Development of the external anal sphincter with special reference to intergender difference: observations of mid-term fetuses (15–30 weeks of gestation)

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Summary: To investigate intergender differences in muscle cleavage and joining during development of the external anal sphincter (EAS), we examined semiserial sections of 16 fetuses between 15 and 30 weeks of gestation (6 males and 10 females). The subcutaneous part of the EAS (EASsc) developed along the male perineal raphe and extended posteriorly. Thus, the male EAS was characterized by anterior protrusion of the subcutaneous muscle, in contrast to the almost circular female EAS. In both genders, the bulbospongiosus anlage (or the levator ani anlage) issued muscle fibers to form the superficial (or deep) part of the EAS. The EASsc communicated with the superficial part in males, whereas the female bulbospongiosus tended to communicate with the levator ani rather than the EAS. In both genders, the longitudinal muscle bundle(s) of the anorectum contributed to perineal body formation. However, the male perineal body also had a thick fascia between the rhabdosphincter and the levator. The bulbospongiosus seems to play a critical role in forming the EAS. A strict intergender difference in subcutaneous muscle development is evident along the perineal raphe, as the raphe is not evident in females. These results help to explain variations in the EAS, including anal malformations.

Introduction

Although recent investigations provided new lights on fetal development of the human anorectum (van der Putte 2006, 2009; Fritsch *et al.* 2007), intergender differences in the external anal sphincter (EAS), especially in their development, appeared to still remain obscure. Classically, intergender differences in the external anal sphincter were examined in detail by Oh and Kark (1972) and Stelzner (1981), who demonstrated schematically that, in females, the deep, superficial and subcutaneous parts of the EAS together form a single small mass anterior to the anorectum. However, according to Fritsch *et al.* (2002) and Rociu *et al.* (2000), a notable sexual difference in fetuses was limited to the “thickness” in the anterior half of the EAS. We hypothesized that classically-postulated differences are likely between genders

and that the presence of the vagina in females is likely to correlate with the differences.

Obviously, fetuses in which each part of the EAS and other perineal striated muscles can be clearly identified topohistologically are likely to provide good clues for clarification of intergender developmental differences. Which stages are adequate for the clarification? Johnson (1914) described that the muscle coat of the anorectum becomes evident around 18 weeks of gestation. It seems to be almost consistent with sonographic observations (Bourdelat *et al.* 2001). Using mid-term fetuses, Fritsch and Fröhlich (1994) demonstrated that fasciae along and around the levator ani became distinct, and that the funnel-shaped, three-dimensional extent of the levator began to show an intergender difference in shape. An excellent review by van der Putte (2005) displayed diagrams including the EAS in fetuses later than CRL 125

mm (around 16 weeks of gestation). In a recent study (Niikura *et al.* 2008), we found that pelvic striated muscle becomes distinguishable from smooth muscle fibers at around 20 weeks of gestation according to transverse striations in muscle fibers under hematoxylin and eosin staining. Consequently, we examined semiserial sections of human fetuses at 15–30 weeks of gestation to identify intergender differences in the development of perineal structures, especially the EAS and perineal body.

Materials and Methods

We examined 16 fetuses between 15 and 30 weeks of gestation (6 males and 10 females, CRL 105–280 mm). The paraffin sections examined were 10–20 micron thick and had been cut at 100-, 200- or 500-micron intervals depending on the site and size of the blocks. Transverse sections of the anorectum were prepared from 11 fetuses (3 males and 8 females) and frontal sections from 5 fetuses (3 males and 2 females). All fetuses had been fixed by immersion in 10% formalin solution in water and preserved for more than 20 years as part of the collection of the Medical Museum of Sapporo Medical University for medical education and research. Most of the sections had been stained with hematoxylin and eosin (HE staining) and stocked as part of the collection. However, other sections were newly prepared from 3 fetuses (2 males and 1 female; included among the aforementioned 16 fetuses). These new paraffin sections (10 micron thick cut at 100-micron intervals) were also stained with HE, but some were stained immunohistochemically for smooth muscle actin. The primary antibody used for immunohistochemistry was monoclonal anti-human alpha 1-smooth muscle actin (Dako M851, Dako, Glostrup, Denmark; 1:50 dilution), as described by Uchimoto *et al.* (2007) and Soga *et al.* (2007), which allowed the EAS to be clearly discriminated from perineal smooth muscle. The secondary antibody was labeled with horseradish peroxidase (HRP), and antigen-antibody reactions were detected using the HRP-catalyzed reaction with diaminobenzidine. Counterstaining with hematoxylin was performed on the same samples.

