Molecular mechanisms of hormone-mediated Müllerian duct regression: involvement of β-catenin

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SUMMARY

Regression of the Müllerian duct in the male embryo is one unequivocal effect of anti-Müllerian hormone, a glycoprotein secreted by the Sertoli cells of the testis. This hormone induces ductal epithelial regression through a paracrine mechanism originating in periductal mesenchyme. To probe the mechanisms of action of anti-Müllerian hormone, we have studied the sequence of cellular and molecular events involved in duct regression. Studies were performed in male rat embryos and in transgenic mice overexpressing or lacking anti-Müllerian hormone, both in vivo and in vitro.

Anti-Müllerian hormone causes regression of the cranial part of the Müllerian duct whereas it continues to grow caudally. Our work shows that this pattern of regression is correlated with a cranial to caudal gradient of anti-Müllerian hormone receptor protein, followed by a wave of apoptosis spreading along the Müllerian duct as its progresses caudally. Apoptosis is also induced by AMH in female Müllerian duct in vitro. Furthermore, apoptotic indexes are increased in Müllerian epithelium of transgenic mice of both sexes overexpressing the human anti-Müllerian hormone gene, exhibiting a positive correlation with serum hormone concentration. Inversely, apoptosis is reduced in male anti-Müllerian hormone-deficient mice. We also show that apoptosis is a decisive but not sufficient process, and that epitheliomesenchymal transformation is an important event of Müllerian regression.

The most striking result of this study is that anti-Müllerian hormone action in peri-Müllerian mesenchyme leads in vivo and in vitro to an accumulation of cytoplasmic β-catenin. The co-localization of β-catenin with lymphoid enhancer factor 1 in the nucleus of peri-Müllerian mesenchymal cells, demonstrated in primary culture, suggests that overexpressed β-catenin in association with lymphoid enhancer factor 1 may alter transcription of target genes and may lead to changes in mesenchymal gene expression and cell fate during Müllerian duct regression. To our knowledge, this is the first report that β-catenin, known for its role in Wnt signaling, may mediate anti-Müllerian hormone action.

Key words: AMH, MIS, β-catenin, LEF1, Müllerian duct regression, Apoptosis, Epitheliomesenchymal transformation, Rat, Mouse

INTRODUCTION

Hormones secreted by the fetal gonads control the differentiation of the reproductive tract and external genitalia. The experiments of Jost (1953) demonstrated that a specific testicular factor is required in the male fetus for the regression of the Müllerian duct, the anlagen of the uterus, oviducts and upper part of the vagina. This factor, called anti-Müllerian hormone (AMH) or Müllerian inhibiting substance (MIS) or factor (MIF), is a homodimeric glycoprotein, secreted by the Sertoli cells of the testes (Blanchard and Josso, 1974; Tran et al., 1977). Testosterone, secreted by testicular Leydig cells, masculinizes the external genitalia and urogenital sinus. Thus, the fetal testes produce two hormones, one that stimulates Wolffian duct differentiation and one that inhibits the development of the Müllerian duct.

The period of sensitivity of the Müllerian duct to AMH, termed the ‘critical period’, is transient and programmed at the end of the ambisexual stage. Before this period, growth and maintenance of the Müllerian duct are not hormone-dependent. After the critical period, Müllerian ducts degenerate in the male, even in the absence of AMH, when AMH no longer inhibits Müllerian ducts in the female (Josso et al., 1977). In the rat, the window of sensitivity to AMH is 14-15 days postcoitum (dpc). Studies carried out in vivo and in vitro have demonstrated that the regression of the Müllerian duct begins at the cranial part of the duct and progresses cranio-caudally from 14 to 16 dpc in the rat (Picon, 1969; Tsuji et al., 1992). An organ culture system was developed to assay for AMH
bioactivity in which 14.5 day female rat urogenital ridges were cultured for 3 days with or without fetal testes. Male Müllerian ducts were maintained when explanted without testes on day 14.5, and regression of female Müllerian duct was obtained when urogenital ridges were co-cultured with a fetal testis whatever the age of the fetal testis (Picon, 1969).

Since then, genetic manipulation of the mouse germline has led to the generation of models for AMH function which have confirmed that the regression of the Müllerian duct is one unequivocal biological activity of AMH. Female transgenic mice overexpressing the human AMH gene under the control of the mouse methallothionein promoter (MT-hAMH) lack uterus and oviducts (Behringer et al., 1990), whereas in male homozygous AMH-deficient (AMH-KO) mice Müllerian ducts do not regress (Mishina and Behringer, 1996).

