Sigma, P-2308) and finally fixed with 4% paraformaldehyde. The sections were incubated with Dig-labeled antisense or sense RNA probes overnight at 45 °C. The sections were treated with RNase A (Sigma, R-5250) and then incubated with alkaline phosphatase-labeled anti-Dig Fab fragment (Boehringer Mannheim). The alkaline phosphatase enzyme activity was detected using 5-bromo-4-chloro-3-indoxyl phosphate and nitroblue tetrazolium.

Results

Morphology

In the 3-week-old DES-mice, the uterine diameter was smaller than that of the controls: the uerine diameter was 0.85±0.13 mm and 0.58±0.06 mm in the control and DES-mice, respectively, when it was calculated from the photographs of 25 cross sections of each experimental group. The uterine lumens were lined with simple columnar epithelium and with pseudostratified columnar

epithelium in some parts, while only simple columnar epithelia were present in the control animals. In the DES-mice, the uterine glands and muscular layers, especially the external longitudinal layer, were poorly developed, compared with the controls.

Effect of E_2 on c-fos expression in 3-week-old mice

Immunohistochemistry

In the vehicle-treated controls and DES-mice, a single E_2 injection induced the expression of c-Fos protein in the endometrial epithelium and endothelial cells of the blood vessels but not in the stromal and myometrial cells. In the control animals, a faint immunoreaction was detectable in the nuclei of the epithelial cells before the E_2 injection (baseline) (Fig. 1a). The intensity of nuclear c-Fos immunostaining gradually increased and peaked at 2 to 3 hours after E_2 administration (Fig. 1c). The luminal epithelium exhibited a stronger immunostaining than the glandular epithelium. In the uteri of DES-mice, a faint c-Fos immunostaining was present in the epithelium at baseline (Fig. 1b). The c-Fos immuostaining was slightly stronger at 1 hour but was clearly weaker at 2 and 3 hours after the E_2 injection, compared with the controls (Fig. 1d). c-Fos protein was localized in the endothelial cells of blood vessels at 2 hours in both the control and DES-mice.

In situ hybridization

E_2 treatment induced the rapid and transient expression of c-*fos* mRNA in the uterine epithelia and vascular endothelia of the control and DES-mice. The expression of the c-*fos* transcript was not evident in any of the uterine cell types of either experimental group at baseline (Fig. 1a,b). In the vehicle-treated controls, the expression of c-*fos* mRNA reached a maximum at 2 hours after the E_2 injection in the epithelial cells (Fig. 2e) and at 1 hour after the E_2 injection in the endothelial cells (Fig. 2c); the glandular epithelium showed a weaker reaction than the luminal epithelium (Fig 2e). The c-*fos* mRNA level in the endometrial epithelium of DES-mice was almost the same at 1 hour (Fig. 2d) and weaker at 2 hours after the E_2 injection (Fig. 2f), compared with the levels in the control animals. Faint c-*fos* hybridization signals were present in the epithelium of both control and DES-mice 3 hours after the E_2 injection (Fig. 2g, h).

