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REGULAR ARTICLE

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Differential expression of c-Jun proteins during Müllerian duct growth and apoptosis: caspase-related tissue death blocked by diethylstilbestrol

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Abstract To elucidate whether the differentiation of the Müllerian duct (MD) is mediated by c-Jun proteins, Western immunoblot with c-Jun/sc-45 antibody was used to investigate these proteins in female chick left and right MDs (LMD and RMD, respectively). The content of these proteins (e.g., the 66-kDa, 45-kDa, and 39-kDa forms) in the LMD or RMD of various stages of embryos was detected by measuring their density in autoradiograms by a Spot-denso-program with Alpha Ease software. In the LMD, the growing embryonic sex tract, the content of the 66-kDa and 39-kDa proteins increased to their highest level in 9th to 12th day embryos and then declined thereafter. In the RMD, the apoptotic embryonic sex tract, the content of these proteins also showed a linear increase from the 9th to 10th day and then declined at the 13th day. When the RMD entered the apoptosis stages (14th to 18th day of incubation), these proteins were persistently overexpressed. Another protein (45 kDa) was detected in both ducts only at the 9th to 13th days, and its content was higher in RMD than in the LMD. In parallel to this finding, high caspase-3 activity (determined by the measurement of the fragmented 85-kDa poly ADP-ribose polymerase) was found in the RMD during apoptosis. The apoptotic death of RMD was prevented by in vivo diethylstilbestrol treatment, which inhibited the overexpression of the 66-kDa and 45-kDa proteins, the fragmentation of DNA, and the activity of caspase-3. No inhibitory effect was found for the 39-kDa protein.

Keywords Müllerian duct · Apoptosis · C-Jun proteins · Caspase · Diethylstilbestrol · Chicken (White Leghorn)

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Introduction

Both female and male chick embryos, after a period of 4–5 days of incubation, possess undifferentiated pairs of gonads, Müllerian ducts (MDs), and Wolffian ducts (WDs). In the male embryo, the MDs undergo involution and disappear by the 12th day of incubation, whereas the WDs are retained and become the vas differentia in the adult male. In the female embryo, the MDs remain undifferentiated at day 8, after which they begin to differentiate. The right MD (RMD) undergoes a relatively slow anterior to posterior involution by the 13th day of incubation and disappears at the time of hatching (Romanoff 1960; Hamilton and Teng 1965). The left MD (LMD) continues to grow and develops into the functional female sex tract, i.e., fallopian tube, isthmus, magnum, uterus, and shell gland. The WDs disappear before hatching (Wolff 1959; Jost 1960).

The regression of the RMD is attributed to the active secretion of Müllerian inhibiting substance (MIS or anti-Müllerian hormone) from the embryonic right ovary (or ovotestis) in the 9th to 14th day of incubation (Teng 1987). The regression of female RMD can be prevented by the administration of the estrogenic hormone diethylstilbestrol (DES) prior to the 5th day of incubation. Full retention of the RMD and both male MDs is accomplished with one single dose of DES treatment, with retention persisting after birth (Teng and Teng 1979, 1985; Teng 1990). MIS, a glycoprotein, is a member of the transforming growth factor-β family, which regulates the differentiation of embryonic sex tracts in vertebrate species (Mishina et al. 1996; Lane and Donahoe 1998). MIS specifically binds to MD mesenchyme and alters its functional activity; this leads to MD regression (Teng 1990; Tsuji et al. 1992). Recently, a type II MIS receptor (MIS II R) has been found to transduct the signal in the mesenchyme surrounding the MD (Baarends et al. 1994; Teixira et al. 1996; Racine et al. 1998; Lee et al. 1999). MIS II R is expressed only in the mesenchyme (instead of the epithelium), suggesting that MIS-induced MD epithelial regression is mediated through the mesenchyme

(Roberts et al. 1999). Terminal dUTP-biotin nick-end-labeling assay has been applied to confirm that the MIS-induced cell death in MD (or fetal rat lung) is apoptotic in nature (Catlin et al. 1997; Roberts et al. 1999). However, the molecular mechanism behind MIS-induced apoptotic death of MDs in avian and mammalian species is as yet unknown.

