Development of the human male urethra: A histochemical study on human embryos

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A R T I C L E   I N F O

Article history:
Received 21 February 2013
Received in revised form 31 December 2013
Accepted 4 January 2014

Key words:
Urethra
Penis
Development
Embryology
Hypospadias

A B S T R A C T

Purpose: Controversy persists regarding the formation of human penile urethra. The classic fusion theory for the development of the spongy urethra and ectodermal ingrowth or endodermal transformation theories for the development of the glanular urethra do not explain the wide spectrum of anomalies seen in patients with hypospadias. This histological study was made to clarify the mechanism of urethral development.

Materials & Methods: 15 human male embryos ranging from 6 to 14 weeks were studied. The phalluses were examined microscopically and photographed. Tissues were prepared as serial histological sections and stained with haematoxylin and eosin and with special immuno-histochemical stains.

Results: 1) The penile urethra: At 6 weeks of gestation, the urethral plate which is solid distally and partially grooved proximally becomes grooved distally and has fused proximally by 8 weeks. At 14 weeks of gestation; the urethral opening migrates only to the middle of the shaft.

2) The glanular urethra: At the 6th week of gestation, a solid epithelial plate reached the tip of the genital tubercle, and a glans cannot be identified. At the 7th week, a central vacuolation appears and the penile urethral groove does not reach the tip of the phallus. At the 8th week; coronal sulcus starts to appear, and a well defined blind central canal was evident in the 13th week. During the 14th week, the floor of the glanular canal degenerated to form a glanular groove.

Conclusions: Our observations suggest that the spongy urethra passes through 3 stages of development: a solid epithelial plate, deep urethral groove, and fused urethra. The glanular urethra passes through 4 developmental stages: a solid epithelial plate, a blind central canal, a deep glanular groove, and the floor from the preputial lamella. There was no evidence of ectodermal ingrowth. These observations raise serious questions to the current theories for human urethra development. Further studies on fresh human embryos are needed.

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The development of the human urethra has been a controversial subject in embryology for years [1]. Glenister stated that the penile urethra arises by fusion of the primitive urethral groove and the secondary urethral groove that develops from the urethral plate [2]. In contrast, Van der Putte and Neeteson concluded that the male penile urethra is formed by a movement in ventral direction of the urogenital opening relative to the growing perineum and not by fusion of the genital folds [3]. Kluth et al. could not find evidence of fusion [4,5].

If the urethra develops through simple fusion (according to the current classic theory), why do we see chordee? How can torsion occur? Why do we see variable width and depth of the urethral plate? Why there is a wide range of corpus spongiosum hypoplasia and dysplasia in hypospadias? Why there is a variable degree of glans clefting (i.e. grooving)? How can simple fusion explain the presence of ano-cutaneous tract on the ventral penis in patients with imperforate anus? And why don’t we see skipped segment of fusion (i.e. fused urethra proximally and distally with a skipped segment in-between) in hypospadias as we see in the case of fusion of the spinal cord (e.g. in meningocoele)?

Similarly, controversy continues to exist regarding the formation of the glanular urethra [1]. Glenister [2] suggested a dual embryological origin with the proximal part of the glanular urethra forming in a similar way to the penile urethra from the endodermal urethral plate, while the terminal part arises from an ectodermal invagination from the terminal epithelial tag at the tip of the glans—a description that remains in contemporary embryology textbooks today [6,7]. An alternative model proposes that the entire urethra forms from endoderm which undergoes differentiation in glanular portion to form squamous epithelium [8,9,2]. Neither model explains the wide spectrum of glanular hypospadias that is encountered in clinical practice with different degrees of glans clefting, blind glanular canal and glanular ridge. A clear understanding of the normal development of the urethra is important in the elucidation of its malformations [10].

The aim of the study is to try to clarify the mechanism of human urethra development, based on the principle that the urethra
The study is based on the principle that the urethra develops from proximal to distal. The aim is to record step by step the dynamic process during the critical active period of urethral development. Examination of the serial individual sections from distal to proximal can serve as single films of a moving urethral development. Development of the glanular urethra goes parallel to that of the spongy urethra:

1) **The spongy urethra**: The study documented the actual fusion point of the spongy urethra (point e). The sections distal to it would show the gradual steps of fusion (point d) and sections proximal to it would show an immediately fused urethra (point f). In this way, histological sections of a single embryo during active development can show several developmental stages of the spongy urethra (embryos 2, 7, 14).

