Experimental contributions to the study of the embryology of the vagina

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BACKGROUND: Acién’s hypothesis, deduced from patients with malformations of the female genital tract, especially those with renal agenesis and ipsilateral blind hemivagina, affirms the embryology of the human vagina as deriving from the Wolffian ducts and the Müllerian tubercle and could explain the embryological origin of all the female genital malformations reported. In this study, we investigated the hypothesis in rats.

METHODS: Twenty-five pregnant rats were used to analyse female embryos (64) from day 15 (stage indifferent) to day 20 postcoitum (vagina completely formed). We performed transverse and longitudinal sections of embryos, haematoxylin–eosin tinction and immunohistochemical staining using markers specific to Wolffian derivatives. We also analysed the presence of these markers in the vagina of four adult rats.

RESULTS: The Müller ducts converge until they fuse into one tube, but caudally they diverge and finally they fuse with the ‘urogenital sinus bulbs’ that are actually the distal portion of the Wolffian ducts according to the immunohistochemical marking with GZ1 and GZ2. The Müllerian tubercle is observed between those elements. Then, the immunohistochemical staining can be seen all along the completely formed vagina, which is also observed in the vagina of the adult rat.

CONCLUSION: We prove the participation of Müller tubercle and Wolffian ducts in the formation of the vagina in rats, so we confirm experimentally Acién’s hypothesis about the human vagina embryology.

Key words: genital malformations/Müllerian ducts/rats/vagina/Wolffian ducts

Introduction
Female genital malformations are frequent (2–3% of fertile women, 3% of infertile women and 5–10% of women with reported miscarriages). Complex malformations are not so frequent, and they are rarely diagnosed or treated correctly. This is due to two important factors. Firstly, gynaecologists may not consider the malformations as a cause of the clinical symptoms, and secondly, the embryological origin of different elements of the genitourinary tract is not considered.

Although the embryological origin of the different genital organs is known, there are controversial ideas about the vagina, whose origin could be Müllerian (Hunter, 1929, 1930; Kurita and Cunha, 2001), Wolffian (Forsberg, 1965), from the urogenital sinus (Jost and Tavernier, 1956; Bulmer, 1957) and from different combinations of these structures (Mjöberg, 1926; Marshall and Beisel, 1978). The hypothesis proposed by Acién suggests that the vagina is formed from the Wolff ducts and the Müller tubercle (Acién and Armíñana, 1986; Acién, 1992). This hypothesis arose from trying to give an embryological explanation for the findings observed in patients with genitourinary malformations. To be exact, a systematic association between unilateral blind vagina and renal agenesis was observed, and it had been widely mentioned in literature (Goldstein et al., 1973; Yoder and Pfister, 1976; Fried et al., 1978; Feins and O'Connor, 1979; Acién and Armíñana, 1986; Acién et al. 1987, 1991; Acién, 1992). The embryological explanation of this constant association could be a common origin: ‘as the origin of the ureteral bud is in the mesonephric duct just at the opening to the urogenital sinus, the anomaly of one of these Wolffian ducts would produce a blind hemivagina and absence of the formation of the ureteral bud with consequent renal agenesis’. Studies by Bok and Drews (1983) had already pointed out that the protrusions of the urogenital sinus, ‘bulbs of the urogenital sinus’ or ‘sinus vagina’, during development could derive from the mesonephric ducts or Wolff ducts. In the present study, we aim to investigate the above-mentioned hypothesis by Acién experimentally in rat embryos.
Materials and methods

Twenty-five pregnant Wistar rats were sacrificed to analyse the female embryos from day 15 (indifferent stage) to day 20 postcoitum (formed vagina). We used four pregnant rats from each gestational age. A total of 125 fetuses were obtained after a Caesarean section on their mothers who were anaesthetised according to protocol for protecting animals from suffering (Real Decreto 223/8 for the protection of animals in experiments). The embryos were sectioned from below the liver to keep the piece, which contains the genital tract. The dissection was then carried out under a magnifying glass to identify the male and female embryos through inspection of the gonads. Once the females were identified (64 embryos), half of them were oriented for transversal sections and the other half for longitudinal sections. Then, they were set in paraffin.