Recently, the involvement of protooncogenes (e.g.,the c-*myc,* c-*fos*, c-*jun*, and *bcl-2* families) in the process of growth or apoptotic death of somatic, cancer, and embryonic cells has been extensively studied (for reviews, see Henriksson and Lüscher 1996; Karin et al. 1997; Adams and Cory 1998; Dang 1999; Teng 2000). c-Jun proteins encoded by c-*jun* are AP-1 transcription factors that dimerize with c-Fos and bind to DNA at AP-1 sites in prokaryotic and eukaryotic genes (Bohmann et al. 1987; Bos et al. 1988). The dimerization is mediated by a carboxy-terminal coiled-coil structure termed the leucine zipper, which is necessary for DNA binding to a palindromic sequence known as the AP-1 site (with base sequence TGACTCA; Teng 2000). This AP-1 site plays an important role in controlling gene expression during embryonic cell differentiation and apoptosis (Rutberg et al. 1997). Because of the intricate molecular interaction required for c-Jun proteins to activate the AP-1 site, especially during growth or apoptosis, an understanding of the function of this protein has become a biomedically important topic.

In this regard, I have used the female embryonic chick genital system as a model to investigate the role of c-Jun protein in mediating the growth or death of MDs and its prevention by DES. In this study, I have found that three c-Jun proteins are expressed in MDs. At various stages of embryogenesis, the expression of these proteins in the LMD and RMD is different. Preceding the apoptotic death of the RMD, a pattern of biphysical and prolonged c-Jun overexpression is found in the duct. The regression of the RMD is characterized by a laddering type of DNA fragmentation and is correlated with the elevation of caspase-3 activity. Treatment of embryos with DES suppresses the overexpression of c-Jun proteins and the activity of caspase-3 in the RMD, and consequently, the death of the RMD is prevented.

Materials and methods

Chemicals and supplies

White Leghorn chick embryos at various stages of development were obtained as described previously (Teng and Teng 1978a, 1978b). Chemicals were obtained from the following sources: phenylmethyl sulfonyl fluoride (PMSF), aprotinin, pepstatin, leupeptin, Triton X-100, DES, and cholamidopropyl-dimethyl-ammoniol-propane-sulfonate (CHAPS) from Sigma (St. Louis, Mo.); precast TRIS-glycine polyacrylamide gel for polyacrylamide gel electrophoresis (PAGE), PAGE sample buffer, sample reducing agent, antioxidant, transfer buffer, nitrocellulose membrane, and protein standard from Novex (San Diego, Calif.); c-Jun/AP-1:sc-45 polyclonal IgG, poly ADP-ribose polymerase (PARP A-20):sc-1562, actin (c-11) polyclonal IgG, and goat anti-rabbit IgG with alkaline phosphatase from Santa Cruz Biotechnology (Santa Cruz, Calif.); peroxidase-labeled anti-rabbit antibody and high-performance autoradiography film from Amersham Life Science (Amersham, Buckinghamshire, England). All other chemicals were of analytical grade.

Preparation of tissue extracts

LMD and RMD obtained from 36–60 female embryos were minced finely with curved iris scissors. c-Jun protein was extracted from total tissues according to Teng and Vilagrasa (1998). Tissues were suspended in 5 volumes RIPA lysis buffer (10 mM, TRIS-HCl, 150 mM NaCl, 0.1% SDS, 1% CHAPS, 1 mM EDTA, pH 7.4, containing 0.5 mM PMSF, 10 μg/ml aprotinin, and 2 μg/ml each of pepstatin and leupeptin) at 4°C and homogenized by 10 strokes with a Teflon homogenizer. The homogenates were then sonicated in an ice bath with a Branson sonifier (Model W 140; Ultrasonics, Plainview, N.Y.) for 15 s, followed by two 10-s pauses. The sonified homogenates were centrifuged at 15,000 *g* for 15 min at 4°C to remove the cellular debris, and the supernatants (or RIPA extract) were saved for analysis.

Western immunoblots and determination of c-Jun proteins

The cellular c-Jun-related proteins and 85 kDa PARP protein levels in LMD and RMD (or RMD with DES treatment) were determined by an immunoblotting technique (Towbin et al. 1979). RIPA extract (30 μg proteins) from various tissue samples was separated on 4%–12% TRIS-glycine PAGE at 125 V for 2 h with a Power Ease 500 power supply (Novex). In some experiments, two identical PAGEs were prepared, one gel being stained with Coomassie brilliant blue to show the total protein bands in the RIPA extract, and the other gel being used for the immunoblot. The proteins in the gel were transferred onto a nitrocellulose membrane by using antioxidant and transfer buffer (Novex). Blots were then incubated in phosphate-buffered saline (PBST) with 0.5% skim milk powder (containing 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h and then transferred to PBST (10 ml) containing 10 μl primary antibody (epitope corresponding to amino acids 95–105 mapping at the amino terminus of p^{39} c-Jun of mouse origin) and incubated at 4°C for 14 h, followed by washing with PBST. Blots were then incubated with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (5000-fold dilution in 10 ml PBST) for 1 h at room temperature, after which they were washed and submerged in Western blotting detection reagents (Amersham Life Science) for 60 s and covered with Saran Wrap. The blots were immediately exposed to high-performance autoradiograph film in a Hypercassette (Amersham, UK) for 1–2 min.