2) **The glanular urethra**: During the critical active period of development, histological sections at the middle of the glans (point b) would document a developmental stage that is later than that of the distal glans (point a) and earlier than that of the proximal glans (point c). The aim is to record step by step the dynamic changes in the glans during active development (embryos 3, 11, 13).

Develops from proximal to distal. The present study was designed to record step by step the dynamic process of urethral development. Examination of the serial individual sections from distal to proximal during the critical active period of development can serve as single films of a moving urethral development (Fig. 1).

With the help of specific immunohistochemical stains, we attempt to observe and document the actual fusion point of the urethra (Fig. 1, point e). The sections distal to it would show the unfused urethral groove (Fig. 1, point d) and the sections proximal to it would show the gradual steps of fusion (Fig. 1, f). Similarly, histological sections at the middle of the glans (Fig. 1b) would document a developmental stage that is later than that of the distal glans (Fig. 1a) and earlier than that of the proximal glans (Fig. 1c). In this way, histological sections of a single embryo during active development can show several developmental stages.

### 1. Materials & methods

A series of 15 human male embryos and fetuses from the routine diagnostic pool of the Department of Pediatric Pathology, Mainz University was studied (Table 1). These were obtained from legal, spontaneous or medically indicated abortions between 2001 and 2009. Embryos less than 6 weeks and fetuses more than 14 weeks were not available for the present study. The sex of the embryos was confirmed by chromosomal analysis.

In this study, age estimations are given in weeks, estimated from an assumed time of fertilization of the egg. This estimation is made on the basis of a comparison of as many embryonic organs as possible with embryos of tolerably exact developmental ages as described by Streeter [11].

All the embryos and foetuses were examined and photographed under magnification using stereomicroscope MZ 16A manufactured by Leica. They were treated in 80% filtered isopropyl alcohol after being fixed in 4% formaldehyde according to the method described by Fahr et al. [12].

Specimens that included the external genitalia, anus and perineum were processed through the standard sequence of dehydration and paraffin embedding. Serial sagittal histological sections were obtained at 3-μm intervals and stained with haematoxylin and eosin and von Gieson stains (Fig. 1). On average, 200 sections were obtained from each specimen. Sections were retained at variable intervals for specific immuno-histochemical stains. The interval depended on the size of the specimens and the importance of the region.

The following antibodies were used according to standard immuno-histochemical methods with the VECTASTAIN Elite ABC PEROXIDASE Kit Standard (VC-PK-6100-KI01 AXXORA):

1) **Vimentin** (Monoclonal Mouse Anti-Vimentin., Clone V9, 1 ml, M072501, DAKO): diluted 1:200 PBS–BSA, used to label cells of mesenchymal origin

2) **MB-1** (monoclonal antibody that reacts with the Ki-67 nuclear antigen, 1 ml, 7240, DAKO): diluted 1:150 PBS–BSA, used to label proliferating cells.

3) **Cytokeratin**: a combination of AE1/3 (Monoclonal mouse anti-human Cytokeratin, clones AE1/AE3 (500 μg MAB 3412, CHEMICON) (diluted 1:100) and CAM 5.2 (BECTON DICKINSON anticytokeratin)(1:25): 100 μl PBS–BSA + 1 μl AE 1/3 + 4 μl CAM 5.2 that is used to label epithelial cells.

Positive and negative controls were done by routine procedures of patients' material. Controls on embryos genital area were avoided in order not to miss serious section slides.

The dye that labels specifically the apoptotic nuclei (terminal transferase nuclear end labelling, TUNEL method) was used in one embryo but was not conclusive probably due to the fact that the specimen was not fresh.

For descriptive purposes, the floor of the urethra means the ventral urethral surface and roof of the urethra means dorsal surface of the urethra in an erect penis (Fig. 1).

### 2. Results & Observations

Examination of the 15 specimens showed the active sequence of developmental changes of the human urethra which was persistent in...
Fig. 2. (penile urethra): Embryo 7, week 8. Serial histological sections through proximal penis in the region of the urethral orifice during penile urethra formation, demonstrate the 3 phases of fusion between epithelial structures (from distal to proximal, stained with haematoxylin and eosin ×10):

1. **Proliferation** of the epithelium and condensation of the underlying mesenchyme (Fig. 2b).
2. **Adherence** of the epithelial edges resulting in formation of an epithelial plate of a double layer of epithelium is demonstrated (Fig. 2c).
3. **Apoptosis** within this epithelial plate results in disruption of the two epithelial layers including their basement membrane after which the process of fusion is completed and the mesenchyme becomes in continuity (Fig. 2d).