The present study was undertaken within the provisions of the Declaration of Helsinki, 1995 (as revised in Edinburgh, 2000); however, the project did not include a specific protocol requiring examination and approval by a suitably constituted institutional ethics committee.

Results

Perineal muscles observed in transverse sections

In transverse sections, an anterior extension or protrusion toward the surface skin consistently characterized the male EAS at the level of the coccyx (Fig. 1A).

Because this anterior extension was located in the subcutaneous tissue, it corresponded to the subcutaneous part of the EAS. However, in females, because the vestibulum of the vagina was adjacent to, and even connected to, the anterior skin cleft of the anus, depending on the individual (not stage), there was little space in which the EAS could extend anteriorly. In cases where a limited space for the female anterior EAS was present (3 of 8 female specimens in transverse section; Fig. 1B), it was located inferior to the coccyx. Thus, the subcutaneous part of the female EAS was poorly developed and lay more inferiorly than in males. In both genders, the anterior distribution of the subcutaneous muscle corresponded to the levels of the transitional zone or intermediate epithelium between the columnar epithelium and stratified squamous epithelium of the anus (Fig. 1).

Another intergender difference seen in transverse sections was evident on the posterosuperior side of the EAS: in males, a cluster of striated muscle fibers with a thick lining fascia (in transverse sections of 2 of 3 specimens) often extended transversely to connect between the internal obturator fascia and the developing perineal body. It was an anlage of the transversus perinei profundus, and was located immediately inferior to the bulbo-urethral or greater vestibular gland. In males, the thick lining fascia consistently provided a border between the rhabdosphincter and the anterior bulge of the levator ani, and medially it connected with the smooth muscle mass derived from the longitudinal muscle layer of the anorectum (Fig. 2; see also the subsection below entitled “Perineal body and other structures”). The male transverse muscle primordium was interposed between the bulbospongiosus (located anteriorly) and the levator (located posteriorly). However, on the inferior side of the transverse muscle, the male bulbospongiosus extended posteriorly along the lateral margin of the developing perineal body and communicated with the EAS (Fig. 2). Notably, in contrast, the female bulbospongiosus usually communicated with the levator on the inferior side of the greater vestibular gland (in transverse sections of 5 of 8 female specimens; Fig. 3). We did not find a continuation between the bulbospongiosus and levator in males.

Perineal muscles observed in frontal sections

In frontal sections of the 3 male specimens, we clearly identified the subcutaneous, superficial and deep parts of the EAS (Fig. 4). The scrotal or perineal raphe (the “dorsal perineal raphe” after van der Putte 2005) extended superiorly and connected to the median septum between the bilateral muscle bellies of the bulbospongiosus. The raphe was composed of subcutaneous thick fibrous tissues showing a tortuous course (Fig. 5), but it was difficult to identify in transverse sections (Figs. 1A and 2E). The raphe did not reach the surface skin, but the subcutaneous muscle occupied a narrow space between the superficial end of the raphe and the surface skin. Notably, the male

Fig. 1. Transverse sections showing an intergender difference in arrangement of the anterior half of the external anal sphincter (20 weeks; a CRL 170 mm male and a CRL 180 mm female).