The c-Jun proteins identified in total tissue by Western blot appeared as three separated bands (designated as the 66-kDa, 45-kDa, and 39-kDa bands). The major PARP fragment was identified as a 85-kDa protein. The density of these proteins was quantitatively determined by a computer-controlled Spot-densoprogram with Alpha Ease software (Alpha Innotech, San Leandro, Calif.). The results are presented as the integrated density value (IDV) of the protein bands after background subtraction.

DES administration

The technique for DES administration was initially reported by Seltzer (1956) and Van Tienhoven (1957) and was modified by Teng and Teng (1979): after incubation for 5 days, fertilized White Leghorn chick eggs were placed in a solution containing 20 mg DES per milliliter 70% ethanol, at 55°C, so that the bottom 1/2–1 inch was immersed in the DES solution for 15 s. The control eggs received the same treatment with a solution that did not contain DES. After the treatment was completed, the eggs were returned to the incubator. When the embryos reached 15th (or 18th) day of incubation, they were dissected midventrally, and the

RMDs were removed for the preparation of the RIPA extract (or DNA).

Analysis DNA fragmentation

MDs were minced finely and suspended in lysis buffer (containing 10 mM TRIS/HCl, 20 mM EDTA, 0.1% Triton X-100, pH 7.4) with brief mechanical agitation, and the resulting lysate was treated with 100 μg/ml proteinase K for 16 h at 55°C. The lysate was centrifuged at 30,000 *g* for 45 min at 4°C. The DNA fragments residing in the pellet were precipitated in 70% ethanol and 0.3 M sodium acetate and vacuum-dried. The DNAs were redissolved in TE buffer (10 mM TRIS, 1 mM EDTA, 0.01% SDS, pH 7.4), treated with 100 μg/ml ribonuclease-A for 4 h at 37°C, and then extracted with chloroform:isoamyl alcohol (24:1) four times. The amount of DNA was determined by the diphenylamine reaction method (Giles and Myers 1965) and resolved by electrophoresis at 85 V/cm for 90 min on 2% agarose gels impregnated with ethidium bromide. A fixed quantity of DNA was loaded and run simultaneously with multiple DNA molecular weight reference preparations (1.35-kb to 0.3-kb ladders). DNA fragments were visualized under UV light.

Biochemical methods

Proteins were determined by using the Bio-Rad protein assay reagent (Bio-Rad Lab, Hercules, Calif.) with bovine serum albumin as a standard. DNA was determined by the diphenylamine reaction (Giles and Myers 1965) with calf thymus DNA as a standard.

Statistical analysis

Mean values were recorded as mean ±SEM. The mean data were tested statistically by using the two-way analysis of variance (ANOVA), and the differences between specific means were tested for significance by Scheffe's multiple range test (Steele and Torrie 1960). In some cases, the Mann-Whitney test was applied. A difference between the two means was considered to be statistically significant at $P<0.05$.

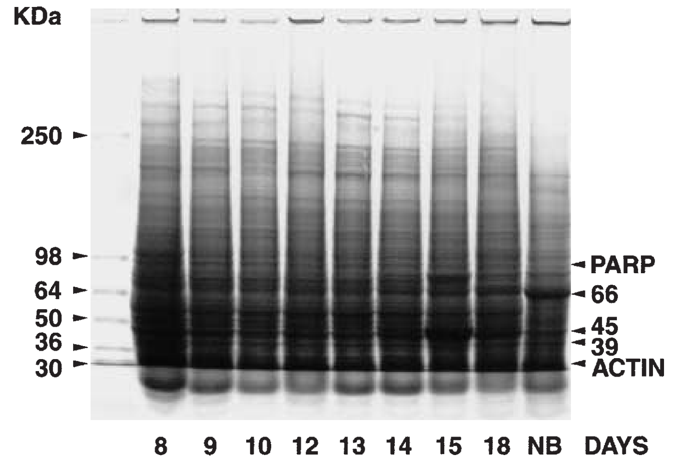


Fig. 1 Electrophoretic detection of c-Jun and other proteins in Müllerian ducts. Total proteins (containing 30 μg RIPA extract) from LMD of various stages (days of incubation) of female embryos were resolved by using 4%–12% TRIS-glycine PAGE. The protein bands, i.e., 85-kDa PARP fragments, 66-kDa, 45-kDa, and 39-kDa c-Jun-related proteins and actin, were identified by their primary antibodies and the Western immunoblot technique. The total protein banding patterns were detected by Coomassie brilliant blue (Bio-Rad Lab). The protein standard markers, from 30–250 kDa, were obtained from Novex (*NB* left oviduct of a 5th-day newborn chick)

