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Hyaluronan and Its Binding Proteins during Cervical Ripening and Parturition: Dynamic Changes in Size, Distribution and Temporal Sequence

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Abstract

The uterine cervix undergoes changes during pregnancy and labor that transform it from a closed, rigid, collagen dense structure to one that is distensible, has a disorganized collagen matrix, and dilates sufficiently to allow birth. To protect the reproductive tract from exposure to the external environment, the cervix must be rapidly altered to a closed, undistensible structure after birth. Preparturition remodeling is characterized by increased synthesis of hyaluronan, decreased expression of collagen assembly genes and increased distribution of inflammatory cells into the cervical matrix. Postpartum remodeling is characterized by decreased hyaluronan (HA) content, increased expression of genes involved in assembly of mature collagen and inflammation. The focus of this study is to advance our understanding of functions HA plays in this dynamic process through characterization of HA size, structure and binding proteins in the mouse cervix. Changes in size and structure of HA before and after birth were observed as well as cell specific expression of HA binding proteins. CD44 expression is localized to the pericellular matrix surrounding the basal epithelia and on immune cells while inter α trypsin inhibitor (I α I) and versican are localized to the stromal matrix. Co-localization of HA and I α I is most pronounced after birth. Upregulation of the versican degrading protease, ADAMTS1 occurs in the cervix prior to birth. These studies suggest that HA has multiple, cell specific functions in the cervix that may include modulation of tissue structure and integrity, epithelial cell migration and differentiation, and inflammatory responses.

Keywords

Parturition; cervical ripening; hyaluronan; CD44; versican

Introduction

The molecular mechanisms controlling cervical remodeling during pregnancy and parturition remain to be elucidated and are critical towards the development of therapies to reduce the

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incidence of preterm birth. The cervix is a collagen-rich structure that must remain tightly closed during pregnancy to keep the fetus in the uterus. In preparation for birth, the cervix undergoes a progressive remodeling throughout pregnancy. The earliest phase of remodeling, termed softening, is characterized by an increase in collagen solubility and tissue distensibility as compared to nonpregnant cervix (Read et al., 2007). As softening progresses and overlaps with the later phase of cervical ripening, there is increased cell proliferation, tissue hydration and a progressive increase in tissue distensibility without any further increase in collagen solubility (Mahendroo et al., 1999; Read et al., 2007). Cervical ripening and dilation of the cervix upon initiation of uterine contractions is characterized by extensive changes in the structure of the extracellular matrix. Changes in collagen fibril structure brought about in part by changes in glycosaminoglycan and proteoglycan composition result in a loss of tensile strength of the cervical matrix and the ability of the cervix to dilate upon generation of forceful coordinated uterine contractions (Leppert, 1995).

One major change in numerous species during this period is the large increase in synthesis of the glycosaminoglycan, hyaluronan (HA) (Anderson et al., 1991; Downing and Sherwood, 1986; El Maradny et al., 1997; Rajabi et al., 1992). Increased HA synthesis in human and mouse cervix at term results from increased transcription of one of three HA biosynthetic enzymes, hyaluronan synthase 2 (HAS2) (Straach et al., 2005). HAS2 is expressed in the cervical epithelia while the majority of HA is located in the matrix surrounding the cervical stroma.

Hyaluronan (HA) is a polymer made up of repeating disaccharides of D-glucuronic acid and β -1, 3-N-acetylglucosamine- β 1, 4. HA exhibits unusual physiochemical properties because of its random coil structure, its large size, and its capacity to interact with water. Thus HA forms solutions with high viscosity and elasticity that provide both space-filling, lubricating functions, serves as a substrate for assembly of proteoglycans, promotes cell movement, regulates cell function and development and is involved in tumor progression, inflammation and wound healing (Almond, 2007; Lee and Spicer, 2000; Tammi et al., 2002).

HA functions are determined in part by the size of HA, structure of HA and HA's interaction with HA binding proteins termed hyaladherins. High molecular weight HA plays a structural role and promotes tissue integrity while low molecular HA may be a signal of tissue injury or wound healing (Almond, 2007). The association of HA with specific hyaladherins can modulate the physical structure and properties of HA within the ECM. For example, viral infection of colon smooth muscle cells or inflammation of the colon result in formation of HA cables structures which are highly adhesive for mononuclear leukocytes (de la Motte et al., 2003). A similar observation has been made in renal proximal tubular epithelial cells (Selbi et al., 2006). In contrast, HA structures described as aggregates or coats are not adhesive for tissue monocytes. The formation of cable-like filamentous structures requires the binding of HA to the heavy chain (HC) subunit of the inter α trypsin inhibitor protein family (ITI). Molecules of the ITI family are composed of a common proteoglycan subunit termed, bikunin (40kDa) and heavy chains (75kDa) which are encoded by 3 distinct genes (Salier et al., 1996). One family member, pre- α -trypsin inhibitor (PaI) contains one heavy chain while inter- α trypsin inhibitor (I α I) contains two heavy chains. The heavy chain (HC) is also pivotal in formation of a stabilized HA-rich cumulus oocyte complex during ovulation (Mukhopadhyay et al., 2001; Sato et al., 2001; Zhuo et al., 2001). The transfer of the HC to HA is catalyzed by TNF stimulated gene 6 (Tsg6) in the ovary but is not required for transfer of HC to HA in the renal proximal tubular epithelial cells (Fulop et al., 2003; Selbi et al., 2006).

The association of hyaluronan with HA-binding proteoglycans such as versican, aggrecan and brevican is essential to the stabilization of HA structure and assembly of several tissues during development and postnatal life. HA interactions with proteoglycans are further strengthened by associations with members of the link protein family (HAPLN 1-4) (Spicer et al., 2003).

