Published in final edited form as:

J Urol. 2013 April ; 189(4): 1588–1594. doi:10.1016/j.juro.2012.09.096.

Impact of Parturition on Chemokine Homing Factor Expression in the Vaginal Distention Model of Stress Urinary Incontinence

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Abstract

Purpose—Human childbirth simulated by vaginal distention is known to increase the expression of chemokines and receptors involved in stem cell homing and tissue repair. We hypothesized that pregnancy and parturition in rats contributes to the expression of chemokines and receptors after vaginal distention.

Materials and Methods—We used 72 age matched female Lewis rats, including virgin rats with and without vaginal distention, and delivered rats with and without vaginal distention. Each rat was sacrificed immediately, or 3 or 7 days after vaginal distention and/or parturition, and the urethra was harvested. Relative expression of chemokines and receptors was determined by real-time polymerase chain reaction. Mixed models were used with the Bonferroni correction for multiple comparisons.

Results—Vaginal distention up-regulated urethral expression of CCL7 immediately after injury in virgin and postpartum rats. Hypoxia inducible factor-1 α and vascular endothelial growth factor were up-regulated only in virgin rats immediately after vaginal distention. CD191 expression was immediately up-regulated in postpartum rats without vaginal distention compared to virgin rats without vaginal distention. CD195 was up-regulated in virgin rats 3 days after vaginal distention compared to virgin rats without vaginal distention. CD193 and CXCR4 showed delayed up-regulation in virgin rats 7 days after vaginal distention. CXCL12 was up-regulated in virgin rats 3 days after vaginal distention compared to immediately after vaginal distention. Interleukin-8 and CD192 showed no differential expression.

^{*}Financial interest and/or other relationship with Juventas Therapeutics, SironRx Therapeutics, Athersys and Aastrom Biosciences.

[†]Financial interest and/or other relationship with Allergan, Astellas and Pfizer.

[§]Financial interest and/or other relationship with Fate Therapeutics, Beech Tree Laboratories and Eli Lilly.

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Conclusions—Vaginal distention results in up-regulation of the chemokines and receptors expressed during tissue injury, which may facilitate the spontaneous functional recovery previously noted. Pregnancy and delivery up-regulated CD191 and attenuated the expression of hypoxia inducible factor-1 α and vascular endothelial growth factor in the setting of vaginal distention, likely by decreasing hypoxia.

Keywords

urethra; delivery; obstetric; vagina; wounds; injuries; cytokines

Stress urinary incontinence is a significant, pervasive disorder. A third of women experience SUI in their lifetime with up to 30% of them undergoing surgical treatment.^{1,2} Despite the incidence of vaginal delivery and the increasing rates of other risk factors, including obesity, not all women have permanent SUI. Up to a third experience peripartum SUI and most recover within 8 weeks of delivery.³ However, SUI will never develop in some women, even when there are multiple risk factors. Taken together, this implicates a variable endogenous repair process that promotes healing and repair during the immediate postpartum state. When stressed by compounding risk factors in predisposed women, this process may be overwhelmed and SUI may subsequently develop.

Circulating stem cells, such as HSCs and MSCs, are multipotent adult progenitor cells. They can home to areas of injury to facilitate repair and regeneration via differentiation, paracrine and/or autocrine mechanisms, and they have significant roles in endogenous tissue repair and inflammation attenuation.⁴ In numerous animal models and in humans therapeutic delivery of MSCs exerts beneficial effects on various host injuries and diseases, including SUI.⁵ Stem cell homing cytokine up-regulation has shown promise in localizing HSCs to areas of tissue ischemia after myocardial infarction.⁶

Stem cells home to areas of injury via gradients of homing cytokines, including chemokines in the CCL and CXCL families.⁷ The corresponding CCR and CXCR receptor families are located on various cell types, including lymphocytes and progenitor cell populations, and they participate in several functions, including inflammation and repair. HIF-1 α and VEGF aid in the angiogenic response vital to tissue repair. In our previous study we characterized expression of a number of these factors and receptors after VD, which is simulated human childbirth injury (see Appendix).^{8–10}

Despite its well characterized, validated use, to our knowledge the effects of VD on stem cell homing and tissue repair in the setting of pregnancy and parturition are yet unknown. We hypothesized that the biochemical milieu of pregnancy and delivery facilitates repair in the VD setting by increasing the expression of chemokines and their receptors to facilitate endogenous stem cell homing and repair. In this study we compared the urethral expression of chemokines and their receptors believed to be critical for repair after pregnancy and parturition in rats with or without VD.