Results

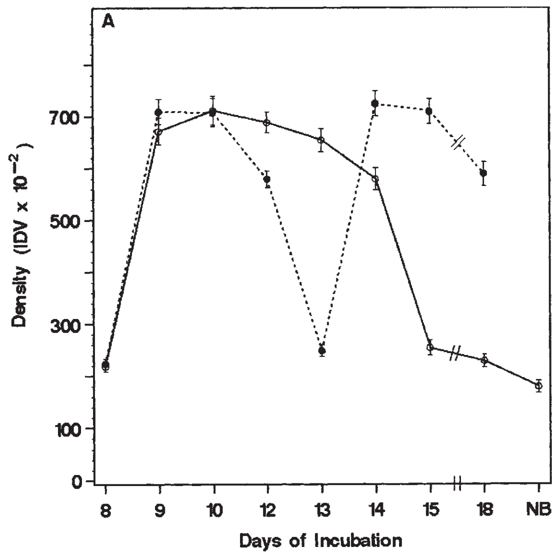
Electrophoretic detection of c-Jun and other proteins

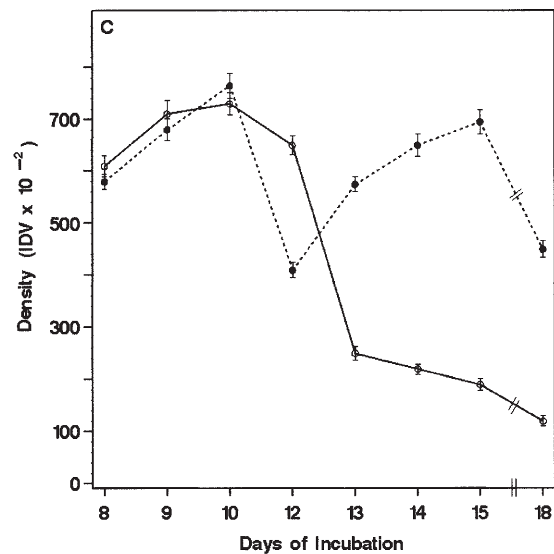
The presence of c-Jun-related proteins, actin, and PARP protein in the RIPA extract was detected by using 4%–12% TRIS-glycine PAGE and then interaction with their primary antibodies. Three positive c-Jun protein bands with molecular weights of 66 kDa, 45 kDa, and 39 kDa were identified in the LMD and RMD. Other proteins (e.g., PARP fragments and actin with molecular weights of 85 kDa and 32 kDa) were also identified (Fig. 1). Total RIPA-extracted proteins were stained with Coomassie brilliant blue and appeared in a molecular-weight range of 30–250 kDa. The overall protein banding pattern from the left and right ducts (data not shown) was similar. However, the quantitative change in the c-Jun and other proteins was distinct (Fig. 2).

Analysis of c-Jun proteins by Western immunoblot

c-Jun proteins were obtained from LMD and RMD extracts and recognized by Western blot with c-Jun/sc-45 antibody (Fig. 2A–C). The intensity of the protein bands was quantitated by a computer controlled Spot-denso-program with Alpha Ease software. The gradient profiles were plotted according to the IDVs of the protein bands averaged from 3–4 separate determinations. In the developing LMD, the quantity of 66-kDa c-Jun protein was relatively low on the 8th day, increased to a high level in the 9- to 10-day-old embryos, and then declined rapidly from the 13th to the 18th days. Consequently, the level of the protein in the left duct of the newly hatched chicks was further reduced (71% less than that of the 10-day-old embryos).