Panels A (male) and B (female) correspond to the border between the columnar and intermediate epithelia of the anus. The two panels are at the same magnification. The male EAS displays a horseshoe-like arrangement with the anterior protrusion (panel A). Arrows indicate subcutaneous muscles developing along the perineal raphe (see also Figs. 4 and 5). In contrast, the female circular EAS does not accompany the distinct anterior protrusion (panel B). Asterisks, developing fat.

subcutaneous part of the EAS developed along the perineal raphe (Figs. 2E, 4A, 4B and 5), although the muscle fibers differed in number in frontal sections among the 3 male specimens. In females, the perineal raphe was not evident, but a few subcutaneous striated muscles were scattered more laterally than in males.

In both genders, the levator ani consistently extended inferomedially and issued muscle fibers to form the deep part of the EAS. Moreover, most muscle fibers of the superficial EAS were derived from the bulbospongiosus (Fig. 4). The anteriorly located bulbospongiosus issued 1–2 large subdivisions extending postero-inferiorly, and entering the middle layer of the EAS (i.e., a superficial

position). In males, some muscle fibers originating from the subcutaneous part of the EAS also contributed to the formation of the superficial part (Figs. 4C–4E). The transversus perinei profundus also developed closely to the bulbospongiosus, but extended superiorly along the lateral surface of the bulbo-urethral or major vestibular gland rather than transversely (Figs. 4B–4D), in contrast to the inferiorly extending bulbospongiosus subdivision joining the EAS. All these muscle cleavages and joinings were clearly seen at around 20 weeks of gestation. In frontal sections, the ischiocavernosus was clearly separated from all of the muscles contributing to EAS formation.

Fig. 2. Transverse sections showing muscle fiber communication between the bulbospongiosus and external anal sphincter (28 week; a CRL 270 mm male).

Panel A (or Panel E) is the superiormost (or the inferiormost) in the figure. Intervals between panels are 3.0 mm (A-B), 1.0 mm (B-C), 2.0 mm (C-D) and 1.0 mm (D-E), respectively. These five panels are at the same magnification. In panel A, a thick fascia (stars) is evident between the rhabdosphincter (RS) and levator ani (LA). Being extended between the bulbospongiosus (BS) and the LA, this fascia (stars in panel C) becomes combined with the developing perineal body (PB). Finally, the fascia (stars in panel E) is located between the BS and the external anal sphincter (EAS). The BS issues a bundle of muscle fibers posteriorly (arrows in panels C and D) and communicates with the EAS. The subcutaneous part of the EAS (sc) is evident near the median septum (arrows in panel E; see also Fig. 5A) between the inferior parts of the bilateral BSs. CSP, corpus spongiosum penis.

Fig. 3. Transverse sections showing muscle fiber communication between the bulbospongiosus and levator ani (18 week; a CRL 105 mm female).

Panel A (or Panel D) is the superiormost (or the inferiormost) in the figure. Intervals between panels are 1.7 mm (A-B), 0.8 mm (B-C) and 0.5 mm (C-D), respectively. Magnifications for panels B-D are greater than for panel A, in order to show muscle fibers. Panel A includes the inferior rectal valve of Kohlrausch. The bulbospongiosus extends posteriorly (arrows in panel C) and communicates with the levator (LA). Immediately inferior to the muscle communication, the bulbospongiosus turns medially to provide a large (possibly superficial) part of the external anal sphincter (EASsp in panel D). The developing perineal body (PB) is derived from an anteromedian part of the longitudinal or outer smooth muscle coat of the rectum (outer). UVS, urethrovaginal sphincter; VB, vestibular bulb.

Fig. 4. Frontal sections showing communication between the male external anal sphincter and bulbospongiosus (22 weeks male; CRL, 170 mm).