Cell biology studies of the Müllerian duct regression have identified several cellular changes. (i) The morphological transformation of the mesenchymal cells surrounding the Müllerian duct which form a continuous layer with scarce intercellular space (Dyche, 1979). (ii) The disruption of the periductal basement membrane, the change of orientation for some epithelial cells and their entry in the mesenchymal compartment (Dyche, 1979; Trelstad et al, 1982; Wartenberg, 1985). (iii) The apoptosis of Müllerian epithelial cells (Price et al., 1977; Catlin et al., 1997; Roberts et al., 1999). AMH acts via a paracrine mechanism, since the mesenchymal cells surrounding the Müllerian duct are those expressing the AMH type II receptor (AMHRII) (Baarends et al., 1994; di Clemente et al., 1994; Teixeira et al., 1996). AMH is a member of the TGF-β family, which signals through a receptor complex formed by two distantly related serine/threonine kinases (Massagué and Weis-Garcia, 1996). The primary receptor, or type II receptor, binds its ligand, but must be coexpressed with an appropriate type I receptor for signal transduction. The cDNA and the gene for the AMH type II receptor have been cloned (Baarends et al., 1994; di Clemente et al., 1994; Imbeaud et al., 1995), and recent in vivo data demonstrated a high specificity for the interactions between the AMH ligand and its type II receptor (Mishina et al., 1999). How AMH binding to its receptor in the periductal mesenchyme triggers the morphological changes in the epithelium is still somewhat obscure.

How does the Müllerian duct exposed to AMH regress at its cranial part and progress at its caudal part? Is the apoptotic process in the Müllerian epithelial cells decisive for ductal regression? How is the effect of AMH signal mediated in the periductal mesenchyme? In this study we demonstrate that AMH receptor protein expression accounts for the cranial to caudal pattern of Müllerian duct regression, and that a wave of cell death spreads along the Müllerian duct as it progresses caudally. We also show that apoptosis is a decisive but not sufficient process, and that epitheliomesenchymal transformation is an important event of the Müllerian duct regression. Finally, our results reveal that AMH action in the peri-Müllerian mesenchyme leads to an increase of the cytoplasmic levels of uncomplexed β-catenin, which may interact with the lymphoid enhancer factor 1 (LEFI) in the nucleus and probably alters transcription of target genes. Thus, we show for the first time that β-catenin, known for its role in Wnt signaling, may mediate AMH action in the peri-Müllerian mesenchyme.

**MATERIALS AND METHODS**

**Animals, fetus collecting and determination of genotypes**

Fetal male and female rats at 14.5 to 16 dpc were obtained from timed pregnant Wistar rats (Centre d’Elevage R. Janvier). Pregnant rats were asphyxiated in a CO₂ chamber, the fetuses quickly removed from the uterine horns, and the urogenital ridges with gonads separated from the rest of the body.

Fetal male and female mice at 14 dpc were obtained from C57BL/6J and transgenic mice either ectopically expressing human AMH throughout development (MT-hAMH), or lacking AMH (AMH-KO). MT-hAMH mice, descended from the transgenic line 1789-1 (Behringer et al., 1990), were genotyped by dot hybridization of DNA, using the species-specific exon 1 of the human AMH gene as probe (Cate et al., 1986). The serum concentration of AMH was assayed by ELISA, using a monoclonal antibody directed against human recombinant AMH (Carré-Eusèbe et al., 1992). Because of the high sensitivity of the assay, it could be performed on 10 μl of blood obtained by cardiac puncture of 14-day-old embryos (Lyet et al., 1995). AMH-KO (The Jackson Laboratory) were genotyped by Southern blot after digestion of tail DNA by EcoRI and EcoRV enzymes, using a 3' probe external to the targeting vector, as previously described (Behringer et al., 1994).

**Organ culture and primary culture of peri-Müllerian mesenchymal cells**

Male and female urogenital ridges were removed from fetal Wistar rats at 14 dpc. The explants were placed on an agar-coated grid in organ-culture dishes (Falcon) in which DMEM (Gibco), either with or without recombinant hAMH at a final concentration of 3 μg/ml, was added up to the level of the grid. Urogenital ridges were cultured for 24, 30 and 38 hours.

Peri-Müllerian mesenchymal tissue was isolated as previously described (Tsujii et al., 1992) with modifications. 15-day-old female urogenital ridges were incubated in 2.5 mg/ml collagenase A (Boehringer) in HBSS medium without Ca²⁺ and Mg²⁺ for 10 minutes at 37°C. The Müllerian mesenchyme was stripped from the epithelial duct and cultured on poly-D-lysine four-chamber Lab-Tek slides (Nunc) in CMRL-1066 medium (Life Technologies) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Eurobio), amino acids (Eurobio) and 10% fetal calf serum. After 24 hours in culture in the presence or absence of AMH (3 μg/ml), cells were fixed 2 minutes in acetonemethanol (v:v), permeabilized in 1× PBS containing 0.5% Triton X-100 and processed for immunohistofluorescence.

**Histology and detection of apoptotic cells**

Male and female urogenital ridges were fixed in 4% phosphate-buffered saline (PBS) paraformaldehyde for 2 hours at 4°C, embedded in paraffin, and serially sectioned transversely at 5 μm. Rat urogenital ridges were obtained every 4-6 hours over a 48 hours period (14 to 16 dpc), whereas mice urogenital ridges were recovered at 14 dpc at 08.00 or 14.00 hours. For histological examination, 1 out of 10 serial 5 μm sections was stained with hematoxylin and eosin.

Apoptotic cells were detected in Müllerian and Wolffian ducts using a fluorescent antibody for the staining of DNA strand breaks by the terminal deoxynucleotidyl transferase deoxy-UTP-nick end labeling (TUNEL) assay. TUNEL labeling was performed using Apoptag FITC kit (Oncor) following the manufacturer’s instructions. Counterstaining with propidium iodide visualized epithelium nuclei for counting the total per section. The count of total nuclei was validated by counting nuclei on adjacent sections stained with 1 μg/ml Hoechst-33258 dye (Sigma). Counting of apoptotic cells was performed at 25 μm intervals in order to ensure that they were counted once only, as shown by preliminary experiments. The results were expressed as a percentage of the number of apoptotic nuclei over the...
The total number of epithelium nuclei. Male and female urogenital ridges were recovered from at least three or four different mothers at each time point, in order to minimize maternal effects.