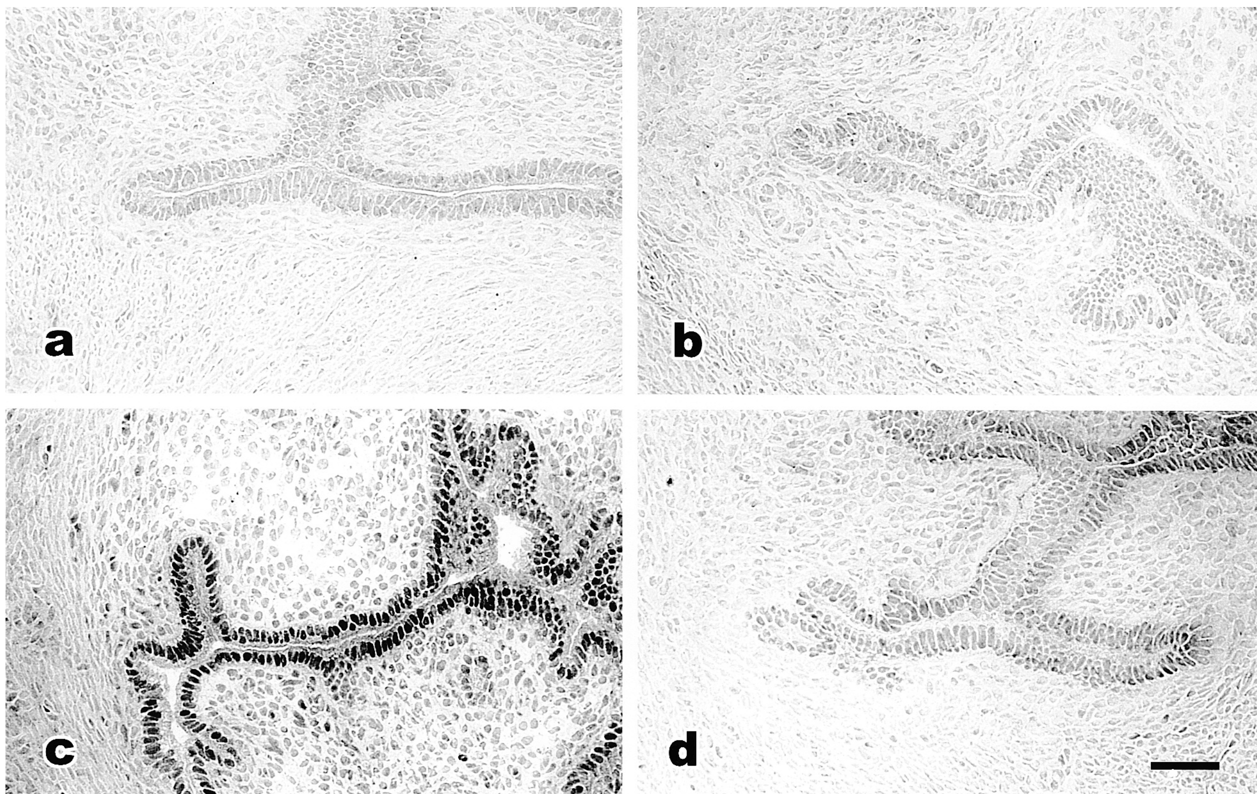

Fig. 1. Immunohistochemistry of c-*fos* protein in the uteri of 3-week-old mice. c-Fos is localized in the uteri of neonatally vehicle-treated controls **(a and c)** and of diethylstilbestrol (DES)-exposed mice, DES-mice **(b and d)**. Mice sacrificed before 17ß-estradiol (E_2) injection **(a and b)** and 2 hours after E_2 injection **(c and d)**. Bar: 50 μm.