Sections e and f were stained with the monoclonal antibody MIB-1 that reacts with the Ki-67 nuclear antigen, to label proliferating cells at the critical fusion point demonstrating 2 phases of fusion between epithelial structures (from distal to proximal):

1. **Adherence** of the epithelial edges resulting in formation of an epithelial plate of a double layer of epithelium is demonstrated × 20 (Fig. 2e).
2. **Fusion and cell death** within this epithelial plate result in disruption of the two epithelial layers including their basement membrane after which the process of fusion is completed and the mesenchyme becomes in continuity × 40 (Fig. 2f).

The area of fusion of the basement membrane was magnified (suggesting cell death before disruption) before the proliferating mesenchymal cells (brown) on either side becomes in continuity.
all the specimens. The glanular urethra appears to start developing in parallel to the spongy urethra. None of the specimens included in the study showed signs of development of the prepuce.

After detailed careful histological and histochemical examination of the 15 embryos and fetuses, embryos 2, 7 and foetus 14 were selected to demonstrate the developmental stages of the spongy urethra and embryos 3, 11 and 13 for the glanular urethra. This is based on age and the quality of the stained sections.

2.1. Penile Urethra

2.1.1. Embryo 2 (6th week)

The genital tubercle has appeared and consisted of a conical structure less than 1 mm long. A glans could not be identified at the tip of the genital tubercle. The undersurface showed a well-marked urethral groove that did not reach the tip. The dorsal surface showed a longitudinal indentation.

Histological examination showed the urethral plate to consist of a short horse shoe lamella extending into the genital tubercle from the converging anterior walls of the urogenital sinus. Dorsal to the urethral plate, the mesenchyme was homogenous and undifferentiated. The glans and corpora cavernosa could not be identified as such. The mesenchyme on either side of the urethral plate was proliferating to raise low folds, covered by surface epithelium. These urethral folds, and the enclosed urethral groove, did not reach the tip of the phallus.

2.1.2. Embryo 7 (8th week) (Fig. 2)

The genital tubercle was about 1.5 mm long and had a distinct ventral curvature (chordee). The shape of the genital tubercle is globular and a faint coronary sulcus has started to appear. Two small scrotal folds could be seen on either side of the base of the phallus.

Sagittal histological sections (Fig. 2a–d) showed that in the penile shaft region, the urethral plate was partly solid and partly grooved to...
form the urethral groove. The urethral groove extended from the base of the penis to the coronary sulcus. The degree of grooving increased as one progressed from distal to proximal. At the distal end of the phallus, the urethral plate was solid.

Microscopic examination of the transverse sections immediately distal to the fused urethra showed active migration and condensation of the mesenchyme at the edges of the urethral plate around the urethral groove. This active migration was associated with proliferation and hyperplasia of the overlying epithelium (Fig. 2a). The active migration and condensation of the mesenchyme brought the edges of the hyperplastic overlying epithelium closer together and they adhered to each other (Fig. 2b). As one progressed proximally, there were gradual apoptosis and regression of the intervening epithelium and the mesenchyme became in continuity (Fig. 2c, d).

Fig. 2e, f documents the 3 steps of fusion of epithelial surfaces using the immuno-histochemical stain MBA1 which is specific for cell proliferation. The Figure shows serial histological sections from the distal penis (unfused urethra) to the proximal penis (fused urethra). Fig. 2e documents the stage of proliferation and adherence, Fig. 2f shows the stage of resorption of the basement membrane.

2.1.3. Fetus 14 (14th week)
The genital tubercle was 2 mm long and had a mild curvature in a caudal direction. The shape of the genital tubercle has become cylindrical and a coronal sulcus has appeared. The terminal epithelial tag could be identified macroscopically at the tip of glans penis. Two well-developed scrotal folds are situated on either side of the base of the phallus.

The urethral groove extended from the base of the penis to the coronal sulcus. The urethral groove appeared deepest and widest in the middle and narrowed towards its distal end. The groove did not extend into the glans but its epithelium was in continuity with that of the solid urethral plate in the glans.