For example, HA forms aggregate structures with aggrecan which provide stable load bearing capabilities to cartilage. Other HA binding proteins include the cell surface receptor, CD44, which mediates cell signaling of HA and internalizes HA for breakdown by hyaluronidases. In addition the assembly and retention of a pericellular matrix of many cells involves interactions between CD44 and HA (Day, 1999).

In the current study hyaluronan size, structure and HA binding proteins that may influence HA function were evaluated during mouse pregnancy and parturition in an effort to better understand the biological roles HA may play in cervical remodeling.

Results

Mice at late stages of gestation were utilized to evaluate temporal changes in HA molecular weight. While total hyaluronan content is increased in the cervix during cervical ripening, changes in the distribution of hyaluronan based on estimated molecular size is unknown (Straach et al., 2005). Cervical extracts from gestation days 12, 15, 16, 17, 18, 18.75, in labor (IL) and postpartum samples at 2–4h pp, 10–12h pp, 24h pp were run on agarose gels along with high and low HA molecular weight standards. Gel was stained with Stains-All as previously described and HA is stained in blue (Fig 1) (Lee and Cowman, 1994). Relatively little HA is present until day 18 when there is an increase in high and to a lesser extent low molecular weight HA with greater amounts by late day 18 (18.75), in labor on day 19 (IL) and 2–4 hours postpartum. Ten to 12 hours after birth while high MW HA is still abundant there is also an increase in medium to low molecular weight HA in the range of 495 –110 kDa. By 24 hours postpartum the majority of HA is medium to small MW HA. These results suggest that during cervical ripening and dilation the majority of HA is large molecular weight and that the breakdown of HA to smaller molecular weight HA begins during postpartum repair of the cervix.

Previous studies have identified an abundance of HA in the matrix surrounding the cervical stroma (Straach et al., 2005). To more accurately assess HA localization and structure in the cervix prior to, during and after cervical ripening fluorescence microscopy was carried out utilizing a biotinylated HA binding protein. In addition the sections were co-stained for the HC of IαI. In Figure 2, the panels on the left show staining for HA in green and IαI HC in red in the cervical stroma while HA staining (green) in the epithelia is shown on the right. Colocalization of HA and IαI HC in the stroma is shown in yellow. HA was present before (d15), during (d18) and after (8–10h pp and 24h pp) cervical ripening in the stromal matrix. Interestingly, the structure of HA appeared different in cervixes before birth as compared to after birth. Prior to birth the HA appears disorganized and randomly oriented while after birth appears as strands or filaments. IαI HC expression indicated in red, was not significant until day 18 and there was little colocalization with hyaluronan until the postpartum period coincident with the change in HA structure. HA was not only present in the stromal matrix as previously described but also localized to the pericellular matrix surrounding the cervical epithelia (Fig 2). Prior to birth the pericellular staining was primarily in the basal epithelia and was more evident on day 18 as compared to day 15. Eight to twelve hours postpartum HA was present in all layers of epithelia and appears to be secreted into the mucus. HA staining was still present 24h pp but was reduced in all layers of epithelia.

To further substantiate the observation that HC of ITI proteins bind HA and IαI and PαI are increased in the cervix shortly after birth, protein blotting experiments were carried out using cervixes from nonpregnant, and gestation days 10, 15, and 18 and 24h postpartum females. Cell extracts representing an equal proportion of cervix were incubated in PBS overnight at 37°C in the absence and presence of *Streptomyces* hyaluronidase as previously described (Mukhopadhyay et al., 2001). Extracts were analyzed by SDS-PAGE under reducing

conditions, followed by blotting and incubation with a biotinylated HA binding protein (HABP) (top panel) or $\text{I}\alpha\text{I}$ HC polyclonal antibody (lower panel). HABP probe analysis revealed the presence of HA by gestation d15 with a gradual increase in HA which peaked 24h post partum (Fig 3, Top panel). Treatment of extracts with *Streptomyces* hyaluronidase resulted in loss of detectable HA. The presence of HC in the cervix is shown in Fig 3, lower panel. In untreated extracts, the polyclonal $\text{I}\alpha\text{I}$ HC antibody recognizes a band at the top of the gel which corresponds to HC covalently anchored to HA (Mukhopadhyay et al., 2001). The two ITI proteins at approximately 250 and 125 kDa are $\text{I}\alpha\text{I}$ and $\text{P}\alpha\text{I}$ respectively which are found circulating in blood. These proteins are increased in cervical tissue at the end of pregnancy most likely due to increased tissue vascularity in the pregnant cervix as compared to the nonpregnant (Mowa and Papka, 2004). As $\text{I}\alpha\text{I}$ and $\text{P}\alpha\text{I}$ are increased in the cervix, more HC becomes available for association with HA. The association of HA with HC appeared to increase with progression of pregnancy and reached a maximum in the postpartum period (Fig 2 and 3). Upon treatment of extracts with *Streptomyces* hyaluronidase, the association of HC with HA is lost resulting in an increase of the 75 kDa band corresponding to the HC. The source of HC in the nonpregnant cervix is unclear since little $\text{I}\alpha\text{I}$ or $\text{P}\alpha\text{I}$ was detectable. These results further confirm an association of HA with HC in the pregnant cervix that is maximal in the postpartum period. The covalent transfer of HC from ITI proteins to HA requires TSG6 in the ovary (Mukhopadhyay et al., 2001). Low expression of transcripts encoding *Tsg6* has been identified in the pregnant cervix by RTPCR (unpublished observations).