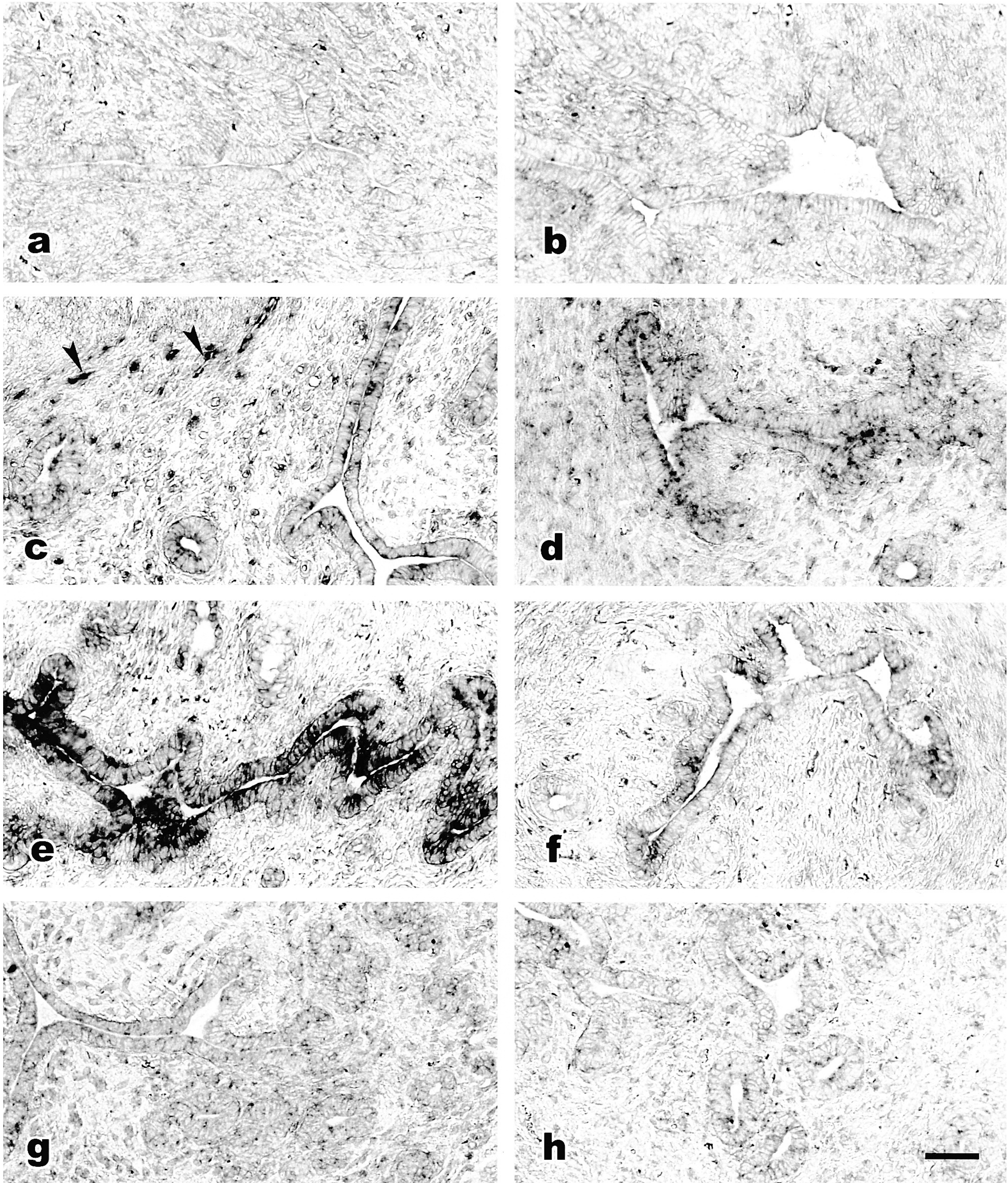

Fig. 2. *In situ* localization of c-*fos* mRNA in the uteri of 3-week-old mice. Uteri of controls **(a, c, e and g)** and DES-mice **(b, d, f and h)**. The c-*fos* transcripts are demonstrated in the uterine sections before E_2 injection **(a and b)**, and 1 hour **(c and d)**, 2 hours (e and f) and 3 hours **(g and h)** after the E_2 injection, using digoxigenin-labeled RNA probes. Arrowheads indicate the reaction products in the endothelial cells of blood vessels. Bar: 50 μm.

Effect of E2 on c-jun expression in 3-week-old mice

Immunohistochemistry

E_2 elicited different c-Jun expression responses in the major uterine cell types: epithelial, stromal and endometrial cells. There were no significant differences in c-Jun expression between the control and DES-mice (Fig. 3a-d). Epithelial cells displayed a weak to moderate c-Jun immunoreaction in the nuclei at baseline. Stromal and muscular cells showed a weak immunoreaction (Fig. 3a,b). The E_2 injections induced no significant changes in the c-Jun immunostaining in the epithelial cells. On the other hand, the intensity of c-Jun immunostaining gradually increased in the stromal and myometrial cells from 1 to 3 hours after E_2 treatment (Fig. 3c,d). Weak c-Jun immunostaining was present in the endothelial cells of the blood vessels at 2 hours after the E_2 injection.

In situ hybridization

The uterine cells of the control and DES-mice exhibited almost the same c-*jun* mRNA expression patterns after E_2 administration (Fig. 4a-d). Strong c-*jun* mRNA signals were present in the luminal epithelium, and a weak reaction was seen in the glandular epithelium in the uteri of both experimental groups before the E_2 injection (Fig. 4a,b). The amount of c-*jun* transcripts in the epithelial cells decreased at 1, 2 and 3 hours. Low levels of c-*jun* mRNA expression were seen in the stromal and muscular cells at baseline, and mRNA expression was transiently induced, peaking at 1 to 2 hours after the E_2 injection (Fig. 4c,d). The endothelial cells of the blood vessels displayed c-*jun* mRNA signals 1 hour after the E_2 injection.

Expression of c-Fos and c-Jun proteins during development

In the control mice, a faint c-Fos immuoreaction was present in the uterine epithelium on days 4, 10 and 15 (Fig. 5a,c). In the DES-mice, the epithelium showed stronger c-Fos immunostaining on days 4 and 10 (Fig. 5b,d), compared with those of the control animals. No significant differences in the c-Fos expression were seen between the two experimental groups on day 15; epithelial cells exhibited a faint immunostaining.