Serial histological sections of 5 μm were obtained through the genitourinary tract. Then, sections were stained with haematoxylin–eosin and incubated with monoclonal antibodies specific to antigens located on the cellular membrane of the Wolffian cells and which do not react with Müllerian cells GZ1 and GZ2 (Dohr and Tarmann, 1987). Both antibodies are produced against the basolateral membrane of epithelial cells from the small intestine of rats and are specific to the membrane of the Wolff duct, recognizing different antigen determinants of the same protein. We used these markers in rat embryos from day 15 to day 20 postcoitum. Through these serial sections, carried throughout the embryogenesis of the genitourinary system, we made a reconstruction of the embryology of the vagina. Samples of small intestine were used as positive control.

Later, we studied four adult rats to analyse whether the markers specific to the Wolffian duct could still be observed in the mature vagina. Of the four pieces obtained, we placed two completely in paraffin to obtain longitudinal sections, whereas we sectioned the other two into three segments (proximal, medial and distal) to obtain transversal sections of the vagina at different levels. Serial sections were obtained from the four pieces and dyed with haematoxylin–eosin, and, afterwards, we applied markers specific to the Wolff ducts (GZ1 and GZ2).

Results

In the embryos of 15 and 16 days of gestation, we observed how a cross is produced at middle level, from proximal to distal, between the Wolff and Müller ducts. As we descend, the lateral Müller duct becomes medial until it crosses ventrally with the Wolff duct and positions itself at distal levels, medial to the Wolff duct. This convergence of the Müller ducts goes to a lower level, and both ducts become so close that they make contact and fuse later. At the same time, this fusion is laterally presided over by the Wolff ducts (Figure 1A), and we can see how they extend distally to form the bulbs of the urogenital sinus (Figure 1B). An interesting point is that after the fusion of the Müller ducts and the common segment, the ducts separate again diverging in a caudal and lateral direction to then fuse with the wall of the Wolff ducts a little before the most distal portion, which is the one that forms the bulbs of the urogenital sinus (Figures 1 and 2). In this way, we could distinguish three portions of the Müller ducts: converging, fusion of both and a diverging portion which then fuses with each of the Wolff ducts. The Müller tubercle remains below. Therefore, the Müllerian tubercle is delimited superior–laterally by the diverging portion of the Müller ducts, laterally by the distal portion of the Wolffian ducts, inferolaterally by the bulbs of the urogenital sinus and inferiorly by the urogenital sinus (Figure 1). During the 17th day, a decrease in the calibre of the Wolff duct was observed. This reduction appears in the middle portion at a near distal position and from there extends upwards and downwards; the distal portion maintains its calibre longer. This distal segment is the one we observed fused with the diverging portion of the Müller ducts. Besides, in the longitudinal sections, we can observe the ureteral bud emerging from the Wolff duct, practically at a level where it reaches the sinuses, that is, the segment where the so-called bulbs of the urogenital sinus are formed (Figure 2B).

Afterwards, through immunohistochemical techniques, we tested what had already been observed with haematoxylin–eosin. To do so, we used monoclonal antibodies to GZ1 and GZ2 markers, specific to cells of the Wolffian ducts. In 15-day embryos, the Wolff ducts are stained (Figure 3B) and not the Müller ducts (Figure 3A). Although this stain is only slightly positive compared with the intensity of positive control (Figure 3C), it is enough to discriminate the Wolffian nature of the structures. In the same way, the sinovaginal bulbs are slightly marked in 15-day embryos (Figures 4A and 4B) and the remaining portion of the urogenital sinus is also marked in 16- and 17-day embryos. This, therefore, demonstrates the Wolffian nature of the urogenital sinus bulbs. These elements can be observed in longitudinal sections and their form in 17-day rat embryos can be seen in Figure 4C. We can see how in embryos of this age, there is a space between the emergence of the ureteral bud and the Wolff ducts (both structures are positive for GZ1 and GZ2), which we called the ‘Wolffian patch’, doubtlessly a product of stretching through the growth of this space (Figure 4C). In the vagina of an adult rat, we detected the GZ1 and GZ2 markers (Figure 4D) which we did not observe in other places (e.g. the urethra). This marking was appreciated in the longitudinal sections as well as in the transversal sections, demonstrating, therefore, the Wolffian nature of the vagina of an adult rat.
Discussion