The amount of 66-kDa protein in the RMD was measured at various stages of differentiation, e.g., from 8- to 18-day-old embryos. During the first stage, from the 8th to the 10th day of incubation, the amount of the protein increased. During the second stage, from the 12th day to the 13th day, the content of the protein decreased. During the third stage, from the 14th to the 18th day, the content of the protein increased and remained consistently at a high level for 4 days. Based on the observations presented in Fig. 2, it was concluded that biphasic c-Jun protein expression occurred in the process of the natural death of chick RMD. The amount of 66-kDa protein detected in the RMD at the late stages of embryonic development (from the 15th to the 18th day of incubation) was higher (2- to 3-fold) than that in the LMD. To establish equal gel loading of MD extracts, the amounts of actin present in the samples were investigated: equal amounts of actin were detected in every sample, whereas the 66-kDa protein was markedly reduced or induced in LMD or RMD, respectively (Fig. 2A).





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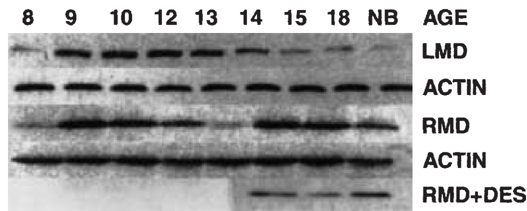

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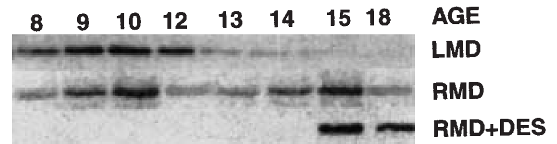

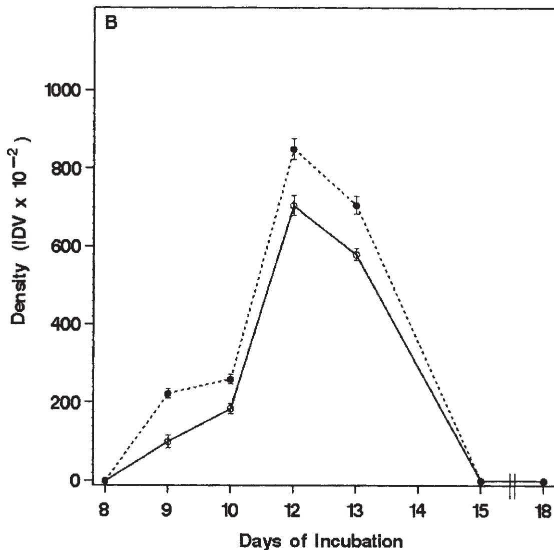

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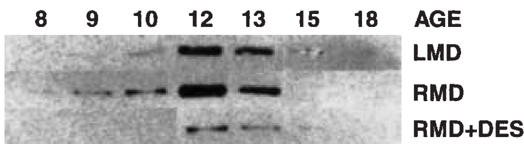

Fig. 2 **A** Expression of the 66-kDa c-Jun protein in LMD and RMD. Equal amounts (30 µg) of the RIPA extracted proteins from LMD and RMD (or actin) were run through the gel (*bottom*). Equal loading was confirmed by monitoring the approximately constant expression of actin. The intensity of the protein bands was quantitated by a Spot-denso-program with Alpha Ease software (*top*). The gradient profiles were plotted according to the integrated density value (*IDV*). *Vertical bars* SEM from 3–4 separate experiments. The protein band pattern of RMD obtained from DES-treated female embryos (age: 14, 15, and 18 days old) is presented *bottom*. The densities of these protein bands are presented in Table 1. **B** Expression of the 45-kDa protein in LMD and RMD. The experimental conditions for the quantitation of the 45-kDa protein were identical to that of the 66-kDa protein (**A**). DES-treated RMDs were from embryos aged 12–18 days. *Vertical bars* SEM from 3–4 separate experiments. **C** Expression of the 39-kDa c-Jun protein in LMD and RMD. The experimental conditions for the quantitation of 39 KDa protein were as in **A**. *Vertical bars* SEM from 3–4 separate experiments. DES-treated RMDs were from embryos aged 15–18 days. *Open circles* LMD, *closed circles* RMD

The amount of 45-kDa protein in both the LMD and RMD at various stages of development (from the 8th to the 18th day of incubation) was also investigated (Fig. 2B). On the 8th and the 10th day of incubation, the amount of 45-kDa protein, which existed in both ducts, was relatively low. However, a marked increase was found in the RMD of 12th and 13th day embryos where the content of protein in RMD was 25% higher than that in the LMD. After 13 days of incubation, the amount of the protein in both ducts became nondetectable. (Fig. 2B).