MATERIALS AND METHODS

Animals

This research was approved by the Cleveland Clinic institutional animal care and use committee. We identified 4 experimental groups, each consisting of 18 age matched female Lewis rats, including group 1—virgin controls without VD, group 2—virgin rats with VD, group 3—rats delivered without VD and group 4—rats delivered with VD. For groups 3 and 4 we obtained 9-week-old primigravid, timed pregnant rats and observed them until delivery. Within 24 hours of delivery the rats in group 4 underwent VD for 4 hours. At the same time the age matched virgin rats in group 2 underwent VD for 4 hours. Group 3 rats were observed until delivery and then underwent anesthesia for 4 hours without VD. Group 1 consisted of age matched virgin rats that underwent anesthesia alone without VD.

Rats were sacrificed immediately, or 3 or 7 days after VD and/or anesthesia alone. The urethra of each rat was harvested under anesthesia, snap frozen in liquid nitrogen and stored at -80C for later analysis by RT-PCR.

Vaginal Distention

Rats undergoing VD were first anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg). The vagina was serially accommodated with lubricated bougies (24Fr up to 32Fr). A modified 10Fr Foley catheter was then inserted into the vagina. The catheter tip was cut off to allow for complete insertion of the balloon in the vagina. A single stitch of 3-zero silk was placed in the skin near the vagina to secure the catheter in place and the balloon was slowly inflated to 3 ml. During VD, the anesthesia level was continuously monitored by response to the toe pinch reflex and supplemental anesthesia was supplied if any response was noted. After 4 hours the stitch was removed, the balloon was deflated and the catheter was removed. For pain control all animals received buprenorphine (0.3 mg/kg) subcutaneously every 12 hours for 24 hours.

Real-Time Polymerase Chain Reaction

RT-PCR was done to evaluate the mRNA expression of 5 chemokines and 5 chemokine receptors associated with stem cell homing and tissue injury (see Appendix). Total RNA was extracted from the tissues using the RNeasy® Mini Kit according to manufacturer instructions. Reverse transcription was performed in a 20 µl reaction volume with 400 ng total RNA, 2.5 µM oligo deoxythymidine reverse transcription primer and 1 U reverse transcriptase at 48C for 45 minutes. Quantitative RT-PCR was performed using the ABI Prism® 7500 sequence detector. The reaction mixture contained TaqMan® Universal PCR Master Mix, each primer (900 nM), probe (200 to 250 nM) and cDNA (50 ng). After activation of AmpliTaq® Gold for 10 minutes at 95C, 40 cycles were performed, consisting of 15 seconds at 95C, followed by 1 minute at 60C. A standard curve method was used for TaqMan RT-PCR. Primer/ probe sets (CXCL12, CXCR4, CD191, CD192, VEGF, CD193 and CD195) for TaqMan RT-PCR were obtained from Applied Biosystems™. TaqMan MGB probes for CCL7, HIF-1α and IL-8 were synthesized. The sequences were 5'-FAM-TGCTGCTCACAGCTG-MGB-3', 5'-FAMAAAGAGCCCGATGCC-MGB-3' and 5'-FAM-CTCATCTTGGTGGTGAACA-MGB-3', respectively. The forward and reverse

primers were 5'-CTGCCGCGCTTCTGTGT-3' and 5'-ACGTGCACGGTGAAAGCA-3' for CCL7, 5'-GCTGCCCTTCGACAAGCTT-3' and 5'-CGCTGGAGCTAGCAGAGTCA-3' for HIF-1 α , and 5'-CGGCTTTGTGGGCAACAT-3' and 5'-AGTCATCTTCTCCCGGAAGCT-3' for IL-8. Relative mRNA levels were determined by normalization to the GAPDH internal control.

Data Analysis

Data are shown as the mean $\pm$ SE of normalized RT-PCR results, standardized to the mean of virgin rats without VD immediately after the end of the 4 hours of anesthesia using SigmaPlot® 10. Data were analyzed with mixed models methods and SAS® 9.2. Groups (combinations of parity and injury status) and time after intervention were treated as categorical variables to assess pairwise differences in population means across groups and time points. Comparisons were made at each time point between groups with similar parity (virgin or delivered) or injury status (with or without VD) as well as in each group across time points. The resultant 24 specific pairwise contrasts were constructed. The Bonferroni correction was applied to adjust for multiple comparisons within each chemokine and/or receptor with adjusted $p < 0.05$ considered statistically significant.