Through this study, we believe to have demonstrated the Wolffian nature of the urogenital sinus bulbs. Koff (1933) was sure that their origin was in the urogenital sinus, and they are so called for this reason. This old theory arose from observing human embryos (Carnegie collection), and it has been accepted until present. For this author, the origin of the vagina was dual; the upper vagina was Müllerian and the lower or sinusal was derived from the growth of the sinuvaginal bulbs in cranial direction. However, previously Hart (1901) had adopted the term ‘Wolffian bulb’ because the epithelial pockets that form the sinuvaginal bulbs keep remnants of the Wolffian ducts. Witschi (1970) re-examined the Koff’s embryos and deduced that the sinuvaginal bulbs were identical to the lower segments of the Wolff ducts. This observation was confirmed by Bok and Drews (1983) in an experimental study with embryo cultures.

We have also demonstrated how the termination of the Müller ducts is produced on the medial wall of the Wolff ducts and the nature and situation of the Müller tubercle. For Marshall and Beisel (1978), who introduced the term ‘Müllerian tubercle’, it is the joining of the Müllerian ducts with the urogenital sinus. Most of the authors accept that the Müllerian tubercle is an accumulation of Müllerian cells which remain at the caudal end of the primordium uterine above, on the dorsal wall of the sinus below and the mesonephric or Wolffian ducts on both sides. We have observed, in contrast to other authors, but as Bok and Drews (1983), that once the Müller ducts fuse they elongate and diverge caudally, so we could distinguish three portions: the first is converging, the second is common (formed by the fusion of both ducts) and the third is diverging, which then fuses with each of the Wolffian ducts before they end in the sinus bulbs. Therefore, the Müller tubercle is not a cap at the end of a tube, but a piece of mesenchymal tissue which is superior–laterally delimited by the diverging portion of the Müllerian ducts, laterally by the Wolffian ducts, inferolaterally by the sinus bulbs and inferiorly by the urogenital sinus.

In rat embryo cultures, Bok and Drews (1983) show the exact role of the Wolff ducts in the formation of the vagina. They observed that after fusion, the Müller ducts diverge and

Figure 2. (A) Longitudinal cut of rat 17 days postcoitum (×5 magnified, haematoxylin–eosin). Lateral view of Müller duct (M) approaching the Wolff duct (W) and of the latter reaching the urogenital sinus (US). (B) Longitudinal cut of rat 17 days postcoitum (×10 magnified, haematoxylin–eosin). See emergence of ureteral bud (UB) from distal segment of the Wolff duct (W) at the level of its opening into the urogenital sinus. B, bladder; R, rectum.

Figure 3. Transversal cut of rat 17 days postcoitum (×40 magnified, immunohistochemical). (A) Detail of the transversal section of the Müller duct (M), without staining. (B) Wolff duct (W), positive staining. (C) Intense staining of positive control which is the intestine (I).
M.L. Sánchez-Ferrer et al.

1626

Elongate caudally to then fuse with the Wolffian ducts. Concomitantly, the Wolffian epithelium acquires the characteristic appearance of the Müllerian epithelium, and, after fusion, the contribution of the Wolffian epithelium to the vagina can be difficult to demonstrate except by localization. By employing a Wolffian specific marker, we could in fact identify the Wolff duct after its fusion with the Müller duct and demonstrate its participation in the ultimate formation of the vagina in adult. This fusion does not surprise us, since according to Marshall and Beisel (1978) it was so from its origin. The Müllerian duct is initially formed by an invagination in the ceolomic epithelium at level of the third thoracic somite, following the course of the existing Wolffian duct. We interpreted that this point of fusion would descend helicoidally, so that it first approaches the lateral side of the Wolffian duct, then the anterior and finally the medial side, where it fuses with the distal and internal portion of the Wolffian duct, as we observed in our embryos. This close relation between Wolff and Müller ducts had already been recognized by most authors. Grüenwald (1941) affirmed that the caudal end of the Müller duct was situated inside the basal membrane of the Wolff duct, and this dependence was demonstrated clearly by the failure of the Müller duct on experimentally interrupting the Wolffian duct. It seems to us that there is no cellular contribution because they are stained in a different way, but there is a close relation between Wolff and Müller (with this permanent and descending fusion), which would justify why in all cases of alteration in the vagina (unilateral blind or atretic hemivagina, Wolffian duct lesions) there are also uterine malformations (i.e. anomalies in the Müllerian development). We believe that the fusion is as Grüenwald (1941) outlines and not as Bok and Drews (1983) present it, since we have not observed light between the two fused ducts, and the diverging end of the Müllerian duct is practically solid when it fuses with the Wolff duct. We think that the difference may be due to the different methodology used, as they used embryo cultures.