**Immunohistofluorescence**

Detection of cytokeratin, laminin and collagen IV was carried out on tissues fixed with 4% paraffin formaldehyde in PBS for 2 hours at 4°C, washed in PBS with increasing concentrations of sucrose (12, 15 and 18%), embedded in Tissue-Tek OCT compound (Miles) and stored at −80°C. Cryostat sections (6 μm thickness) were fixed in acetone for 10 minutes at −20°C and stored at −20°C until assay. Detection of β-catenin was performed on tissue flash-frozen in Tissue-Tek OCT compound in dry ice and stored at −80°C. 6 μm cryostat sections were fixed in acetone: methanol (1:1) for 10 minutes at −20°C.

Frozen sections were blocked in Block Buffer (1× PBS, 10% normal goat serum [Dako], 5% bovine serum albumin [Sigma]) for 1 hour at room temperature. For immunodetection, sections were incubated overnight at 4°C with primary antibodies: rabbit polyclonal anti-keratin wide spectrum screen antibody (1:800, Dako), mouse monoclonal anti-vimentin antibody (1:100, Dako), rabbit polyclonal anti-laminin antibody (1:100, Dako), rabbit polyclonal anti-collagen IV antibody (1:250, Institut Pasteur, Lyon), mouse monoclonal anti-β-catenin antibody (1:100, Transduction Laboratories), rabbit polyclonal anti-LEF1 antibody (1:500, generous gift from R. Grosscheld) and rabbit polyclonal anti-AMHRII antibody (10 μg/ml; Gouédard et al., 2000). Fluorescein and/or Texas red goat either anti-rabbit or anti-mouse IgG conjugates were used at a 1:250 dilution as secondary antibodies. The coverslips were mounted in Vectorshield (Vector) and the tissues examined and photographed on a Zeiss Axioskop microscope equipped for epifluorescence.

**In situ hybridization**

In situ hybridization was performed as previously described by Oosterwegel et al. (1993) with some modifications. Male urogenital ridges from fetal Wistar rats at 14 and 15 dpc were fixed in 4% buffered-parafomaldehyde for 2 hours at 4°C, embedded in paraffin wax, sectioned transversely at 5 μm thickness and mounted on superfrrost plus slides. Sections were incubated with 35S-labeled antisense or sense RNA probes (18750 cpm/ml) at 50°C. High stringency washing were performed in 5x SSC/10 mM DTT and 50% formamide/20°C. Cryostat sections were fixed in acetone:methanol (v:v) for 10 minutes at −20°C.

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**RESULTS**

**Timing and incidence of apoptosis during Müllerian duct regression**

Fluorescence labeling of DNA fragmentation with TUNEL assay was performed on male and female urogenital ridges between embryonic days 14 to 16 in the rat. Fig. 1 shows the temporal changes in the Müllerian and Wolffian ducts apoptosis. TUNEL-positive cells were observed in the epithelium of both ducts in the male (Fig. 1C) and female (Fig. 1F). In both ducts, the nuclear fragments and the cytoplasm condense with extensive protrusion of cell surface, and apoptotic bodies of various sizes are produced. These bodies, specifically detected by TUNEL technique, are then rapidly phagocytised by neighbouring cells. In male Müllerian ducts, apoptotic bodies were also present in basal regions of epithelial cells and protruded in the surrounding mesenchyme.

In the male, there appeared to be no difference (P>0.05) in the percentage of apoptotic cells (apoptotic index, percentage of TUNEL-positive nuclei per total nuclei) between the Müllerian and the Wolffian ducts until 15 dpc 04.00 (Fig. 1A). Thereafter this percentage increased significantly in the Müllerian duct, was maintained at a constant level (approximately 10%), and was significantly higher than either in the Wolffian duct (P<0.001) or in the female Müllerian duct (P<0.001) (Fig. 1A-F). In female littermates, the epithelial cells of the Müllerian duct displayed levels of apoptotic cells similar or lower than those of the Wolffian duct (Fig. 1B,F,G).

**Spatiotemporal analysis of apoptosis in the Müllerian duct**

A detailed spatiotemporal analysis of apoptosis was performed on Müllerian ducts of male 15- and 16-day-old rat embryos. The Müllerian duct was divided into three parts along its craniocaudal axis (Fig. 2A). The Müllerian duct, limited on day 15 at 04.00 to the cranial part, grew towards the urogenital sinus, and the caudal part appeared only on day 15 pc after 13.00. Thus, its length increased threefold in less than 15 hours from 15 dpc 04.00 to 15 dpc 19.00 (Fig. 2B). On day 15, the highest incidence of apoptosis was observed in the cranial and intermediate parts of the Müllerian duct (15 dpc 09.00 and 15.00, Fig. 2A). On day 16 (16 dpc 10.00), the apoptotic index was back to a very low level in the cranial part (2.6±1.8%) whereas it was significantly higher in the intermediate (10.6±2.0%) and caudal (16.8±2.9%) parts (Fig. 2A).