Neonatal DES treatment elicited a decrease in c-Jun immuoreaction in the uterine epithelium on days 4 and 10 (Fig. 6c,d). The stromal cells in DES-mice showed a

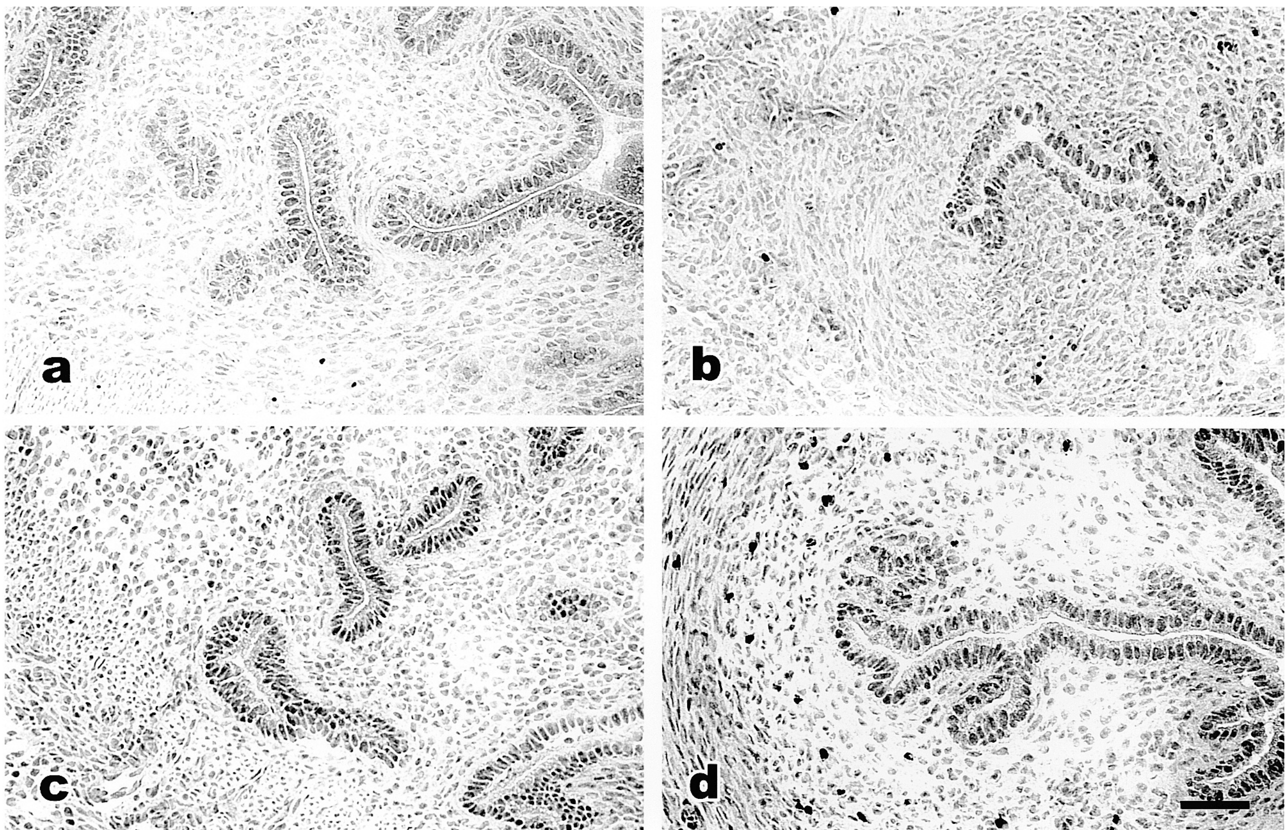

Fig. 3. Immunohistochemistry of c-Jun protein. c-Jun protein is immunostained in the uteri of 3-week-old controls **(a and c)** and DES-mice **(b and d)**. Mice were killed before E_2 injection **(a and b)** and 3 hours after the injection **(c and d)**. Bar: 50 μm.

stronger c-Jun immunostaining on day 4 and a slightly weaker reaction on days 10 and 15 (Fig. 6b,d), compared with those of the controls (Fig. 6a,c). In the muscle cells, the induction of c-Jun protein was evident on day 4 (Fig. 6b), whereas both experimental groups revealed similar weak reactions on days 10 and 15.