Panel A (or Panel F) is the most anterior (or the most posterior) section of the figure. Intervals between panels are 0.7 mm (A-B), 0.5 mm (B-C), 0.3 mm (C-D), 0.6 mm (D-E) and 0.9 mm (E-F), respectively. The left-hand side of the figure corresponds to the right side of the pelvis (anterior view). Magnifications for panels D-F are slightly higher than for panels A-C, in order to show muscle fiber directions (scale bar, panels A and D). The subcutaneous part of the external anal sphincter (EASsc) appears at the inferior end of the perineal raphe (arrow in panel A; higher magnification views, see Fig. 5). The EASsc issues muscle fiber bundles (arrows in panels C-E) toward the bulbospongiosus (BS) or the superficial part of the EAS (EASsp). The right marginal part of the BS becomes divided (asterisk in panels A and B) and, posteriorly, this subdivision changes in fiber direction from an anteroposterior to a transverse course (asterisk in panels C-E). However, in the left side of this specimen, the BS tends to maintain its mass-like arrangement at posterior sites (BS in panels C-E). The levator ani (LA) issues muscle fibers postero-inferiorly (star in panels D and E) to form the deep part of the EAS (EASdp). In panels B-D, a smooth muscle core of the developing perineal body (PB) is seen as a column situated between the bilateral bulbo-urethral glands (G in panel C) or between the bilateral transversus perinei profundus (TPP in panel D). CL (panel F), conjoining longitudinal muscle coat; CS (panel A), colliculus seminalis of the ductus deferens; IAS (panel F), internal anal sphincter; PR, prostate (panels A-C).

Fig. 5. Frontal sections showing the perineal raphe and subcutaneous muscle (22 weeks male; CRL, 170 mm).

Same specimen as in Fig. 4. Panel A (B) corresponds to a level 1.0 mm (0.1 mm) anterior to Fig. 4A. In panel A, subcutaneous muscle fibers (EASsc) appear to originate from the superficial or inferior end of the perineal raphe. The raphe regresses more deeply or superiorly, and the much more subcutaneous muscles become aggregated beneath the skin (panel B). asterisks, developing fat.

Perineal body and other structures

In transverse sections (Figs. 1–3), on the inferior side of the inferior rectal valve of Kohlrausch, the longitudinal smooth muscle layer of the rectum formed a continuous muscle coat (Figs. 1A and 3) or was composed of multiple bundles resulting in a rosette-like appearance around the rectal circular muscle layer (Figs. 1B and 2). The presence of the continuous coat or rosette-like appearance was not dependent on gender, or even stage, and was considered most likely to vary among individuals. Along the median line, the continuous outer muscle coat protruded anteriorly and, at a more inferior level, this tongue-like protrusion was separated from the rectum to form an independent smooth muscle mass (Fig.

3C; immunohistochemistry, not shown). Likewise, when the multiple bundles comprised the outer muscle coat of the rectum, one to three of the bundles became large or thick at a more inferior level and formed one or multiple independent muscle mass(es) (Figs. 2A–2C). Each of the latter masses was smaller than that originating from the tongue-like protrusion. We regarded the smooth muscle mass or protrusion as a primordium of the adult perineal body. In frontal sections, the developing perineal body was identified as a “column” extending supero-inferiorly. This column was sandwiched by the bilateral anteromedial ends of the levator slings, the bilateral bulbo-urethral or greater vestibular gland, or the developing transversus perinei profundus (Figs. 4C and 4D). Nerves sometimes

Fig. 6. Schematic representations showing development of the external anal sphincter and its intergender difference: a hypothesis based on the present observations.

Panel A (or Panel B) displays the male (or the female) morphology. The left-hand side of the figure represents the hypothetical origins of each part of the external anal sphincter (EAS) according to patterns of muscle cleavage and joining. The right-hand side of the figure exhibits the adult morphology. The longitudinal muscle coat of the anorectum is defined by a series of muscle bundles arranged circularly around the lumen. The male EAS is divided into deep, superficial and subcutaneous parts (dp, sp and sc) according to the hypothetical origins. The subcutaneous muscle (sc) develops along the perineal raphe and seems to migrate posteriorly. In the female, the sc is few in number and the bulbospongiosus (BS) communicates with the levator ani (LA). TPP, transversus peprinei profundus; UVS, urethrovaginal sphincter.

penetrated the developing perineal body and communicated between the bilateral nerve plexuses in an angular area between the rectum and urethra (or vagina).