The interaction of HA with cell surface receptors such as CD44 can mediate cell signaling events as well as serve to internalize HA where it can be degraded or perhaps carry out other functions. Relative to nonpregnant, transcripts for CD44 were not significantly different in pregnant cervixes from gestation day 8 onwards as determined by quantitative real time PCR (Fig 4). Though not significant, highest expression was observed a few hours after birth. The cell specific expression of CD44 was detected by immunohistochemistry and immunofluorescence utilizing a rat anti-mouse CD44 antibody and paraffin embedded cervical sections at gestation days 15 and 18, and 8–10 or 24 hours after birth (Fig 5). At both gestation day 15 and 18 (Fig 5, panels A and B respectively), CD44 staining indicated in pink is evident in the pericellular matrix surrounding the basal cervical epithelia and in immune cells embedded in the matrix of the cervical stroma. The intensity of staining or number of CD44 positive cells did not appear to change between the two time points. Regulated changes in the cell specific localization of CD44 is reported to influence HA binding affinity (Sleeman et al., 1996). Immunofluorescence staining of CD44 was carried out to better evaluate changes in cell localization. Changes were identified in the CD44 positive immune cells within the stroma. CD44 staining, shown in red or pink, appeared on the cell surface of immune cells on gestation day 15 (Fig. 5, panel C). On gestation day 18 CD44 staining was on the cell surface of most positive cells as well as clustered on one side of the cell in other cells (Fig. 5, panel D). Shortly after birth the majority of CD44 moved to one pole of the cell, a process termed clustering or capping which may increase the binding affinity of CD44 to HA (Sleeman et al., 1996) (Fig 5, panel E). Twenty four hours after birth a new population of immune cells appears as evidenced by the presence of cells expressing CD44 on the cell surface along with CD44 capped cells (Fig 5, panel F). CD44 expression in both immune cells and basal epithelia suggest multiple functions for this receptor in the cervix.

In addition to CD44, the mRNA expression of proteoglycans that may interact with HA was evaluated in the cervix by RTPCR. Transcripts for versican were expressed in the nonpregnant and pregnant cervix while transcripts for aggrecan and brevican were low to undetectable (Fig 6). Brain cDNA was used as a positive control. Expression of link protein family members (HAPLN 1-4) which form a complex with proteoglycans and HA were also evaluated. HAPLN 1-4 expression was measured and only HAPLN1 and 4 transcripts were detectable at low levels (data not shown). The expression of versican was further characterized to determine mRNA

isoforms expressed in the cervix and the temporal expression during pregnancy and parturition from gestation days 8 to the day of birth (day 19). Isoform specific primers were utilized in RTPCR to estimate the relative abundance of V0, V1, V2 and V3 isoforms as previously described (Russell et al., 2003b). In the cervix the major isoform was V1 with lowest expression of isoforms V2 and V3 (unpublished observations). Using quantitative real time PCR, the expression of only the V0 and V1 isoforms or all isoforms was measured (Fig 7A). Versican was expressed throughout gestation with little change in expression until shortly after birth. The increased postpartum expression was not statistically significant. The temporal pattern of expression was similar using V0/V1 isoform specific primers as well as primers that recognize all isoforms consistent with our observation that the V1 isoform is the major product in the pregnant cervix.

To determine the cell specific expression of versican, immunohistochemical detection of versican was carried out utilizing a polyclonal antibody against mouse versican (Fig 7B). At both gestation days 15 and 18 the majority of protein was localized in the matrix surrounding the stroma similar to the pattern of expression of HA in Figure 2.

In a recent study in which genes upregulated during cervical ripening were identified in the mouse, a protease that cleaves versican and other proteoglycans such as aggrecan was found to be upregulated on gestation day 18. (Timmons and Mahendroo, 2007). This protease, ADAMTS1 (a disintegrin and metalloprotease with thrombospondin-like repeats-1), cleaves versican V0 and V1 resulting in loss of versican cross-linking to HA. The expression of the precursor and mature form of the ADAMTS1 enzyme was determined in the cervix during pregnancy and parturition utilizing antibodies specific to each form of the protein (a gift from Dr JoAnne Richards, Baylor College of Medicine) (Russell et al., 2003a). Both the precursor protein at 110 kDa and mature form of the protein at 85 kDa were transiently upregulated on gestation day 18 and 10 to 12 hours postpartum (Fig 8) suggesting that versican breakdown and loss of versican-HA cross-links may facilitate or precede the final stages of cervical ripening or cervical dilation once uterine contractions are initiated.

Discussion

Successful birth is critical for the survival of species and in ovoviviparous animals requires both coordinated uterine contractions and remodeling of the cervix to allow passage of a term fetus through the birth canal. Progressive changes in the biomechanical properties of the cervix begin during pregnancy and are accelerated during the phases of cervical ripening and dilation in the final days or hours of pregnancy. After birth the cervix completes the loop of remodeling via rapid and extensive changes that take the cervix back to a rigid, undistensible structure in which collagen fibrils are tightly packed. Our studies in the mouse cervix suggest that HA has multiple functions in the cervix and many of the characteristics are similar to remodeling or tissue repair processes in other biological systems.

With respect to HA size and perhaps structure, notable differences were apparent before parturition as compared to the post partum period. The predominance of high molecular weight HA during cervical ripening suggest that HA functions during this phase in the matrix are predominantly structural – providing viscoelasticity and space filling properties as collagen fibrils become spread out and disorganized. Similar to HA function in other biological systems, we hypothesize that the change in HA structure, binding of HC to HA and reduction in HA size may serve as signals for tissue damage and proinflammatory responses that are activated during postpartum repair (Almond, 2007; de la Motte et al., 2003; Timmons and Mahendroo, 2006). The decline in HA size is supported by previous observations that the mRNA expression of hyaluronidase 1 and 2 is upregulated in the postpartum cervix (Straach et al., 2005).