Representative apoptosis profiles of single urogenital ridges of equal length and stage of regression in Müllerian ducts of both gender were compared. The temporal changes in the Müllerian and Wolffian ducts apoptosis were comparable (16.2±2.7%) to the
Fig. 1. (A,B) Percentage of apoptotic cells (apoptotic nuclei/total nuclei ×100) in Müllerian and Wolffian ducts of male (A) and female (B) rat embryos at different times of days 15 and 16 post-coitum (pc). The percentages were expressed as the mean±s.e.m. for four animals. Statistical analyses were performed using ANOVA followed by Turkey’s test. Four independent samples were analyzed for each tissue at each time. Statistical difference **$P<0.01$ between 15 dpc 04.00 and 09.00 groups, ***$P<0.001$ between 15 dpc 04.00 and 15.00, 19.00 and 16 dpc 10.00. (C-G) Cross sections of male (C, 15 dpc 15.00; D, 15 dpc 19.00; E, 16 dpc 10.00) and female (F, 15 dpc 19.00; G, 16 dpc 10.00) genital ridges were analyzed for apoptosis by TUNEL staining. Bright green TUNEL-positive cells (arrowheads) are observed in the epithelium of the Müllerian (C-F) and Wolffian (C,F) ducts in male and female. No labeling is seen in ducts of the female genital ridge at 16 dpc 10.00 (G). MD, Müllerian duct; WD, Wolffian duct. Bar, 40 mm in all panels.

Fig. 2. (A) Percentages of apoptotic cells in the cranial, intermediate and caudal parts of the Müllerian duct of male rat embryos at different times of days 15 and 16 pc. The percentages were calculated and expressed as the mean±s.e.m. for four animals. Significance was determined by Student’s t-test (*$P<0.03$, **$P<0.006$). (B) Representative apoptosis profiles in single urogenital ridge during Müllerian regression.
percentages obtained in the male Müllerian ducts (16.8±2.9%) (Fig. 3A). The apoptotic indexes were significantly lower in the epithelium of the Wolffian duct in males (6.8±1.8%) and females (7.1±1.7%) (not shown). Untreated female urogenital ridges in vitro exhibited only a few apoptotic cells in the Müllerian duct (not shown), as did female urogenital ridges in vivo (Fig. 1B,F,G).

In the mouse, the critical period for Müllerian duct regression occurs between 13 and 14 days of gestation. To determine the effects of AMH upon Müllerian duct apoptosis, male and female 14-day-old embryos from wild-type and transgenic MT-hAMH or AMH-KO mice were tested for the presence of apoptotic cells. The percentage of apoptotic cells in the Müllerian duct epithelium was significantly higher in male (11.0±1.6%) than in female embryos (2.3±0.5%; Fig. 4A,B). In AMH-KO male embryos, in which the Müllerian ducts do not regress due to the absence of AMH, few TUNEL-positive cells were detected (Fig. 4C), and the apoptotic index (2.8±0.6%) was quite comparable with that of control females (2.3±0.5%; Fig. 4A,B). In male and female transgenic mice overexpressing human AMH gene, a large number of apoptotic cells were observed in the epithelium of the Müllerian duct (Fig. 4F), and their percentages were correlated with serum AMH concentration (Fig. 4A,B).

Cranial to caudal AMHRII expression in the peri-Müllerian mesenchyme

The expression of the rat AMHRII was analyzed in the peri-Müllerian mesenchymal cells by in situ hybridization with a labeled AMHRII antisense probe and quantified using an image analyzer. Specificity was confirmed using a sense probe (not shown). Analysis of AMHRII mRNA expression was carried out at 4- to 6-hour intervals. At 14 dpc 20.00, a marked AMHRII mRNA expression was first detected in the peri-Müllerian mesenchymal cells and was maintained at the same level until 15 dpc 10.00 when it slightly increased (Fig. 5A).

Conversely, a detailed spatiotemporal analysis showed clear changes in the AMHRII mRNA expression. As the Müllerian duct grew caudally towards the urogenital ridge, AMHRII appeared in the adjacent mesenchyme, first in the mesenchyme underlying the cranial part of the Müllerian duct, then in the intermediate part, and finally in the caudal part (Fig. 5B-F). The cranial to caudal AMHRII expression is illustrated by the micrographs of paraffin transverse sections of a male urogenital ridge at 15 dpc 04.00 (Fig. 5G-L). The pattern of AMHRII expression was also analyzed by immunofluorescence on cryostat transverse sections using an antibody directed against AMHRII protein. AMHRII protein was detected in the peri-Müllerian mesenchymal, starting at 15 dpc 07.00. At this time, a slight reactivity was observed in the mesenchymal cells...
located between the Müllerian duct and the surface epithelium (Fig. 5M). Later, a strong staining was obtained in all mesenchymal cells surrounding the Müllerian duct (Fig. 5O). Serial cryostat cross sections from a male urogenital ridge at 15 dpc 11.00 showed that AMHRII protein appeared progressively in a cranial to caudal direction (Fig. 5N-Q). It should be noted that the treatment of the tissue affected the length of the Müllerian duct (tissue fixed and embedded in paraffin wax as compared to flash-frozen tissue). MD, Müllerian duct; WD, Wolffian duct; T, testis. Bars, 100 μm (G-L); 40 μm (M-Q).