Discussion

The present study demonstrated that neonatal DES-treatment elicits changes in the expression pattern of c-*fos* in the uteri of 3-week-old mice. The transient increase in c-*fos* expression after E_2 stimulation was lower in the DES-mice than in the control animals (Figs. 1, 2). This result agreed with that obtained in the uteri of mature DES-mice ovariectomized postpuberally (Yamashita et al., 2001). The insensitivity of c-*fos* expression to exogeneous E_2 was also reported by Kamiya et al. (1996) in the uteri of adult DES-mice ovariectomized prepuberally. These results clearly indicate that the change in the c-*fos* expression pattern after E_2 stimulation is independent of the postpuberal secretion of ovarian steroids. Estrogen-independent persistent c-*fos* gene activation was shown in the uteri of adult DES-mice that were ovariectomized prepuberally and postpuberally; the c-*fos* mRNA levels at baseline were 2 to 6-fold higher in the DES-mice than in the control mice (Kamiya et al., 1996; Yamashita et al., 2001). Therefore, c-*fos* expression at baseline may also be activated in the 3-week-old DES mice, although this was not evident in the present studies using histochemical techniques. According to a Northern blot analysis, c-*jun* expression was shown to be slightly low at baseline and after E_2 stimulation in the uteri of adult ovariectomized DES-mice, compared with that in control animals (Yamashita et al., 2001). However, no significant differences in c-*jun* expression between the two experimental groups were seen in the 3-week-old mice in the present semiquantitative studies (Figs. 3, 4).

Normal regulation of genital tissues through gonadal steroids appears to be permanently impaired in neonatally estrogenized animals. In general, perinatal estrogen treatment reduces responses to estrogen, such as cell growth and secretory activities, in the uteri and vaginae of both immature and mature rodents and rather persistently stimulates these activities in an estrogen-