Identification of hyaladherins expressed during cervical remodeling also provided insights into potential functions of HA. mRNA expression of the cell surface receptor CD44 is relatively constant during pregnancy and postpartum and is expressed both in basal epithelia as well as immune cells within the stromal matrix. CD44 is colocalized with HA in the pericellular matrix surrounding the basal epithelium which may be required for assembly and retention of pericellular coats. The retention of HA at the cervical epithelial surface by binding to CD44 may be important for cell migration, differentiation and permeability barrier homeostasis similar to HA-CD44 interactions in keratinocytes (Bourguignon et al., 2007; Kaya et al., 1997). Dynamic changes in cervical epithelial cell differentiation and barrier properties are reported to be initiated by late gestation day 18 in the mouse (Timmons et al., 2007). Further studies are required to link these changes with activation of cell signaling pathways initiated by the binding of HA to CD44.

The cell localization of CD44 in the immune cells appeared to change over time as on day 15 the majority of staining was on the cell surface while on day 18 there were fewer stained cells and CD44 protein appeared to be clustered at one pole of the cell in a few cells. Shortly after birth the majority of cells had CD44 clustered to one end of the cell. Within 24 hours post partum cells with CD44 localized to the cell surface reappeared as would be expected when new immune cells are recruited into the tissue. We hypothesize that the changes in the distribution of CD44 during the parturition process along with the changes in HA organization may be correlated with changes in binding affinity of HA and the increased clustering of CD44 after birth may facilitate the uptake and internalization of HA as part of the normal process of postpartum cervical repair. Further studies are required to confirm this possibility.

During cervical ripening an increase in staining for heavy chain of IaI was detectable though the majority appeared to be in the blood vessels and not associated with HA. Within hours following birth IaI and PaI protein are increased in the cervix and the majority of HC is colocalized with HA (Fig 2 and 3). Transcripts for genes encoding HC 1, HC 2 and HC 3 were undetectable and transcripts for bikunin were very low (unpublished observations) suggesting that IaI and PaI from the blood is the source of HC in the cervix. The colocalization of HC with HA after birth coincides with the change in HA structure to strands or filaments (Fig 2) and the activation of proinflammatory responses we have previously observed in the mouse cervix after birth (Timmons and Mahendroo, 2006). Taken together these studies suggest that in the presence of HC, HA forms structures that are conducive to activation of inflammation in response to tissue “injury” and a need to rapidly modify the matrix back to the nonpregnant state.

The proteoglycan versican was expressed in the stroma at a constant level. Similar to HA, versican has numerous functions that include cell adhesion, proliferation, migration, and extracellular matrix assembly (Wight, 2002). Loss of versican expression in mice prevents appropriate migration of embryonic cells required for heart development (Mjaatvedt et al., 1998). This phenotype is identical to mice deficient in HAS2, suggesting the importance of HA-versican cross-links in matrix stabilization (Camenisch et al., 2000).

Within the cervix, the increased expression of ADAMTS1 late on gestation day 18 and postpartum may result in cleavage of HA-versican cross-links. ADAMTS1 null mice are reported to have normal parturition though litter sizes are smaller due to a reduced ovulation rate (Mittaz et al., 2004). We speculate that HA-versican cross-links promote the progressive increase in tissue distensibility and compliance yet maintain tissue integrity during cervical ripening. Upon increase of the ADAMTS1 protease, HA-versican cross-links are disrupted and tissue integrity is lost. The cervix is then able to dilate and upon initiation of uterine contractions opens sufficiently to allow passage of young through the birth canal. Future studies in our laboratory will test this hypothesis.

These studies highlight the diverse cell and temporal specific functions HA may play in the cervix during parturition and post partum repair. The dynamic changes in HA size and structure as well as cell and temporal specific expression of CD44, IaI and versican allow this fascinating molecule to play numerous roles that may influence structure, cell migration, epithelial barrier function and proinflammatory responses. Future studies will better identify the molecular mechanisms of these processes.

Experimental Procedures

Mice

Animals were housed under a 12L:12D photoperiod (lights-on, 0600–1800 h) at 22°C. Mice used in the present studies were of mixed strain (C57BL/6 x 129SvEv). The C57BL/6 x 129SvEv mice were generated and maintained as a breeder colony at the University of Texas Southwestern Medical Center (Dallas, TX). In general, mice in these studies were 3 to 6 months old and nulliparous. Female mice were housed overnight with males and checked at midday for vaginal plugs in order to obtain accurately timed pregnant mice. The day of plug formation was counted as day zero, and birth occurred in the early morning hours of day 19. Cervices collected before noon on the day of birth were estimated to be 10 hours postpartum and those collected before noon on the day after birth were estimated to be 24 hours postpartum. All studies were conducted in accordance with the standards of humane animal care as described in the NIH Guide for the Care and Use of Laboratory Animals. The research protocols were approved by the institutional animal care and research advisory committee.

HA Molecular Weight Gels

One cervix per time point was lyophilized overnight and wet/dry weight was determined by weighing the sample before and after lyophilization. Single cervixes were digested in 950ul 0.0005% Phenol Red, 100mM ammonium acetate, pH 7.0 containing 125 mg proteinase K (Roche, Indianapolis, IN) for 2 hours at 60°C. Another 125mg of proteinase K was added to each tube and incubated for another 2 hours. The sample was boiled to inactivate the proteinase K and pelleted by centrifugation to remove any undigested material. The sample was aliquoted equally into 8 tubes.

One aliquot of each sample was treated with 3ul of DNase (Ambion, Austin, Texas) and 3ul of RNaseA 1.28mg/ml stock (Roche, , Indianapolis, IN) and incubated overnight at 37°C. The enzymes were inactivated by boiling for 5 minutes. Glycosaminoglycans were precipitated in ethanol at –20°C overnight. GAGs were pelleted by centrifugation, and pellets were air dried, resuspended in 20ul 1X TAE and run on a 1% agarose gel (Seakem HGT Cambrex, Rockland, ME,).