**β-catenin and LEF1 expression in the urogenital ridges**

To examine β-catenin and LEF1 expression during Müllerian duct regression, immunofluorescence staining was performed on serial cryostat transverse sections of urogenital ridges prepared from male and female 14 to 15-day-old rat embryos. In female and male embryos, at all times, membrane-associated β-catenin reactivity was observed in the surface epithelium, in the epithelial cells of the Wolffian duct and to a lesser degree of the Müllerian duct (Fig. 6A-F). After 15 dpc 07.00, a strong cytoplasmic β-catenin reactivity was observed in peri-Müllerian mesenchymal cells, whereas cells of the surface epithelium and the Wolffian duct continued to be membrane-associated β-catenin reactive (Fig. 6C-G). A progressive cranial to caudal expression characterized the pattern of β-catenin expression (Fig. 6C-F). In female embryos, mesenchymal cells of the Müllerian duct did not demonstrate cytoplasmic β-catenin reactivity at any time period studied (Fig. 6A). Specific antibody directed against the architectural transcription factor LEF1 detected AMH-independent nuclear expression of LEF1 in the epithelial and mesenchymal cells of the Müllerian duct and diffusely throughout the urogenital ridges of male and female embryos (Fig. 6G), consistent with the LEF1 mRNA expression noted previously during murine embryogenesis by Oosterwegel et al. (1993). Nuclear β-catenin immunoreactivity was observed in several peri-Müllerian mesenchymal cells of male urogenital ridges, co-localized with LEF1 (Fig. 6G, high magnification insert).

**Effects of AMH upon stromal β-catenin expression**

To determine whether AMH triggers stromal β-catenin...
expression, immunostaining was performed on cryostat transverse sections of urogenital ridges with gonads from male and female 14-day-old embryos cultured for 30 hours. Female urogenital ridges were cultured with or without human recombinant AMH at a final concentration of 3 μg/ml. A cytoplasmic accumulation of β-catenin was observed in peri-Müllerian mesenchymal cells of female rat urogenital ridges at 15 dpc 09.00 cultured for 24 hours in the presence of AMH. These cells, obtained by microdissection and characterized by vimentin and AMHR II immunoreactivities (Fig. 8A,B), exhibited a strong cytoplasmic accumulation of β-catenin in the presence of AMH; in several of these cells, β-catenin was also detected into the nucleus, co-localized with LEF1 (Fig. 8D). Neither nuclear nor cytoplasmic β-catenin immunoreactivity was seen in untreated cells which showed only a membrane-associated β-catenin immunostaining (Fig. 8C).

**Epitheliomesenchymal transformation during Müllerian duct regression**

By day 15.5 in the male rat embryo, the basement membrane of the Müllerian duct became patchy and

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Fig. 6. (A,B) Immunostaining showing the expression of β-catenin in urogenital ridges from rat embryos. (A,B) In the female at 15 dpc 10.00 (A) and the male at 15 dpc 07.00 (B), a strong membrane-associated β-catenin reactivity is observed in the surface epithelium and the epithelial cells of the Wolffian duct (arrows). (C-F) Serial cryostat transverse sections of a male rat urogenital ridge at 15 dpc 09.00 showing the progressive cranial to caudal expression of β-catenin (C: 100 μm; D: 350 μm; E: 600 μm; F: 950 μm from the cranial tip of the Müllerian duct). (G) 15 dpc 09.00: male urogenital ridge stained with anti-β-catenin (orange) and anti-LEF1 (green) antibodies. Cross section adjacent to the section illustrated in D showing a nuclear LEF1 immunoreactivity and an intense cytoplasmic accumulation of β-catenin in peri-Müllerian mesenchymal cells (arrow). The high magnification insert shows nuclear β-catenin immunoreactivity colocalized with LEF1 (arrowheads). MD, Müllerian duct; WD, Wolffian duct. Bar, 40 μm (A,B); 55 μm (C-F); 20 μm (G).