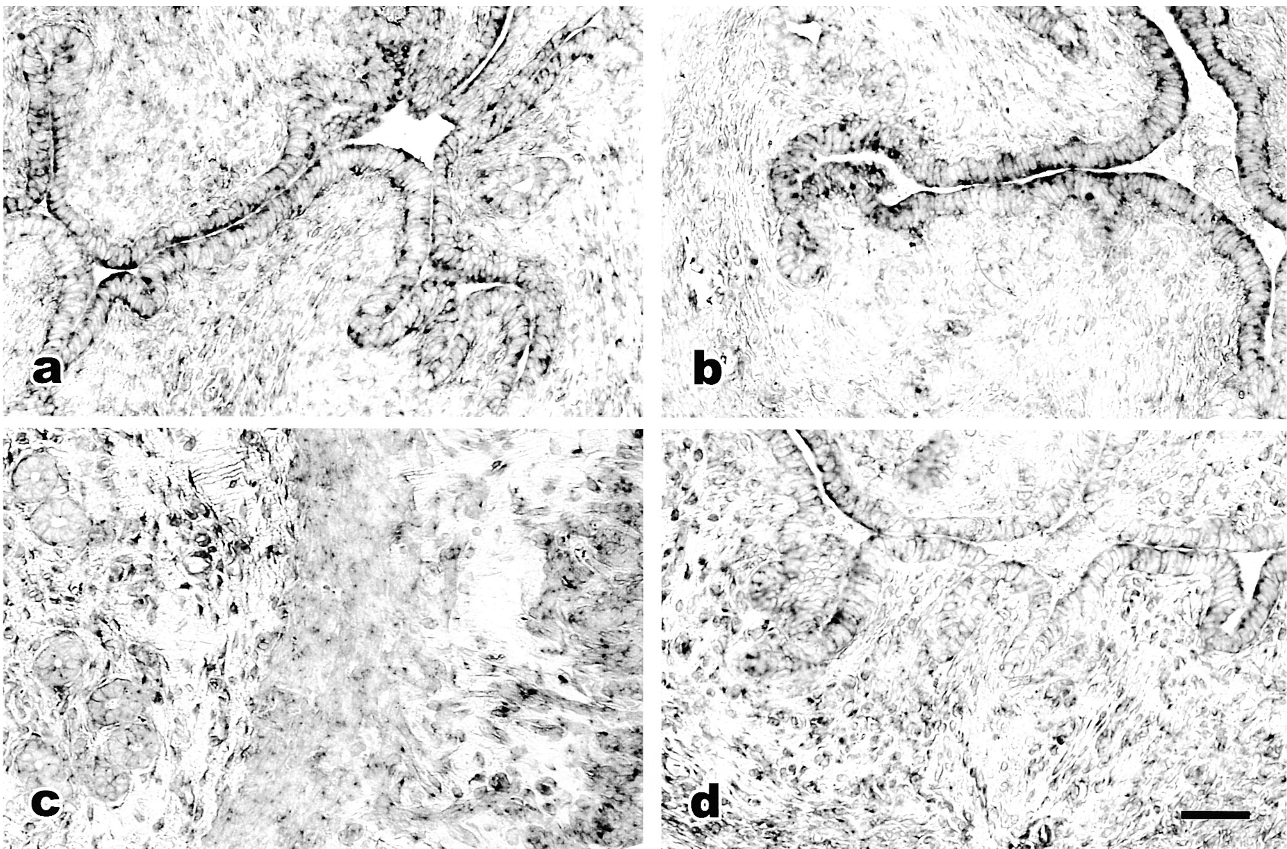

Fig. 4. Expression of c-*jun* mRNA in the uteri of 3-week-old mice. The c-*jun* mRNA is detected in the uterine tissues of controls **(a and c)** and DES-mice **(b and d)**, before E_2 injection **(a and b)** and 2 hours after E_2 stimulation **(c and d)**. Bar: 50 μm.

independent manner (Mori, 1975; Iguchi et al., 1985, Maier et al., 1985, Suzuki et al., 1996). Previous studies have strongly suggested that the stage of cellular differentiation at the time of DES exposure is critical for the imprinting of permanent changes in cell characteristics and the final formation of a wide range of abnormalities (Iguchi et al., 1985; Newbold et al., 1985). The reproductive tract of newborn mice is responsive to exogenous estrogens, although neonatal development of the genital tract is considered to be independent of ovarian hormones until at least day 15 (Ogasawara et al., 1983). The present study revealed that estrogen-regulated protooncogenes in the uteri of immature mice are able to respond to estrogen as well as those of adult rodents and that c-*fos* expression is up-regulated and c-*jun* expression is down-regulated in the uterine epithelium on days 4 and 10, while the c-*jun* gene is activated in stromal cells on day 4 (Figs. 5, 6). These DES-induced abnormal expressions of protooncogenes during the early postnatal period probably cause a permanent alteration in the expression of these estrogen-regulated genes.

Recent studies employing tissue recombination experiments between stroma and epithelium obtained from the uterus and vagina of wild-type and ERα-knockout mice, have shown that epithelial proliferation in the uterus and vagina is mainly regulated through the stromal ERα system in both neonatal and adult mice (Cooke et al., 1997; Buchanan et al., 1998; Kurita et al., 2000). In addition, stromal and epithelial ERα was shown to be required for epithelial differentiation, including E_2-inducible protein synthesis. We have recently reported that ERα protein is undetectable within a few days after birth in the uterine epithelium of control mice but highly induced during the period of DES treatment in DES-mice, remaining at a high level until at least 10 days postpartum; ERα levels in the stromal cells tended to decrease with DES-exposure (Yamashita et al., 1990; Yamashita, 2001b). In contrast, ERß was an undetectable level using immunohistochemical method during postnatal development in the uteri of both control and DES-mice (Yamashita, 2001b). These results indicate that permanent changes in the c-*fos* and c-*jun* expression patterns in the uteri of DES-mice may be mainly mediated through the ERα systems of both epithelial and stromal cells.

Membrane receptor-mediated signaling pathways have been shown to crosstalk with the ER-mediated pathway; the phosphorylation of ER proteins seems to induce an estrogen-independent activation of ERs.