The gel was prerun for 4–6 hours at 80V. Buffer was replaced with fresh TAE solution prior to loading samples. Four µl of loading buffer (0.2% Bromophenol Blue, 1ml 1X TAE, 8.5ml glycerol) was added to each sample. Samples were loaded onto the gel and run at 100V. Five microliters of high molecular weight ladder (Hyalose, Oklahoma City, OK) and low molecular weight ladder (Hyalose) were loaded onto the gel. After electrophoresis, the gel was equilibrated in 30% ethanol for 1 hour at room temperature. Bands were visualized by incubating the gel in 0.01mg/ml Stains All Solution (Sigma, St Louis, MO) overnight at room temperature in the dark. Dye was then removed and replaced with water and exposed to light briefly to reduce background. Gel was then scanned into Adobe Photoshop using a flat bed scanner.

Fluorescence histochemistry for confocal microscopy

Paraffin sections (5 μ m thick) were deparaffinized and preincubated with Hanks' BSS containing 2% FBS (30 min, 25°C). After removing the medium, the tissue sections were incubated with a solution containing biotinylated hyaluronan binding protein (Seikagaku) (5 μ g/ml) and either anti-mouse CD44 rat monoclonal antibody (5 μ g/ml) or rabbit polyclonal inter-alpha inhibitor antiserum (1:100) in Hanks' BSS containing 2% FBS for ~16 h at 4°C. The slides were washed three times with Hanks' BSS, and then incubated with a solution containing fluorescein-tagged streptavidin (1:500) or Alexa 568 conjugated anti-rat Ig (H+L) (1:1000) in Hanks' BSS containing 2% FBS. This secondary incubation was done for 1 h at 25°C. The slides were washed three times in Hanks' BSS and cover slips mounted to the slides in Vectashield mounting medium containing DAPI (Vector Labs, Inc.). The slides were then sealed with nail polish and stored at -20°C.

Confocal images were obtained using a Leica TCS-SP laser scanning confocal microscope (Leica, Heidelberg, Germany), which is equipped with three lasers and photodetectors that permit detection of three distinct fluorochromes.

IaI Western

Two cervixes were collected for each time point, pooled together, and crushed using a tissue pulverizer followed by homogenization in 300 μ l cold 0.1M PBS plus 1% protease inhibitor (Sigma, St. Louis, MO). Homogenization was done with a Polytron tissue homogenizer. Samples were divided into two 100 μ l aliquots, one of which was treated with 10 TRU *Streptomyces* Hyaluronidase (Seikagaku, Japan) and the other was untreated. Both aliquots were incubated at 37°C for 18 hours followed by centrifugation at 12,000 RPM for 10 minutes at 4°C.

Twenty microliters of the supernatant was boiled for 5 minutes in reducing Laemmli buffer and analyzed by SDS-PAGE on 4–20% Tris HCl precast gels (Bio-Rad) along with protein standards (Precision Plus Protein Kaleidoscope, Bio-Rad, Hercules, CA). Proteins were transferred onto nitrocellulose membrane (Biotrace Pall Life Sciences, Pensacola, FL) at 100V for 1 hour at 4°C. Membrane was blocked overnight at 4°C in blocking solution (10% skim milk, 0.2% Tween in TBS). IaI HC was detected with rabbit polyclonal anti-human IaI HC after incubating for 2 hours at RT (1:1000, Dako, Carpinteria, CA,) followed by HRP-conjugated anti-rabbit IgG for 45 minutes at RT (1:10,000, Jackson ImmunoResearch Laboratories, West Grove, PA). This antibody recognizes the HC of both IaI and PaI in human and mouse. Hyaluronan was detected by incubating the membrane with biotinylated HAP (0.5 μ g/ml) for 2 hours at RT (Seikagaku, Japan) followed by HRP conjugated streptavidin. Positive bands were visualized using ECL detection reagent (Amersham Biosciences, Buckinghamshire, UK) according to manufacturer's specifications. Proteins were visualized and photographed using a Fuji film LAS-3000 chemimager (Tokyo, Japan).

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from frozen tissue using RNA Stat 60 (Tel-test B, Friendswood, TX). Total RNA was treated with DNase I (DNA free; Ambion, Austin, TX) to remove any genomic DNA contamination. Complementary DNA was synthesized using a TaqMan cDNA synthesis kit (Applied Biosystems, Foster City, CA). RT-PCR was performed using SYBR Green and a PRISM 7900HT Sequence Detection System (Applied Biosystems). Aliquots (20 ng) of total cDNA were used for each PCR reaction and were performed in triplicate. Expression of each gene was expressed relative to that of the housekeeping gene cyclophilin (*Ppib*). Relative levels of gene expression were determined by the ddCt method (Applied Biosystems User Bulletin #2).

Immunohistochemistry

CD44 staining was carried out using an anti-mouse CD44 rat monoclonal antibody (BD Pharmingen, San Diego, CA) while versican was identified using a polyclonal rabbit anti-mouse versican antibody (gift from Dr. Richard Lebaron at the University of Texas San Antonio, San Antonio, TX). Tissues were fixed in 4% paraformaldehyde. Paraffin embedded sections were deparaffinized and hydrated in xylene and a series of graded ethanol solutions, followed by three washes in phosphate buffered saline (PBS). For CD44 staining, the slides were subjected to citrate buffer antigen retrieval (BD Retrieval A, BD Pharmingen). Non-specific binding was blocked with 1.5% donkey serum (Jackson Laboratories, Westgrove, PA). Slides were incubated with CD44 antibody (6.25 μ g in PBS) for 2 hours at 37°C. Biotinylated donkey anti-rat (1:500 in PBS) (Jackson Laboratories, Westgrove, PA) was applied, proceeded by an incubation with an alkaline phosphatase conjugated avidin-biotin complex for 25 minutes (Vector Laboratories, Burlingame, CA). The tissues were then counterstained with filtered methyl green for 5 minutes and dehydrated before mounting.