We interpret posterior evolution as follows: the joined portion of the Müller ducts (after medial wall re-absorption of both fused ducts) will form the uterine body, which is bicornuate in rats because the joining is more distal. The beginning of

Figure 4. (A) Transversal cut of rat 15 days postcoitum (×10 magnified, immunohistochemical). Detail of positive although weak marking (*) from the sinus bulbs demonstrating its Wolffian nature. (B) Enlarged detail of A (×40). (C) Longitudinal cut of rat 17 days postcoitum (×20 magnified, immunohistochemical). Lateral view of Wolff duct (W) reaching urogenital sinus which is forming the bladder (Bl) and emergence of ureteral bud (UB) from the Wolff duct, both structures with slightly positive marking. (D) Longitudinal cut of adult rat (×10 magnified, immunohistochemical). We see positive marking (*) when the vagina begins, once again demonstrating the contribution of Wolff to its formation.
positive for Wolffian specific markers. We have called this ducts and the ureter have a Wolffian nature, as both tissues are (Figure 5).

Figure 5. (A) Classical diagram of the formation of the vagina from the Müller ducts and the urogenital sinus. (B) Representative diagram of the evolution of the Wolff and Müller ducts according to our study. Müller ducts fuse in the middle line to form the uterus, with a single cavity in women. The Wolff ducts are shown on both sides. See how the diverging portion of Müller fuses with the distal segment of Wolff, which forms the sinus bulbs and how the Müller tubercle remains as an accumulation of cells between these structures. The diverging portion of the Müller ducts is solid and will form the external cervical orifice, so that the vagina will be formed from the beginning to the hymen by cells from the distal segments of the Wolff ducts. Finally, a diagram of the formation of the vagina from the Wolff ducts internally lined with cells from the Müller tubercle is shown.

We can affirm that both the urogenital sinus bulbs as the segment delimited between the opening of the mesonephric ducts and the ureter have a Wolffian nature, as both tissues are positive for Wolffian specific markers. We have called this segment the ‘Wolffian patch’ that could be neoformed by the growth or stretching of the space existing between the Wolffian duct and its ureteral bud. Thus, these studies demonstrate that the rat vagina has a Wolffian origin (although there is also participation of the Müller tubercle) as well as the portion of the Müllerian ducts divergence will correspond to the internal cervical orifice, and the distal end of the diverging portion will correspond to the external cervical os. All vaginal walls will be formed by cells from the Wolffian ducts and then covered by cells of the Müllerian tubercle. Therefore, we believe that the Müllerian ducts do not contribute to the vagina formation. The Müllerian tubercle does, however, in that later a cavitation is formed so that light in the vagina remains and the vaginal walls are internally covered by cells from the Müllerian tubercle (Figure 5).

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To sum up, through our study, it can be inferred that the nature of the urogenital sinus bulbs in rats is Wolffian, concluding that the vagina in rats is formed from the Wolff ducts and the Müller tubercle. Therefore, although there are logical differences to human embryology and anatomy, we believe that the theory put forward by Acién could be correct and consequently a clinical-embryological classification, as we propose (Acién et al., 2004a,b) should be considered as it permits an embryological justification of all genitourinary malformations, including the most complex ones.

Acknowledgements

This study was granted by Institutio de Cultura Juan Gil Albert, Alicante.

References


Submitted on August 31, 2005; resubmitted on November 26, 2005; accepted on January 13, 2006