Fig. 7. (A-F) Effects of AMH in vitro upon β-catenin and LEF1 expression in urogenital ridges from 14.0-day-old male and female rat embryos cultured for 30 hours. As in vivo, a strong membrane-associated β-catenin reactivity is observed in the surface epithelium and the epithelial cells of the Wolffian duct (arrows) (A,B), whereas β-catenin reactivity is located in the cytoplasm of the peri-Müllerian mesenchymal cells in males (A) and in females exposed to recombinant hAMH (B) (arrowheads). (D,E) A nuclear LEF1 immunoreactivity (green) and an intense cytoplasmic accumulation of β-catenin (orange) are observed in peri-Müllerian mesenchymal cells using double immunostaining (arrowheads). (C,F) In untreated females, no cytoplasmic accumulation of β-catenin is detected in the peri-Müllerian mesenchyme. A membrane-associated β-catenin reactivity is observed in the surface epithelium and the epithelial cells of Müllerian duct and Wolffian duct (arrow). MD, Müllerian duct; WD, Wolffian duct. Bar, 40 μm in all panels.
discontinuous. The immunostaining for collagen IV and laminin showed a disruption of the basement membrane of the Müllerian duct (Fig. 9A for laminin). This disruption was associated with apoptosis of the epithelial cell (Fig. 9B) which protruded into the periductal mesenchyme (Fig. 9C). Apoptotic cells were detected in the mesenchymal compartment (Fig. 9D) only after the disruption of the basement membrane. The percentage of apoptotic cells associated with the discontinuities in the basement membrane increased from 18.4±5.1% at 15 dpc 11.00 to 37.5±2.0% at 15 dpc 19.00. The loss of an intact basement membrane was an early event in epithelial-mesenchymal transformation. Once the epithelial-mesenchymal interface was broached, viable cells in the epithelium changed their orientation for entry into the mesenchymal compartment as described previously by Trelstad et al. (1982). Epithelial cells entering the mesenchymal compartment appeared as a ‘mesenchymal-like cell layer’ surrounding the Müllerian duct epithelium (Fig. 9E) and overexpressing vimentin (not shown). Apoptosis due to the disruption of the basement membrane followed by epithelial-mesenchymal transformation was a major event in Müllerian duct regression as demonstrated by the reduction in cell number of the Müllerian duct epithelium. Whereas at day 15 in the morning (04.00) the cell number per cranial cross section was estimated at 21.7±1.7 cells, late in day 15 (19.00) it was still 17.3±0.9 cells, and then strikingly decreased in day 16 (09.00) with an average of 10.3±0.4 cells, as illustrated in the Fig. 9G,F.

DISCUSSION

Previous studies have investigated only selected stages of regressing Müllerian duct. In this work we describe the sequence of cellular and molecular events involved in AMH-induced Müllerian duct regression, combined with the progressive growth of the Müllerian duct along the craniocaudal axis (Fig. 10). AMH induces the regression of the Müllerian epithelium by a paracrine mechanism originating in the adjacent mesenchyme where β-catenin/LEF1 appears as a possible mediator of AMH action. Thus, we demonstrate that AMH induces accumulation of cytoplasmic β-catenin in peri-Müllerian mesenchyme, immediately following the appearance of AMHRII protein.

Apoptosis is a decisive event of AMH-induced Müllerian duct regression. Two types of apoptosis can be distinguished: (i) type I, observed in the epithelium of Müllerian and Wolffian ducts of both sexes, and characterized by a rapid phagocytosis of apoptotic bodies by neighbouring cells; (ii) type II, detected only in the male Müllerian duct exposed to AMH, resulting from disruption of basement membrane, and in which apoptotic bodies protrude into the mesenchyme where they are probably phagocyted. The number of type I apoptotic cells significantly increases in Müllerian duct approximately 2 hours after AMH interacts with its receptor, first in the cranial part, and thereafter all along the Müllerian duct as it grows caudally. Type II apoptotic cells are observed only 5 hours later and allow migration of healthy epithelial cells throughout the connective tissue and their transformation into mesenchymal-like cells, another important event of Müllerian duct regression.

AMH induces apoptosis in the Müllerian duct

Apoptosis or programmed cell death is a process through which individual cells carry out a suicide program in a healthy tissue. It occurs in embryonic development, reproductive function, and also during involution of organs (Gosden and Spears, 1997; Hsueh et al., 1994; Jacobson et al., 1997). AMH has been shown to induce apoptosis in the Müllerian duct in vivo (Price et al., 1977) and in vitro (Price et al., 1979; Roberts et al., 1999). Our results are in agreement and extend the findings of other groups. The apoptotic process during Müllerian duct regression can be reproduced in an organ.
culture system, and recombinant hAMH can induce apoptosis in the epithelium of the female Müllerian duct. Furthermore, the action of AMH on apoptosis induction was confirmed in transgenic mice. Apoptosis is reduced in male AMH knockout mice and inversely is increased up to threefold in Müllerian epithelium of male and female mice overexpressing the hAMH gene. Interestingly, the percentages of apoptotic cells (this study) and the degree of regression (Lyet et al., 1995) were positively correlated with the serum hAMH concentration. Thus, it is now well established that the AMH-induced regression of the Müllerian duct is associated with apoptosis. AMH-induced apoptosis is the primary event of the Müllerian duct regression, but the nature of upstream signaling pathways that regulate apoptosis remains to be investigated.

**AMH-receptor expression correlates with a cranial to caudal wave of type I apoptosis and with the pattern of Müllerian duct regression**

AMH induces Müllerian duct regression indirectly through mesenchymal tissue. Indeed, AMH has no effect either on isolated Müllerian epithelial cells (Tsuji et al., 1992) or on epithelial ducts cultured with heterologous mesenchyme lacking AMH receptors (Roberts et al., 1999). We have analyzed and quantified the AMHRII mRNA expression during the period of sensitivity to AMH. AMHRII transcripts are detected in peri-Müllerian mesenchyme of both male and female rat embryos as early as 14 dpc 20.00. They appear first in the cranial part of the urogenital ridges, and then in the intermediate and caudal parts as the Müllerian duct grows caudally. The availability of a specific antibody directed against AMHRII confirms a similar cranial-caudal gradient of the receptor protein which appears approximately 10 hours after the transcripts. Differences in the spatial distribution of the mesenchymal AMHRII are observed between male and female. In the male, AMHRII expression levels varied along the cranial-caudal axis of the duct due to the decrease of the mesenchymal expression of AMHRII as Müllerian duct regression proceeds. In the female, the mesenchymal AMHRII expression is maintained along the length of the Müllerian duct, consistent with persistence of the Müllerian duct (Teixeira et al., 1996 and this work). Expression of AMHRII requires an intact epithelium and depends on an epithelial-derived Wnt7a