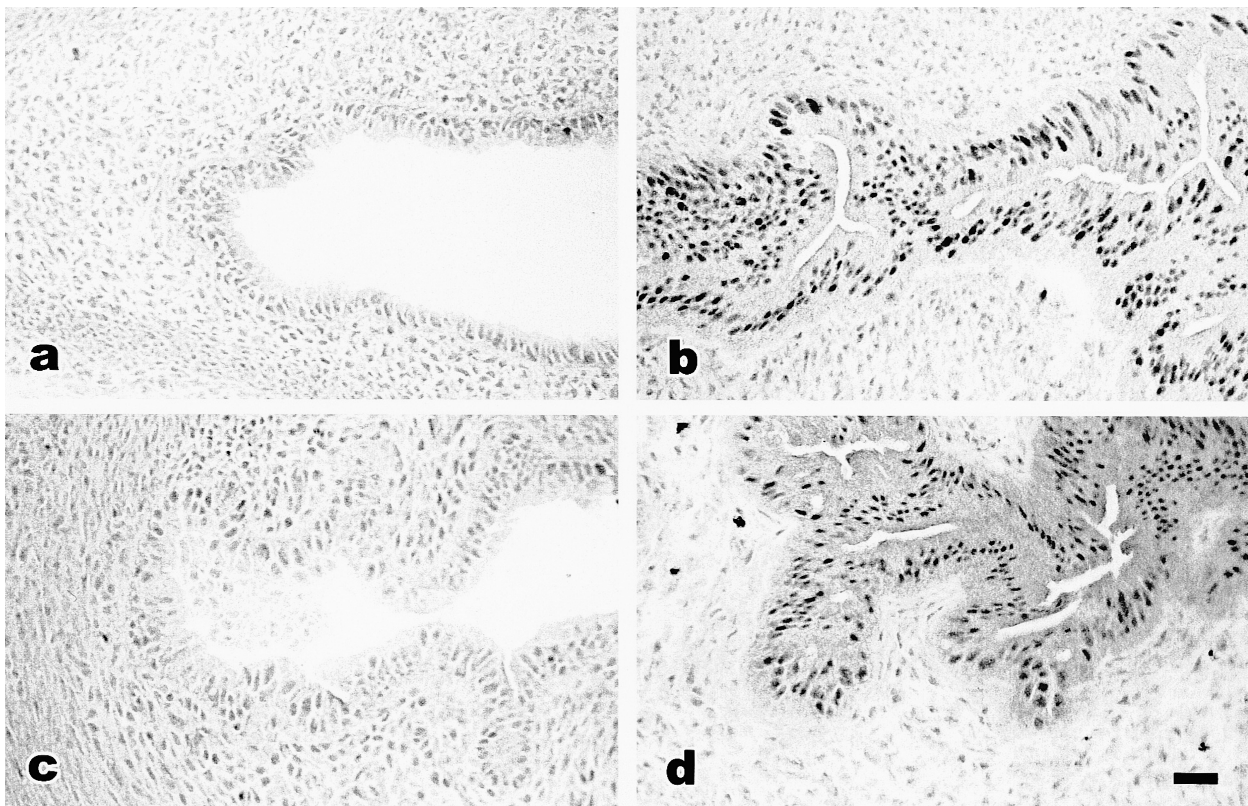

Fig. 5. c-Fos immunostaining in the uteri of neonatal mice. Neonatally vehicle-treated controls **(a and c)** and DES-mice **(b and d)** were killed on day 4, **(a and b)** and on day 10 **(c and d)** and then the uteri were immunostained with anti-c-Fos antibody. Bar: 50 μm.

Several peptide growth factors, such as epidermal growth factor (EGF), insulin-like growth factor-1 and transforming growth factor, are induced by estrogen treatment in different cell types of female reproductive tracts (DiAugastine et al., 1988; Murphy and Chahary, 1990; Takahashi et al., 1994). These growth factors are postulated to act as autocrine or paracrine mediators of estrogen-induced cell proliferation and differentiation in these tissues, since EGF administration can mimic estrogen action such as uterine epithelial proliferation and the synthesis of lactofferin (LF), an estrogen-induced glycoprotein in adult ovariectomized mice (Nelson et al., 1991; Ignar-Trowbridge et al., 1992; Curtis et al., 1996). Nelson et al. (1994) reported that a persistent ovary-independent activation of estrogen-regulated genes (*LF* and *EGF* genes) is seen in the uterus and vagina of DES-mice. The persistent expression of EGF may also be responsible for the constitutive c-*fos* gene activation in the uterus of both mature and immature DES-mice, since Curtis et al. (1996) revealed that the expression of c-*fos* mRNA in the mouse uterus is stimulated by exogenous EGF in the absence of estrogen. These results suggest that the interactive and continuous stimulation of ER, AP-1 and membrane receptor-mediated signaling systems modifies the cell characteristics of the female reproductive tract in DES-mice and causes serious abnormalities.

One of the possible mechanisms which modify estrogen-regulated genes is the demethylation or methylation of DNA in DES-mice. Neonatal DES treatment was shown to imprint an abnormal, site-specific demethylation immediately upstream from the ERE of the LF promoter (Li et al., 1997). Continuous estrogen secretion from the ovary of DES-mice, which do not have corpora lutea, presumably causes further expression modifications in these ER-regulated genes, since hormonal deprivation by ovariectomy decreases the incidence of serious lesions.