For versican staining, endogenous peroxidases were blocked for 10 minutes at room temperature (3% H₂O₂ in methanol). Sections were washed 3 times in PBS followed by chondroitinase ABC treatment (Seikagaku Corp., Tokyo, Japan) (0.5mU/ul diluted in 1M Tris pH 7.4) for 30 minutes at room temperature. Non-specific binding was blocked with 1.5% Donkey Serum (Jackson Laboratories, Westgrove, PA) in PBS. Sections were incubated overnight at 4°C with primary antibody (1:25) diluted in PBS. Biotinylated donkey anti-rabbit (1:1000 in PBS) (Jackson Laboratories, Westgrove, PA) was applied, proceeded by an incubation with a peroxidase standard ABC complex for 45 minutes (Vector Laboratories, Burlingame, CA). Sections were washed in PBS and incubated with DAB substrate (Invitrogen, Carlsbad, CA) for 5 minutes. The sections were then counterstained with hematoxylin (Biomedex, Foster City, CA) and dehydrated in ethanol and xylene before coverslipping with Permount (Fisher, Fair Lawn, NJ).

ADAMTS1 Protein Blots

Two cervixes were collected for each time point and homogenized in 300ul cold RIPA buffer (150mM NaCl, 10mM Tris pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% DOCA, and 5mM EDTA) plus 1.5% protease inhibitor (Sigma, St Louis, MO). Samples were centrifuged at 14,000 RPM for 10 minutes at 4°C. Protein concentration of supernatants was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL).

Forty μ g of protein was boiled for 5 minutes in reducing Laemmli loading buffer and analyzed by SDS-PAGE on 4–20% Tris HCl precast gels (Bio-Rad Criterion gel) and transferred to PVDF Membrane (Biotrace Pall Life Sciences, Pensacola, FL) at 100V for 1 hour at 4°C. The membrane was blocked using NAP-Blocker in TBST (G Biosciences, St. Louis, MO) for 1 hour at room temperature, then probed with anti-mouse ADAMTS-1 α -prodomain antibody (1:3000 dilution NAP blocking solution) for 2 hours at room temperature. The secondary antibody- HRP-conjugated donkey anti-rabbit IgG antibody (Jackson, Westgrove, PA) was used at a 1:10,000 dilution in blocking solution and incubated for 45 minutes. Positive bands were visualized using ECL Western blotting detection reagents (Amersham Biosciences). Proteins were visualized and photographed using a Fuji LAS-3000 chemimager.

The same blot was stripped using BlotFresh Western Blot Stripping Reagent (Signagen, Gaithersburg, MD) and reprobed with anti-mouse ADAMTS α -metalloproteinase domain (1:1000). The membrane was stripped a second time then probed with calnexin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) to assess protein loading.

RT-PCR

CDNA was synthesized from 1ug total RNA from brain or cervix (nonpregnant, gestation d15 and d18) using the Taqman cDNA synthesis kit (Applied Biosystems, Foster City, CA). Five microliters of cDNA was used as a template for PCR. PCR was carried out using Platinum Taq polymerase (Invitrogen, Carlsbad, CA) and 30 cycles at the following conditions (94°C –30sec, 51°C - 40sec, 72°C - 40 sec). Primers for versican: forward-5'CAT GCA CTA CAT CAA GCC AA3' and reverse-5'TGC ATC ACA CTG CTC AAA TC3', aggrecan: forward-5'AGG ACT GTC TAT CTA CAC GA3' and reverse-5'GGG CGA TAG TGG AAT ACA AC3', and brevican: forward-5'CAA GGT AAA CGA AGC CTA CC3' and reverse-5'ATA GCC ATC CAT GTC TCC AG3'. PCR products were visualized on a 1.5% agarose gel in 1X TBE.

Statistics

Data were analyzed using one-way analysis of variance with pairwise multiple comparisons performed with Tukey test for data normally distributed. Non-parametric methods were employed for non-normal data. This included the Kruskal-Wallis test for one-way analysis of variance with multiple comparisons using Dunn's method. $P < 0.05$ are considered statistically significant. Data are displayed as mean + SEM. Data analyses were performed using SigmaStat V2.03 (SPSS, Chicago, IL).

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Abbreviations

ITI	inter α trypsin inhibitor protein family
IαI	inter α trypsin inhibitor
PaI	pre- α -trypsin inhibitor
ADAMTS1	a disintegrin and metalloprotease with thrombospondin-like repeats-1
HA	hyaluronan
HC	heavy chain
HAPLN	link protein family
TSG6	TNF stimulated gene 6
RTPCR	real time polymerase chain reaction