Throughout the stages of LMD development and growth, the expression of the 39-kDa protein followed the same pattern as that of the 66-kDa protein (Fig. 2A,

Table 1 Effect of DES on the expression of c-Jun-related proteins in RMD (*ND* not detectable, *IDV* contents of proteins expressed by integrated density values)

c-Jun proteins	Embryonic age (days of incubation)					Density
	12	13	14	15	18	
66 kDa						
–DES	–	–	72,500	71,000	59,000	IDV
+DES	–	–	37,000	26,000	38,500	IDV
Inhibition (%)	–	–	49	63	35	
45 kDa						
–DES	85,000	70,600	ND	ND	ND	IDV
+DES	26,500	22,000	ND	ND	ND	IDV
Inhibition (%)	68.8	68.8				
39 kDa						
–DES	–	–	69,000	45,000		IDV
+DES	–	–	69,500	45,000		IDV
Inhibition (%)			None	None		

C). The content of the protein increased gradually from the 8th day to the 10th day and then was drastically reduced at the 13th day to an undetectable level (Fig. 2C). In the RMD, this result agreed with a previous finding that had revealed a biphasic response of 39-kDa protein, viz., a down- and up-regulation of the protein was observed at the 12th and 15th days, respectively. The content of 39-kDa protein in the RMD undergoing apoptosis was consistently 2- to 3-fold higher than that of the LMD (Fig. 2C).

c-Jun protein expression after exposure to DES

After the chick embryos had received one treatment of DES on the 5th day of incubation (sexually undifferentiated stage), the amount of 66-kDa and 45-kDa proteins in the RMD of the 14- to 18-day-old and 12- to 13-day-old embryos, respectively, was significantly reduced (Fig. 2A, B). The amount of the proteins from the DES-treated RMDs was compared with that obtained from RMDs without DES treatment: the reduction of 66 kDa ranged from 35% to 63%, whereas a 69% reduction was found in 45-kDa protein (Table 1). No obvious difference for the 39-kDa protein was found in the RMD from the 15- and 18-day-old embryos (Fig. 2C, Table 1).

Assay for caspase activity with or without DES

The LMD and RMD extracts shown in the above section were assayed for caspase-3 activity, by assessing the activity to cleave an intact form of DNA repairing enzyme PARP (116 kDa) to an inactive 85-kDa fragment. The amount of 85-kDa PARP fragments appearing in the RMD extract of the 14-, 15-, and 18-day-old embryos was respectively 74%, 132%, and 217% higher than that of the LMD (Fig. 3). This indicated that the endogenous caspase-3 in RMDs was significantly higher than that of the LMDs. After prenatal DES exposure to the embryos at day 5 of incubation, the caspase activity in LMDs and RMDs was reduced by an average of 67% and 60%, respectively (Fig. 3).

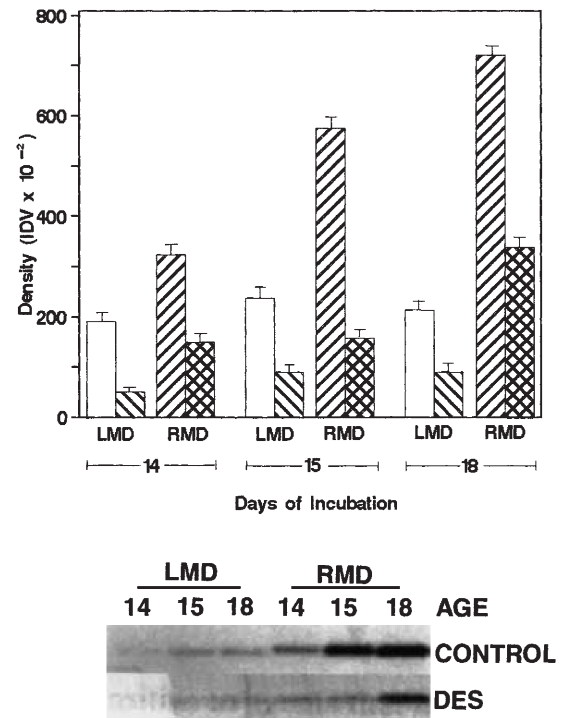

Fig. 3 Caspase-3 activity in LMD and RMD with or without the treatment of DES. The activity of caspase-3 in the control or DES-treated LMD and RMD of the 14-, 15-, and 18-day-old embryos was detected by measuring the content of the fragmented PARP (85-kDa). *Vertical bars* SEM from three separate experiments. *Open bars* PARP content in LMD without DES, *hatching angled left* PARP content in LMD with DES, *hatching angled right* PARP content in RMD without DES, *cross-hatching* PARP content in RMD with DES

Electrophoretic detection of DNA fragmentation

Qualitative analysis of DNA fragmentation was performed by using agarose gel electrophoresis to resolve