Histological examination of the transverse sections showed a shallow urethral groove with signs of vacuolation and degeneration of the caudal cells. There were migration and proliferation of the mesenchyme dorsal to the urethral plate to form undivided corpus cavernosum that seemed to be continuous with the mesenchyme of the glans. The migration and proliferation of the mesenchyme around the perimeter of the urethral groove gave shape to the urethral folds which formed the margin of the groove.

The mesenchyme showed more proliferation and differentiation in histological sections of the proximal penis. The differentiation was less pronounced as one progressed distally.

Tracing the structure of the urethral plate from distal to proximal suggested the mechanism of fusion of the urethral plate. In the middle of the shaft, the urethral plate had the shape of a bilamellar structure that was partially solid and partially vacuolated and grooved. As one progressed proximally, there was gradual proliferation of the mesenchyme that gradually brought the overlying thickened hyperplastic epithelium to adhere together. More proximally, there were signs of apoptosis and regression of the epithelium and the mesenchyme became continuous with one another.

2.2. Glanular Urethra

2.2.1. Embryo 3 (7th week)
The genital tubercle is conical in shape, less than 1 mm long. A glans could not be identified at the tip of the genital tubercle. The undersurface showed a well-marked urethral groove that did not reach the tip.

Histological examination showed a solid urethral plate at the tip of the phallus with a fine vacuole in the centre. As one proceeds proximally, there was gradual vacuolation of the solid urethral plate. The urethral plate was completely vacuolated and deeply grooved in the middle of the phallus. The mesenchyme was still diffuse and not differentiated into glans and corpora cavernosa.

2.2.2. Embryo 11 (13th week)
The embryo is 25 mm in length and the genital tubercle is about 1.5 mm long and had a mild curvature in a caudal direction (chordee). The genital tubercle is globular in shape. A glans penis could be identified clearly with a well defined blind central canal. The urethral groove is diamond in shape with the widest diameter in the middle of the penis. The edges of the urethral groove narrow distally before ending at the coronal sulcus.

Microscopic examination of the transverse sections showed proliferation and differentiation of the mesenchyme to form undivided corpus cavernosum dorsal to the vacuolated urethral plate. Around the urethral groove, the mesenchyme has differentiated into a vascular corpus spongiosum which is more defined proximally.

2.2.3. Fetus 13 (14th week, (Fig. 3)
The genital tubercle is 2 mm long with mild chordee. The glans penis could be identified and a coronary sulcus has appeared. There is a fine indentation at the tip of the glans penis which appears to be solid.

The urethral groove extends from the base of the penis to the coronary sulcus. The urethral groove appears shallower than in embryo 11. The urethral groove appears not to extend into the glans but its epithelium is in continuity with that of the glans plate.

Immunohistochemical staining of selected sections of the penis in this fetus showed 3 different developmental processes of the glanular urethra:

1) A nearly solid urethral plate at the tip of the glans (cytokynes stain that stains the epithelial lining.
2) A central canal at the level of mid glans.
3) A central canal with a large macrophage or scavenger cell starting the degeneration of the floor of the glanular canal at the level of proximal mid glans.

It was interesting to observe that the floor of the central glanular canal started to disappear to form a deep glanular urethral groove at the same time of fusion of the edges of the proximal urethral groove to form the floor of the proximal penile urethra.

Haematoxylin and Eosin staining of histological sections through the glans region in this fetus showed the first 3 stages of glanular urethra development (Fig. 3);

1) A solid glanular epithelial plate at the distal glans (Fig. 3 a).
2) A blind central glanular canal (Fig. 3b). The floor of the blind central canal shows a large macrophage or scavenger cell starting the degeneration at the proximal midglans(Fig. 3c).
3) A deep glanular urethral groove at the proximal glans (Fig. 3d).

3. Discussion
The embryology of the penis and urogenital region has been controversial and confusing. This is partly related to the fact that in the literature, observations have been extrapolated to the human from many different species. There are reports in the literature describing sheep, dogs, rats [13], mice [8], red squirrels [14], pigs [3] and humans [15]. Contrary to human, the mouse, rat and dog phallus have a short bone called “os penis” [16,17]. Therefore, it is possible that the developmental process in humans differs from that in other animal species. For this reason, only relevant studies on human embryos and fetuses are discussed in detail here.

3.1. The spongy urethra
The classic fusion theory of the human spongy urethra formation proposed that the spongy urethra was formed by fusion of the urethral folds like closing a zip in the midline [15]. This theory was greatly enhanced by the investigations of Glenister [2].
Fleishmann [18] and others [19] could not find evidence for fusion in their studies on human embryos. Their ideas were recently supported by Van der Putte [20] in man and Kluth [4,5] in rats as these investigators also failed to find any sign of midline fusion.