The levator ani was separated from the posterior half of the rectum by a narrow fascial space (Fig. 3C). However, at the 1–3 and 9–11 o'clock positions (i.e., the anterior half except for the median area), the epimysium of the levator ani ran anteriorly and internally, passed between the longitudinal muscle bundles, and joined the intermuscular connective tissue layer of the anorectum (Figs. 2A and 3D). Frontal sections (Fig. 4F) demonstrated that the longitudinal smooth muscle layer of the rectum extended inferiorly between the EAS and the internal anal sphincter to form a conjoining longitudinal coat. However, this was still loose around 30 weeks of gestation.

We were unable to find the pubo-analis, i.e., the innermost part of the puborectalis (Macchi *et al.* 2008; Fröber *et al.* 2001), even though an intergender differ-

ence has been reported. The present fetuses did not show a tight connection between the internal anal sphincter and conjoining longitudinal coat, in contrast to the adult morphology described by Arakawa *et al.* (2004). In addition, the middle rectal artery was formed as a branch of the artery running along one of the pelvic splanchnic nerves and ending at ganglion cell clusters in the angular portion between the urethra (or vagina) and the rectum. In females, this artery supplied the anterior vaginal wall rather than the rectum (figures, not shown).

Discussion

It is well known that the deep portion of the EAS forms a complex with the levator ani (Fritsch *et al.* 2002). However, to our knowledge, it has not been clarified what constitutes the portion superficial to the levator-EAS complex. Our present observations suggested that

the bulbospongiosus plays a critical role in connecting or “cementing” the levator-EAS complex to the subcutaneous muscle (Fig. 6A). The “cement” material originated from a mixture of subcutaneous muscle along the perineal raphe and a large subdivision of the bulbospongiosus, the ratio of the combined elements in the cement apparently showing a close relationship to the interindividual and intergender variations of the adult EAS.

In contrast, the female bulbospongiosus appeared to communicate with the levator, rather than the EAS, presumably because the transversus perinei profundus was restricted to the area around the developing perineal body and did not interfere with the posterosuperior extension of the bulbospongiosus. Moreover, the female subcutaneous muscle was poorly developed because of absence of the perineal raphe and the insufficient space for muscle development. Therefore, although analysis of the posterior EAS near the coccyx is still insufficient, the female EAS seems to be composed of 1) a fragmented subcutaneous part, 2) a small superficial part due to the suggested reduction of muscle fibers from the bulbospongiosus and, 3) a deep part as large as in males. In Fig. 6B, morphology of the female perineal membrane, which connects the levator to the distalmost vagina (invisible), is based on our recent paper (Kato *et al.* 2008). The transversus perinei profundus is omitted in Fig. 6B because our previous work (Nakajima 2007) showed that a sheet-like muscle is absent. Although previous reports did not state (Sato 1980; Wallner *et al.* 2008), a detailed study on the perineal muscle innervation may reveal the intergender difference in a contribution of the bulbospongiosus to the EAS or levator.

The subcutaneous muscle developing along the perineal raphe was one of the striking observations in the present study. van der Putte (2005) described details of the perineal raphe, which was considered to differ from the ventral perineal raphe (i.e., the so-called scrotal raphe); the present dorsal raphe appeared to be made up of stroma originating from the anus-related posterior parts of the labia. Strangely, however, he did not describe the subcutaneous muscle. The morphology of the perineal raphe is a critical feature for discrimination of anorectal malformations (Murphy *et al.* 2006): in short, the hypertrophic raphe covers the anus, and this results in stenosis of the anus. A remnant of the raphe provides uncommon congenital lesions (Scelwyn 1996; Krauel *et al.* 2008). However, to our knowledge, there has been no description of the subcutaneous muscle in pathological conditions involving the raphe (van der Putte 2006; Stephens 2006). Therefore, the EAS in association with anorectal malformations may be derived from the levator and/or bulbospongiosus, not from the subcutaneous muscle. van der Putte (2006) described that, in specimens with ectopic anocutaneous canal, the EAS sometimes merges with a dorsally open bulbospongiosus. In addition, the subcutaneous muscle was not associated with smooth

muscle, even though Fritsch *et al.* (2007) reported that distinct smooth muscle spread to and around the developing human EAS.