Reference List

- Almond A. Hyaluronan. *Cell Mol Life Sci* 2007;64:1591–1596. [PubMed: 17502996]
- Anderson J, Raynes J, Fitzpatrick R, Dobson H. Increased hyaluronate synthesis and changes in glycosaminoglycan ratios and molecular weight of proteoglycan: synthesised by cultured cervical tissue from ewes at various stages of pregnancy. *Biochimica et Biophysica Acta* 1991;1075:187–190. [PubMed: 1932074]
- Bourguignon LY, Peyrollier K, Gilad E, Brightman A. Hyaluronan-CD44 interaction with neural Wiskott-Aldrich syndrome protein (N-WASP) promotes actin polymerization and ErbB2 activation leading to beta-catenin nuclear translocation, transcriptional up-regulation, and cell migration in ovarian tumor cells. *J Biol Chem* 2007;282:1265–1280. [PubMed: 17092940]
- Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro A Jr, Kubalak S, Klewer SE, McDonald JA. Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest* 2000;106:349–360. [PubMed: 10930438]
- Day AJ. The structure and regulation of hyaluronan-binding proteins. *Biochem Soc Trans* 1999;27:115–121. [PubMed: 10093718]
- de la Motte CA, Hascall VC, Drazba J, Bandyopadhyay SK, Strong SA. Mononuclear leukocytes bind to specific hyaluronan structures on colon mucosal smooth muscle cells treated with polyinosinic acid:polycytidylic acid: inter-alpha-trypsin inhibitor is crucial to structure and function. *Am J Pathol* 2003;163:121–133. [PubMed: 12819017]
- Downing SJ, Sherwood OD. The physiological role of relaxin in the pregnant rat. IV. The influence of relaxin on cervical collagen and glycosaminoglycans. *Endocrinology* 1986;118:471–479. [PubMed: 3943482]
- El Maradny E, Kanayama N, Kobayashi H, Hossain B, Khatun S, Liping S, Kobayashi T, Terao T. The role of hyaluronic acid as a mediator and regulator of cervical ripening. *Hum Reprod* 1997;12:1080–1088. [PubMed: 9194670]
- Fulop C, Szanto S, Mukhopadhyay D, Bardos T, Kamath RV, Rugg MS, Day AJ, Salustri A, Hascall VC, Glant TT, Mikecz K. Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. *Development* 2003;130:2253–2261. [PubMed: 12668637]
- Kaya G, Rodriguez I, Jorcano JL, Vassalli P, Stamenkovic I. Selective suppression of CD44 in keratinocytes of mice bearing an antisense CD44 transgene driven by a tissue-specific promoter disrupts hyaluronate metabolism in the skin and impairs keratinocyte proliferation. *Genes Dev* 1997;11:996–1007. [PubMed: 9136928]
- Lee HG, Cowman MK. An agarose gel electrophoretic method for analysis of hyaluronan molecular weight distribution. *Analytical Biochemistry* 1994;219:278–287. [PubMed: 8080084]
- Lee JY, Spicer AP. Hyaluronan: a multifunctional, megaDalton, stealth molecule. *Current Opinion in Cell Biology* 2000;12:581–586. [PubMed: 10978893]
- Leppert PC. Anatomy and physiology of cervical ripening. *Clin Obstet Gynecol* 1995;38:267–279. [PubMed: 7554594]
- Mahendroo MS, Porter A, Russell DW, Word RA. The Parturition Defect in Steroid 5 α -Reductase Type 1 Knockout Mice Is Due to Impaired Cervical Ripening. *Mol Endocrinol* 1999;13:981–992. [PubMed: 10379896]
- Mittaz L, Russell DL, Wilson T, Brasted M, Tkalecic J, Salamsen LA, Hertzog PJ, Pritchard MA. Adamts-1 is essential for the development and function of the urogenital system. *Biol Reprod* 2004;70:1096–1105. [PubMed: 14668204]
- Mjaatvedt CH, Yamamura H, Capehart AA, Turner D, Markwald RR. The *Cspg2* gene, disrupted in the *hdf* mutant, is required for right cardiac chamber and endocardial cushion formation. *Developmental Biology* 1998;202:56–66. [PubMed: 9758703]
- Mowa CN, Papka RE. The role of sensory neurons in cervical ripening: effects of estrogen and neuropeptides. *J Histochem Cytochem* 2004;52:1249–1258. [PubMed: 15385571]
- Mukhopadhyay D, Hascall VC, Day AJ, Salustri A, Fulop C. Two Distinct Populations of Tumor Necrosis Factor-Stimulated Gene-6 Protein in the Extracellular Matrix of Expanded Mouse Cumulus Cell-

- Oocyte Complexes. Archives of Biochemistry and Biophysics 2001;394:173–181. [PubMed: 11594731]
- Rajabi M, Quillen EW, Nuwayhid BS, Brandt R, Poole AR. Circulating hyaluronic acid in nonpregnant, pregnant, and postpartum guinea pigs: elevated levels observed in parturition. Am J Obstet Gynecol 1992;166:242–246. [PubMed: 1733201]
- Read CP, Word R Ann, Ruscheinsky Monika, Timmons Brenda C, Mahendroo Mala S. Cervical remodeling during pregnancy and parturition: molecular characterization of the softening phase in mice. Reproduction 2007;134:327–340. [PubMed: 17660242]
- Russell DL, Doyle KM, Ochsner SA, Sandy JD, Richards JS. Processing and localization of ADAMTS-1 and proteolytic cleavage of versican during cumulus matrix expansion and ovulation. J Biol Chem 2003a;278:42330–42339. [PubMed: 12907688]
- Russell DL, Ochsner SA, Hsieh M, Mulders S, Richards JS. Hormone-Regulated Expression and Localization of Versican in the Rodent Ovary. Endocrinology 2003b;144:1020–1031. [PubMed: 12586779]
- Salier JP, Rouet P, Raguenes G, Daveau M. The inter-alpha-inhibitor family: from structure to regulation. Biochem J 1996;315(Pt 1):1–9. [PubMed: 8670091]
- Sato TA, Gupta DK, Keelan JA, Marvin KW, Mitchell MD. Expression of interleukin-1beta mRNA in murine uterine and gestational tissues: relationship with gestational age. Am J Reprod Immunol 2001;46:413–419. [PubMed: 11775011]
- Selbi W, de la Motte CA, Hascall VC, Day AJ, Bowen T, Phillips AO. Characterization of hyaluronan cable structure and function in renal proximal tubular epithelial cells. Kidney Int 2006;70:1287–1295. [PubMed: 16900089]
- Sleeman J, Rudy W, Hofmann M, Moll J, Herrlich P, Ponta H. Regulated clustering of variant CD44 proteins increases their hyaluronate binding capacity. J Cell Biol 1996;135:1139–1150. [PubMed: 8922392]
- Spicer AP, Joo A, Bowling RA Jr. A Hyaluronan Binding Link Protein Gene Family Whose Members Are Physically Linked Adjacent to Chondroitin Sulfate Proteoglycan Core Protein Genes: THE MISSING LINKS. Journal of Biological Chemistry 2003;278:21083–21091. [PubMed: 12663660]
- Straach KJ, Shelton JM, Richardson JA, Hascall VC, Mahendroo MS. Regulation of hyaluronan expression during cervical ripening. Glycobiology 2005;15:55–65. [PubMed: 15317739]
- Tammi MI, Day AJ, Turley EA. Hyaluronan and Homeostasis: A Balancing Act. Journal of Biological Chemistry 2002;277:4581–4588. [PubMed: 11717316]
- Timmons BC, Mahendroo MS. Timing of neutrophil activation and expression of proinflammatory markers do not support a role for neutrophils in cervical ripening in the mouse. Biol Reprod 2006;74:236–245. [PubMed: 16237151]
- Timmons BC, Mahendroo MS. Processes Regulating Cervical Ripening Differ from Cervical Dilation and Post Partum Repair: Insights from Gene Expression Studies. Reproductive Sciences 2007;14:53–62. [PubMed: 18089611]
- Timmons BC, Mitchell SM, Gilpin C, Mahendroo MS. Dynamic changes in the cervical epithelial tight junction complex and differentiation occur during cervical ripening and parturition. Endocrinology 2007;148:1278–1287. [PubMed: 17138657]
- Wight T. Versican: a versatile extracellular matrix proteoglycan in cell biology. Current Opinion in Cell Biology 2002;14:617–623. [PubMed: 12231358]
- Zhuo L, Yoneda M, Zhao M, Yingsung W, Yoshida N, Kitagawa Y, Kawamura K, Suzuki T, Kimata K. Defect in SHAP-Hyaluronan Complex Causes Severe Female Infertility. A STUDY BY INACTIVATION OF THE BIKUNIN GENE IN MICE. Journal of Biological Chemistry 2001;276:7693–7696. [PubMed: 11145954]