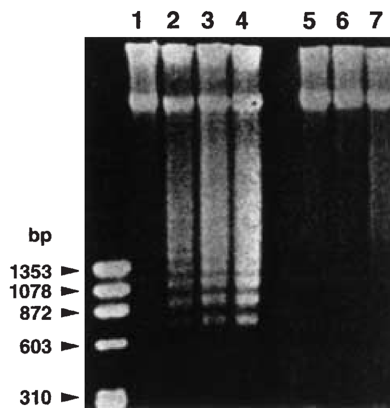

Fig. 4 Agarose gel electrophoresis of DNA fragments from LMD and RMD. DNA analysis by 2% agarose gel electrophoresis of the total DNA extracted from the Müllerian ducts with or without in vivo DES treatment. The DNAs were prepared from the Müllerian ducts of the following female embryos: *lane 1* LMD from 15-day-old embryo, *lane 2* RMD from 15-day-old embryo, *lane 3* RMD from 17-day-old embryo, *lane 4* RMD from 18-day-old embryo, *lane 5* RMD from 15-day-old embryo after DES treatment at 5th day of incubation, *lane 6* RMD from 17-day-old embryo after DES treatment, *lane 7* RMD from 18-day-old embryo after DES treatment

the oligonucleosomal DNA fragments recovered from the LMD and RMD. No visible sign of DNA fragmentation was found in the DNA of the LMD from the 15-day-old embryo (Fig. 4, lane 1). A laddered electrophoretic pattern of oligonucleosomal DNA fragmentation was observed in the RMD of 14-, 15-, and 18-day-old embryos (Fig. 4, lanes 2, 3, and 4). In contrast, there were no DNA fragments found in the RMDs after prenatal DES exposure (Fig. 4, lanes 5, 6, and 7). The results were consistent with the studies presented in Fig. 3 where the treatment of DES inhibited the activity of caspase-3.

Discussion

The present results show that c-Jun/sc-45 antibody against a peptide that corresponds to amino acids 95–105 mapping within the amino terminal domain of mouse c-Jun p^{39} recognizes three c-Jun-related antigens in chick MD. This finding parallels observations from chick and rat brain in which three identical c-Jun proteins of 66 kDa, 45 kDa, and 39 kDa have been identified (Ferrer et al. 1997a, 1997b; Pozas et al. 1997; Ayala et al. 1999). The results support an earlier suggestion that more than one protein (39 kDa) is at present described as c-Jun (Harlan and Garcia 1995). On the other hand, it indicates that c-Jun/sc-45 antibody cross-reacts with epitopes that are specifically associated with apoptosis but that are distinct from c-Jun p^{39}. However, it has to be determined whether these c-Jun proteins are isoforms of the same c-*jun* gene.

In female chick LMD and RMD, a steady increase in the content of c-Jun proteins (66 kDa and 39 kDa) has been found in embryos at 8–12 days of incubation when both ducts are engaging in a linear rate of growth (Teng 1987). This confirms that the expression of c-Jun is involved in the growth of MD. Participation of c-Jun in female reproductive tract differentiation and growth has been demonstrated in rat and hamster (Weisz et al. 1990; Zheng and Hendry 1997). Similar findings in this regard have also been reported in other tissues indicating that c-Jun plays a key role in cell cycle progression (Cater el al. 1991), embryonic stem cell growth and hepatogenesis (Hilberg and Wagner 1992; Johnson et al. 1993), and normal embryonic development (Hilberg et al. 1993).

Previously, in my laboratory, we have discovered that, prior to the apoptotic death of rat spermatocytes induced by gossypol, cell death is implemented by an altered (or biphasic) expression of c-*myc* and c-*fos* genes (Teng 1995, 1998; Teng and Vilagrasa 1998). This present study also reveals a similar biphasic response (down- and up-regulation) of c-Jun proteins (both of the 66-kDa and 39-kDa proteins) prior to the apoptotic death of the RMD. In the late developmental stages of female chick embryo, the content of these c-Jun proteins in the dying RMD rise to a level that is 2- to 3-fold higher than that of the growing LMD. This sustained overexpression of c-Jun in the RMD lasts for 4 days and could contribute to the death of the right duct. Another c-Jun-related 45-kDa protein or apoptosis-specific protein (ASP) has been found in both ducts, and a higher content of this protein has also been observed in the RMD than in the LMD. Above all, a high level of ASP in the RMD exists in the 12- to 13-day-old embryos when the right ducts are just starting to regress (Teng 1987). ASP has also been identified in Burkitt lymphoma cells and other human and rat cell lines with an antibody similar to c-Jun/sc-45. During normal or induced apoptotic death of these cells, ASP expression is up-regulated at a point immediately prior to an irreversible step in the process of apoptosis (Grand et al. 1995). Similar results have been obtained by Ayala et al. (1999). They have discovered that, in chick embryonic spinal cord, excess ASP expression coincides with naturally occurring or experimentally stimulated apoptotic motorneuron death. Unfortunately, the function of ASP is still unknown and remains to be investigated.