**Fig. 9.** (A-D) Cross sections of urogenital ridges of male embryos obtained from a pregnant rat female at 15 dpc 19.00. (A) Cryostat cross section stained with anti-laminin antibody showing the disruption of the Müllerian duct basement membrane (arrowhead). (B,C) Neighboring paraffin cross-sections showing an apoptotic epithelial cell associated with disruption of the basement membrane (TUNEL-positive cell, arrow; B), which protrudes into the adjacent mesenchyme (Hoechst staining, arrowhead; C). (D) Arrows show TUNEL-positive cells in the Müllerian duct and the arrowhead a TUNEL-positive cell in the adjacent mesenchyme. (E) Epitheliomesenchymal transformation leads to a ‘mesenchymal-like cell layer’ surrounding the Müllerian duct epithelium as shown in the cross-section of a male urogenital ridge at 15 dpc 24.00 (arrow, dotted circle). (F,G) Immunostaining of the Wolffian and Müllerian duct epithelial cells with an anti-keratin antibody showing the reduction of Müllerian epithelium between 15 dpc 19.00 (F) and 16 dpc 10.00 (G) (arrows). MD, Müllerian duct; WD, Wolffian duct. Bar, 40 μm (A-C, E-G) 20 μm (D).

**Fig. 10.** Chronology of molecular and cellular events during cranial Müllerian duct regression in the rat. Hours shown indicate the beginning of the process. β-cate, β-catenin.
signal. Parr and McMahon (1998) have shown that male Wnt7a-deficient mice fail to undergo regression of the Müllerian duct as a result of the absence of AMH receptor. The loss of Wnt7a expression due to the regression of the Müllerian epithelium may account for the progressive loss of AMHRII expression in the Müllerian mesenchyme during the craniocaudal regression of the Müllerian duct.

Using the sensitive in situ TUNEL method to detect and to quantify apoptotic cells, we have precisely determined the timing and the spatiotemporal changes of the apoptotic process along the whole length of the Müllerian duct. Increasing apoptosis was detected on and after the 15th dpc past 09:00. The percentage of apoptotic cells was thereafter maintained at a mean level of 10%, but could be twofold higher locally. This study, carried out at 4-6 hour intervals during the period of sensitivity to AMH, demonstrates for the first time that apoptosis is a progressive process along the Müllerian duct. Apoptosis restricted first to the cranial part was then detected progressively in the intermediate and caudal parts of the Müllerian duct. Thus, we establish clearly that a wave of cell death spreads along the Müllerian duct as it progresses caudally, and that there is a strong spatiotemporal correlation between the cranial to caudal expression pattern of AMHRII, the initiation of the apoptosis process and Müllerian duct regression. Our results are contrary to the data reported by Roberts and colleagues (1999), who did not find any cranial to caudal gradient of AMHRII expression and apoptosis. This discrepancy is probably due to the longer time interval of examination (24 hours, from 14.5 to 15.5 dpc) used by this group.

**Apoptosis of type II due to disruption of basement membrane precedes epitheliomesenchymal transformation**

During the latter half of day 15 discontinuities increasingly appear at multiple sites in the basement membrane of the Müllerian duct, as previously described by Trelstad et al. (1982). Here we show that disruption of the basement membrane is associated with apoptosis of the epithelial cells, allowing healthy neighbouring epithelial cells to migrate throughout the connective tissue and transform into mesenchymal cells. Müllerian duct regression has been the subject of a number of morphological studies which demonstrate changes in the peri-epithelial extracellular matrix (Dyche, 1979; Hayashi et al., 1982; Iwaka et al., 1984; Parenko et al., 1986). Müllerian duct regression is triggered by or is closely associated with changes in the surrounding mesenchyme as well as changes in the extracellular matrix at the epithelial-mesenchymal interface. Thus, the dissolution of the Müllerian duct basement membrane could be a major event in ductal regression (Trelstad et al., 1982). Basement membrane, composed predominantly of laminin and type IV collagen, preserves the differentiated morphology of epithelial cells. Degradation of the laminin and the type IV collagen may initiate the process of apoptotic cell death, as described in rat amnion (Lei et al., 1997, 1999), or in mammary gland during involution (Strange et al., 1992). Furthermore, basement membrane extracellular matrix was shown to suppress apoptosis of mammary epithelial cells in tissue culture and in vivo (Boudreau et al., 1995). Indeed, loss of contact with specific extracellular matrix molecules is recognized as a proapoptotic signal (Frisch and Francis, 1994; Meredith et al., 1993).