Figure 1.

Assessment of changes in hyaluronan molecular weight during pregnancy, parturition and post partum. Sizes of low and high molecular weight HA standards are indicated to the left of the gel. Equal fractions of cervical extracts from gestation days 12–18.75 (d12–d18.75), in labor (IL), and approximately 2, 10 and 24 hours after birth (2hpp, 10hpp, 24hpp) are indicated.

Figure 2.

Immunofluorescence staining of hyaluronan (green) and inter α trypsin inhibitor (I α I) (red) in cervical stroma (left panels) and immunofluorescence staining of hyaluronan (green) only in cervical epithelia (right panels) from cervixes at gestation days 15 (d15), late d18 (d18) and postpartum (8–10hpp and 24hpp). Nuclear (DAPI) staining of cells is indicated in blue. Colocalization of HA and I α I results in yellow staining as noted in the postpartum samples. Epithelia (E), and stroma (S). Three to four cervixes were analyzed for each time point and a representative section is presented.

Figure 3.

Detection of hyaluronan (top panel) and IαI HC (lower panel) in cervical extracts collected from nonpregnant, (NP), gestation days 10, 15, 18 (d10, d15, d18) and 24 hours after birth (24hpp). Equal fractions of extracts were incubated with or without (+ or -) hyaluronidase. HA (top panel) and IαI HC (lower panel) were detected using a biotinylated HA binding protein and anti-mouse IαI antibody, respectively. Heavy chain of IαI is labeled as "HC". All samples were run on a single gel but are shown as 3 panels so that time points are in a logical order.

Figure 4. CD44 mRNA expression in nonpregnant (NP), pregnant (d8–d18) and postpartum cervix (10hpp). QRT-PCR was performed using primers specific for the CD44s isoform. The data represents the average of 3 animals $\pm$ SEM for each time point. Gene expression is expressed relative to gestation day 18.

Figure 5.

Immunohistochemical staining of CD44 (pink) in cervix at gestation day 15 (panel A) and gestation day 18 (panel B). Magnification is 10X. Immunofluorescence staining of CD44 (red) in cervical stroma at gestation day 15 (panel C), gestation day 18 (panel D), 8–10h post partum (panel E) and 24h post partum (panel F). Nuclear (DAPI) staining of cells is indicated in blue. In some cases staining is whitish pink when there is overlap of CD44 staining with DAPI. Three to four cervixes were analyzed for each time point and a representative section is presented.

Figure 6.

Expression of proteoglycans in the nonpregnant and pregnant mouse cervix. Lanes labeled “-” indicate PCR amplification in the absence of cDNA, “+” indicates amplification from brain cDNA as a positive control. Nonpregnant (NP), and pregnant day 15 (d15) and day 18 (d18) cervix was evaluated. Expected PCR product size was 550, 440, and 500 base pairs for versican, brevican and aggrecan respectively.

Figure 7.

Panel A, Versican mRNA expression in nonpregnant (NP), pregnant (d8–d18) and postpartum cervix (10hpp). QRT-PCR was performed using primers specific for all versican isoforms and for the predominant cervical isoforms V0/V1 and 20 ng of cDNA per reaction. The average and standard error of three animals per time point is presented. Gene expression is expressed as expression relative to expression of gestation day 18. Panel B, Immunohistochemical detection of versican in cervical paraffin sections collected at gestation days 15 and 18. Versican expression is in the matrix surrounding the cervical stroma.

Figure 8.

Protein expression of the protease, ADAMTS1 is increased in the cervix during cervical ripening and shortly postpartum. Cervical proteins from nonpregnant (NP), gestation day 9–18 and 10–12 or 24 hours after birth (10–12h pp and 24h pp) were run on an SDS PAGE gel and western blotting carried out using anti-pro-domain ADAMTS1 antibody which recognizes the precursor protein (top panel), anti-metalloprotease domain ADAMTS1 antibody which recognizes the mature, processed protein (middle panel) and a protein loading control- calnexin (lower panel).