Fusion is defined as a process during which two separate elements come together and form a single new structure. This occurs frequently in mesenchyme-derived structures e.g. muscle and fat. However, fusion as such does not normally occur when the opposing elements are covered by epithelium. Apposed epithelia may stick together but do not fuse normally.

Apoptosis (programmed cell death) is a phenomenon that has been described as early as the nineteenth century as reviewed by Clark and Clark [21] and is considered to be important for normal embryonic development [22]. Many developmental processes have been postulated to be partly regulated by the time and area dependant death of cells [16,17,23,24].

Fusion of epithelial structures may occur through the following three phases:

1) **Hyperplasia**: there are condensation of mesenchyme and proliferation of the overlying epithelial edges which expand and move towards each other.

2) **Adhesion**: the two epithelial structures show signs of adhesion of their epithelial linings forming a double layer of epithelium.

3) **Apoptosis**: there is disruption of the two epithelial layers including their basement membranes and the mesenchyme becomes in continuity. Examples of tubular structures formed through this process include the neural tube and the nasal cavity [25].

Histochemical stains (vimentin, MiB-1 and cytokeratin) were helpful to document the 3 phases required for fusion of epithelial structures in the present study on human embryos [26].

Baskin et al. conducted a study on mice and hypothesised that the normal urethra in mice is similar to human and forms by fusion of an epithelial seam [27]. We could not identify in their study clear objective evidence to support their hypothesis of the similarity between the development of human and mouse urethra. They suggested that “the middle of the epithelial seam narrows and subsequently pinches into a centrally located urethra and a ventrally discarded excess epithelial cells that are remodelled into ventral skin of the penis”. They hypothesised that seam transformation occurs via cellular migration and not by epithelial–mesenchymal transformation or epithelial apoptosis. This is different than the findings in our study which showed clearly the 3 steps (proliferation, adherence and apoptosis) necessary for fusion between epithelial surfaces. They also proposed that hypospadias results from an arrest in urethral seam formation. This does not explain the wide range of anomalies associated with hypospadias as mentioned before.

Felix et al. stated that the urethral opening moves gradually along the anal surface of the genital tubercle to the tip of the genital tubercle [28]. The important role of migration of mesoderm in the formation of urethral was proposed by van der Putte [20] and Altemus and Hutchins [29] on human embryos. The study of Seifert et al. on mice suggested that the urorectal septum forms the perineum and its cellular migration and not by epithelial urethral was proposed by van der Putte [20] and Altemus and [28]. The important role of migration of mesoderm in the formation of the anal surface of the genital tubercle to the tip of the genital tubercle associated with hypospadias as mentioned before.

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Van der Werff et al. (2000)[24] suggested that the urethral plate is present even before rupture of the cloacal membrane. Interestingly, they proposed that the urethral plate in the glans region of ectodermal region which becomes later on in continuity with the endodermal urethral plate of the rest of the penis.

Re-examination of Fig. 6 in the Kurzrock et al. study [35] showed that the roof and lateral walls of the glanular urethra were vertically oriented, lined with stratified squamous epithelium and stained positively for cytokeratin K14. In contrast, the floor of the glanular urethra was horizontally oriented, lined with pseudo stratified columnar epithelium and stained positively for cytokeratin K8 as was the case with the spongy urethra and the urinary bladder epithelium. This strongly suggests that the floor of the glanular urethra has a different origin than the roof and lateral wall [36].

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The present study showed that the development of the glanular urethra differs from that of the penile urethra. There was no evidence of ectodermal ingrowth. The glanular urethra seems to develop.
through four stages of; a solid epithelial plate, blind central canal and a glanular groove. The floor of the glanular canal seems to be formed through distal migration of the glanulo-preputial lamella which forms the prepuce and fuses with the edges of the glanular groove to form the floor of the urethral orifice.

One limitation of the present study is that it did not include fetuses after 14 weeks of gestation due to ethical regulations in Germany. However, this was compensated for by examination of serial sections proximal and distal to the actual fusion point. Another limitation was that there was no way to ensure that the urethral development would have continued to develop a normal urethra at birth. A third limitation was that the embryos were not fresh. This may explain why the Tunnel method, which stains apoptotic cells, was not conclusive.

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