The finding of a prolonged high level of c-Jun expression in the dying RMD is not unprecedented. Similar results have been documented in lymphoid cells, sympathetic neurons, and fibroblasts undergoing apoptotic cell death (Colotta et al. 1992; Ham et al. 1995; Bossy-Wetzel et al. 1997; Karin et al. 1997). In this regard, it has been suggested that c-jun is required for de novo protein synthesis and to serve as an apoptotic regulator. On the other hand, the presence of an excess amount of c-Jun proteins in the RMD may repress the expression of genes, such as the *bcl-2* gene families, that code for proteins that exhibit anti-cell-death activities (Park et al. 1997; Srivastava et al. 1999). It may also initiate a ladder pattern of internucleosomal DNA fragmentation in RMD. Similar observations in this aspect have been reported in other cell types (Kastan et al. 1991; Manome et

al. 1993; Nelson and Kastan 1994). In addition, workers in this field have been able to correlate the consequences of DNA fragmentation with the activation of p^{53}, a potent death gene. Elevated p^{53} activity in response to DNA damage has been attributed to an increase of DNA-dependent protein kinase activity (Woo et al. 1998).

Sustained overproduction of c-Jun in the RMD may accelerate the Fas-Daxx signal pathway, which interacts either directly or indirectly with the receptor-associated protein that recruits procaspases to the receptor complex (Darnay and Aggarwal 1997). This subsequently leads to the activation of an aspartate-specific cysteine protease, i.e., caspase-3 (Nicholson and Thomberry 1997). In the present report, the presence of an active caspase-3 in RMD undergoing apoptosis has been verified by demonstrating a rapid elevation of the 85-kDa PARP fragments and the fragmentation of DNA. This assumption is based on the following: (1) cleavage of an intact form of PARP (116 kDa) into the inactive 85-kDa fragment and (2) production of a ladder pattern of DNA fragments through cleavage at the linker regions of the nucleosomes require the presence of active caspase-3 (Lazebnik et al. 1994; Tewari et al. 1995; Liu et al. 1996; Jänicke et al. 1998).

Estrogen and its related compounds are considered as mitogens for the normal differentiation and development of the vertebrate female sex tract where they induce transient activation of c-Jun expression (Weisz et al. 1990). However, excessive amounts of these compounds have adverse effects. The association of pre- or neonatal DES (a synthetic stilbene estrogen) exposure with genital-tract abnormalities is one of the best-known examples of such effects. In avian and mammalian species, it has been confirmed that excess DES exposure causes an alteration in target gene expression (Teng and Teng 1985; Di Augustine et al. 1988; Nelson et al. 1994). My current observations agree with these findings and suggest that the content of c-Jun proteins (45 kDa and 66 kDa) in the RMD is suppressed by prenatal DES exposure. This is accompanied by a series of chain reactions, e.g., a reduction of caspase-3 activity, an inhibition of DNA fragmentation, and the reversal of the programmed death of RMD. The molecular action of the synthetic estrogen DES in chick MD differentiation can be interpreted in terms of the hormone down-regulation of MIS that has been suggested by Stoll et al. (1993). It can also be regarded as a weakened MIS interaction with its target sites in mesenchymal cells, making it insensitive to the regressive effects of MIS (Wang et al. 1990). The mechanism of hormonal action mediating the expression of c-*jun* protooncogene is little known and needs to be investigated.

In conclusion, the present data provide evidence that differential expression (quantitative and qualitative) of c-Jun proteins takes place in chick MDs at various stages of differentiation. In the growing MD, the content of c-Jun proteins is up-regulated and then declines, whereas in the dying MD, c-Jun proteins are overexpressed and are accompanied by caspase-3 activation and DNA fragmentation. These apoptotic events involved in MD death can be inhibited by in vivo DES treatment. The system described here should provide a tool for investigating the molecular mechanism by which c-Jun triggers the gene products that are capable of stimulating growth or apoptosis and for identifying their downstream targets.

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