Patterns of muscle cleavage and union have been one of the major focuses of research into muscle development (e.g., Kieny *et al.* 1986). Recently, Sasaki *et al.* (2004) and Yamaguchi *et al.* (2008), studying mouse embryos, demonstrated an intimate topographical relationship between the bulbospongiosus and the EAS promordium. Valasek *et al.* (2005), using chick and mouse embryos, confirmed experimentally that all perineal muscles including the EAS are derived from the ventral muscle mass of the hind limb. Moreover, van der Putte (2005), in his comprehensive review, described that the human bulbospongiosus primordium is combined with that of the “deep portion” of the EAS, although he classified the EAS into superficial and deep portions. The present observations suggested that the EAS had multiple origins: 1) the levator ani, 2) the bulbospongiosus, and 3) the subcutaneous muscle along the perineal raphe (Fig. 6). The reliability of the classical tripartite subdivisions of the EAS has recently been questioned (Fritsch *et al.* 2002). However, conversely, the above three suggested embryologic origins are likely to redefine the three subdivisions.

The primordium of the perineal body seemed to correspond to 1) a tongue-like protrusion of the longitudinal smooth muscle layer of the rectum, or 2) one to three of the multiple longitudinal muscle bundles of the rectum. Which of these patterns, 1 or 2, was chosen did not seem to depend on gender or stage. van der Putte (2005) did not consider that the perineal body originated from the rectal smooth muscle, but from the deep dorsal urogenital stroma. In fact, in the present male fetuses, a thick fascia between the rhabdosphincter and levator also contributed to the formation of the perineal body. Soga *et al.* (2007, 2008) described that the adult perineal body could potentially be composed of variable combinations of the following three structures: 1) the inferior part of the recto-urethralis (in males), 2) a smooth muscle mass communicating with the longitudinal smooth muscle layer of the anorectum; 3) a venous plexus communicating with the genital cavernous tissue. Notably, the aforementioned pattern 2 was quite similar to the present observations of fetuses. We hypothesized that the perineal body was, to a greater or lesser degree, likely to be made of the anterior median parts of the longitudinal muscle coat of the rectum. This might correspond to the primitive perineal body described by Levi *et al.* (1991) in a 7-week fetus. Oerlich (1980) considered the perineal body primordium to be the recto-urethralis in the male fetus. However, in the present study, a smooth muscle mass was usually seen communicating with the rectal longitudinal muscle coat even in females. In addition, the transversus perinei profundus seemed to develop near the developing perineal body in both genders.

Study limitation: Observations of the stages earlier

than the present fetuses would have demonstrated a beginning of the contribution of the bulbospongiosus primordium to the EAS or levator formation (and its intergender difference) more clearly. The terminology “EAS” was convenient but it seemed to be inadequate for a muscle complex of multiple origins such as the subcutaneous muscle, bulbospongiosus and levator.

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Common abbreviations for figures

BS: bulbospongiosus
CCP or CCC: corpus cavernosum penis or clitoridis
EAS: external anal sphincter (dp, deep part; sp, superficial part; sc, subcutaneous part)

G: gland (bulbo-urethral or major vestibular gland)
IC: ischiocavernosus
IPR: ischiopubic ramus
LA: levator ani
OI: obturator internus

PB: developing perineal body
RS: (urethral) rhabdosphincter
UR: urethra; outer (inner), inner circular (or outer longitudinal) smooth muscle layer of the anorectum.