In conclusion, the present study demonstrates that neonatal DES treatment induces permanent changes in estrogen-regulated protooncogenes in the uteri of immature mice. The changes in the cell characteristics appear to be elicited during postnatal development, particularly during the period of DES exposure, which may correspond to a critical period of differentiation in the uterus. In the reproductive tissues perinatally exposed to estrogens, the result in alternative and continuous stimulation of genes, such as c-*fos*, c-*jun* and peptide growth factors participating in cell proliferation and differentiation, should play important roles in the

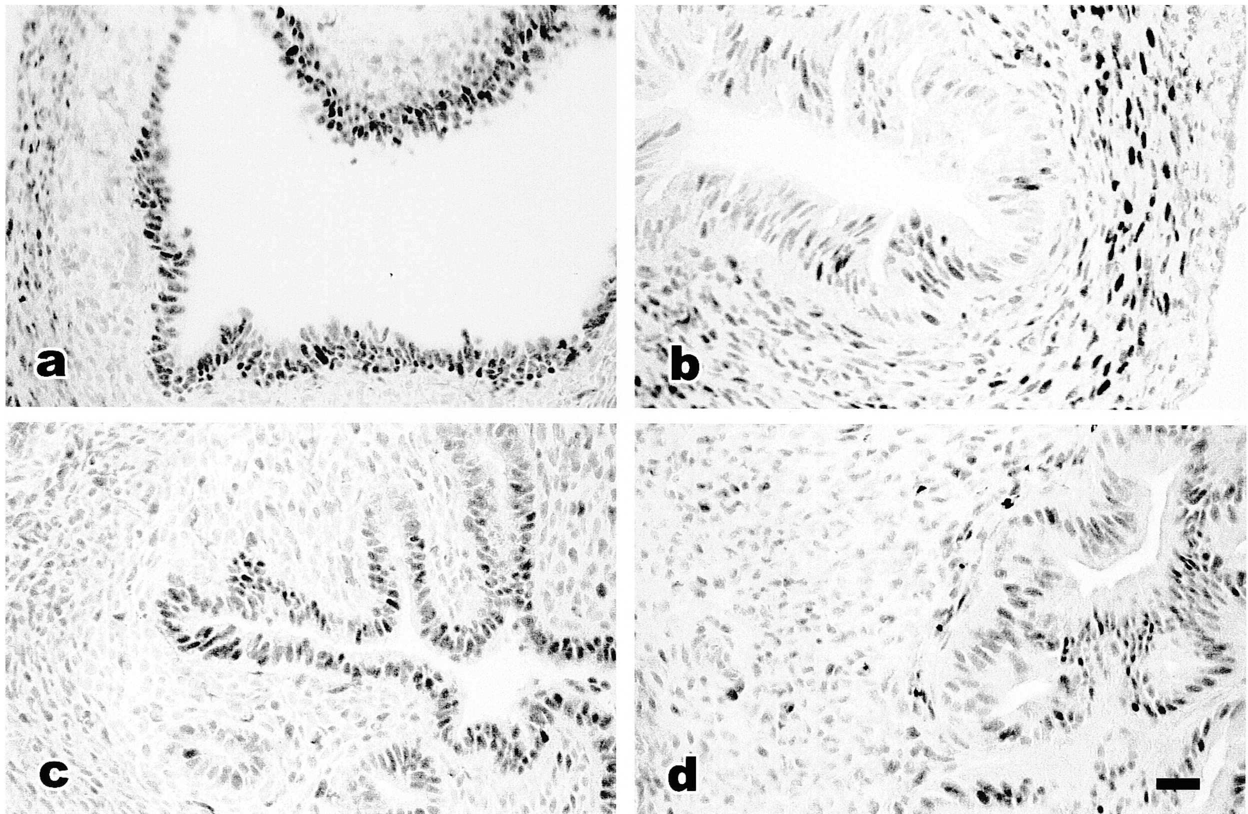

Fig. 6. Localization of c-Jun protein in the uteri of neonatal mice. Controls **(a and c)** and DES-mice **(b and d)**. c-Jun protein is immunostained in the uteri on day 4 **(a and b)** and on day 10 **(c and d)**. Bar: 50 μm.

production of a wide range of abnormalities.

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