Once portions of the basement membrane have been lost and the cells have become apoptotic, changes occur in the organization of the epithelium. A number of viable epithelial Müllerian cells transform into mesenchyme-like cells able to migrate through the connective tissue. Interestingly, Dil-labeled Müllerian epithelial cells were observed migrating out of the duct during regression in male alligator embryos (Austin, 1995). The primary change in cytoskeletal proteins of the new mesenchymal cells is a loss of cytokeratin, which is abundant in epithelial cells, and an increase in vimentin, a marker for mesenchymal cells (van de Klundert et al., 1993). Thus, epitheliomesenchymal transformation is used to remodel unwanted epithelia, as described also for the palate medial edges (Fitchett and Hay, 1989). Epitheliomesenchymal transformation is a major event, which principally accounts for the loss of Müllerian epithelial cells during regression.

**β-catenin as a mediator of AMH-induced Müllerian duct regression**

β-catenin is a multi-functional protein that can affect both cell adhesion and signal transduction (Resnik, 1997; Willert and Nusse, 1998). This dual role involves the association of β-catenin with different partners in distinct subcellular compartments. Thus, β-catenin is found at the plasma membrane, in the cytoplasm and in the nucleus. The role of β-catenin in adhesion is well established. β-catenin associates with cell surface proteins involved in cell adhesion, linking transmembrane cadherin cell adhesion molecules to the actin filament network (Aberle et al., 1996; Kemler, 1993). These interactions allow the adhesion of cells to one another, which induces cell polarity and the acquisition of cell-cell junctions (Miller and Moon, 1996). β-catenin can also act as a transcriptional regulator and it is known to be involved in transducing Wnt signal.

Upon Wnt signaling, a cascade is initiated that results in the stabilization and the accumulation of cytoplasmic β-catenin (Papkoff et al., 1996). Indeed Wnt signal inactivates the down regulation of cytoplasmic β-catenin via the ubiquitin-proteasome pathway (Aberle et al., 1996). There is a concomitant translocation of β-catenin to the nucleus, where its interacts with the LEF/TCF (T cell factor) family of transcription factors to generate an efficient complex able to activate or repress gene transcription (Behrens et al., 1996; Waltzer and Bienz, 1999; Wodarz and Nusse, 1998). Wnt are secreted signaling molecules that have been linked to morphogenetic events. Only Wnts that have a strong cell transforming potential, such as Wnt1, Wnt2, Wnt3 and Wnt3a, have been shown to induce accumulation of β-catenin. The other Wnts, which had no or minimal effect on morphology, increased cytoplasmic β-catenin levels weakly or not at all (Shimizu et al., 1997). Wnt4 and Wnt7a, which belong at the last group, are present in the murine urogenital ridges during the period of sexually dimorphic development of the reproductive tract. Wnt4, expressed in mesenchyme cells, is required in both sexes for formation of the Müllerian duct (Vainio et al., 1999), and Wnt7a, expressed in the Müllerian epithelium, allows sexually dimorphic development in rendering the mesenchyme competent to respond to AMH through the AMH receptor (Parr and McMahon, 1988).
During the period of sexually dimorphic development of the reproductive tract, β-catenin is found at the plasma membrane in the epithelia, and in the cytoplasm and the nucleus in the Müllerian mesenchyme. The localization of β-catenin at the plasma membrane is seen, at all times, in epithelial cells of the surface epithelium, of the Wolffian duct and to a lesser degree of the Müllerian duct. The slight β-catenin immunoreactivity observed at the plasma membrane of the Müllerian epithelium, in both male and female, could be related to an autocontrol action of Wnt7a, which has been shown to have some weak effect on plasma membrane β-catenin expression (Shimizu et al., 1997).

AMH induces in vivo and in vitro a cytoplasmic accumulation of β-catenin in the peri-Müllerian mesenchymal cells. In vivo, cytoplasmic β-catenin accumulation is observed in the periductal mesenchyme of the male urogenital ridges during the period of sensitivity to AMH, and the pattern of the mesenchymal β-catenin accumulation along the cranial-caudal axis of the Müllerian duct correlates with the pattern of AMHRII expression. Cytoplasmic β-catenin reactivity is never detected in the periductal mesenchyme of the female in vivo, but recombinant hAMH may induce an accumulation of free β-catenin in peri-Müllerian mesenchymal cells in both organ and primary cell culture systems. Does the effect of AMH involve Wnt signaling in the Müllerian mesenchyme? This hypothesis appears unlikely in relation to Wnt7a because: (1) cytoplasmic accumulation of β-catenin is observed a long time after (15 dpc 09.00) the action of Wnt7a on the AMHRII gene expression (14 dpc 20.00); (2) recombinant hAMH increased cytoplasmic β-catenin levels in primary cultures of Müllerian mesenchymal cells in the absence of any epithelial Wnt7a signal. This hypothesis seems also unlikely for Wnt4, which has been shown unable to elevate cytosolic β-catenin levels (Shimizu et al., 1997).

The most striking result of this study is that β-catenin may be involved in AMH signaling. AMH induces in vivo and in vitro stabilization and accumulation of cytoplasmic β-catenin in peri-Müllerian mesenchymal cells, and β-catenin cytoplasmic accumulation correlates with increasing apoptosis in regressing Müllerian duct. Furthermore, β-catenin is also detected in the nucleus co-localized with LEF1. Overexpressed β-catenin in association with LEF1 probably leads to changes in mesenchymal gene expression and cell fate during regression of the Müllerian duct. The data reported here will serve as a starting point for the evaluation of the role of β-catenin in mediating AMH action in the peri-Müllerian mesenchyme as well as in other target organs.

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