RESULTS

Three chemokines and 2 receptors showed immediate up-regulation after VD and/or parturition (figs. 1 and 2). CCL7 was significantly up-regulated 8.9 and 7.6-fold immediately after VD in virgin and postpartum rats, respectively, vs controls (each $p < 0.001$). By 3 days expression was significantly lower and no different than in uninjured virgin controls. HIF-1 α was also significantly up-regulated 2.1-fold in virgin rats but not in postpartum rats immediately after VD ($p < 0.001$). It normalized by 3 days. VEGF was up-regulated 2.9-fold in virgin rats immediately after VD but not in postpartum rats after VD ($p < 0.001$). Again, by 3 days these levels had normalized. Expression of the receptor CD191 was increased in postpartum rats without VD compared to virgin rats without VD ($p = 0.03$) but it normalized by 7 days. CD195 expression was increased 3.1-fold in virgin rats 3 days after VD compared to virgin rats without VD.

Two chemokine receptors showed significant upregulation that was delayed beyond 3 days (fig. 3). CD193 was up-regulated 3.7-fold in virgin rats 7 days after VD ($p = 0.002$). CXCR4 expression was similarly increased 3.1-fold in virgin rats 7 days after VD ($p = 0.001$). CXCL12 was up-regulated in virgin rats 3 days after VD compared to the same group immediately after VD ($p = 0.045$). IL-8 and CD192 showed no differential expression in any group at any time point analyzed (fig. 4).

DISCUSSION

Animal studies suggest that the urethra, vagina and supporting pelvic tissues undergo complex biomechanical and biochemical changes before parturition.^{11,12} This process of adaptation likely evolved to facilitate passage of the fetus and protect against injury that may lead to pelvic floor disorders. In this study we identified a second potential mechanism by which the urethra may counteract injury during parturition. We tested the hypothesis that

pregnancy and vaginal delivery alter the expression of chemokines and receptors to promote endogenous stem cell homing and/or limit urethral injury after delivery.

Our results validate our previous study of VD in rats in terms of CCL7 and CXCL12 expression, which are ligands critical for MSC and HSC homing, respectively. VD up-regulates urethral CCL7 expression approximately eightfold compared to controls regardless of parous status, consistent with the results of Wood et al, despite using a different strain of rat.¹⁰ On the other hand, Woo et al reported an approximate twenty-fold increase in the urethral expression of CCL7 after VD.^{8,9} Variations in dissection technique may account for these differences between investigations. However, consistent with the findings of Woo et al, we noted slight but significantly decreased expression of CXCL12 immediately after VD in virgin rats compared to levels at 3 days. A possible explanation is that the decreased compliance of the virgin rat vagina increased the amount of stretch on the tissue during VD. This increased occlusion of the vessels, and hypoxia of the vagina and urethra, a known stimulant of CXCL12, leading to the delayed expression observed at 3 days.¹³

We also identified significant, immediate up-regulation of HIF-1 α and VEGF in the urethra of virgin rats after VD. Up-regulation was attenuated in postpartum rats after VD and did not significantly differ from that in postpartum rats without VD. Two possible hypotheses exist. 1) Mentioned changes in the rat during pregnancy to increase vaginal compliance may also apply to the urethra.¹² Therefore, in postpartum rats increased compliance decreases pressure against the tissue walls and also decreases vessel occlusion, leading to reduced hypoxia. However, in virgin rats no such accommodation occurs and vessel occlusion during VD leads to hypoxia during distention.¹³ Hypoxia is known to stimulate the expression of HIF-1 α , a transcription factor for VEGF, explaining the immediate up-regulation of HIF-1 α and VEGF in virgin rats but not in postpartum rats after VD. Chemokines such as CCL7 are apparently up-regulated after VD regardless of tissue accommodation secondary to pregnancy, potentially due to increased sensitivity to the mechanical injury of VD. 2) Other systemic (eg cortisol) or local (eg relaxin) factors produced during pregnancy and parturition but not assayed in our experiment may modify the expression of HIF-1 α and/or VEGF.¹⁴

The chemokines and receptors that we evaluated represent a subset of those known to be expressed at high levels during cervical effacement and dilatation as well as in amniotic fluid, cervical mucus and contracting myometrial cells at human delivery.¹⁵⁻¹⁷ However, when comparing delivered rats to virgin rats without VD, we did not see a significant effect on the urethral expression of most chemokines and receptors analyzed. In contrast, we noted an effect of parturition alone on the expression of CD191, which increased immediately after delivery and returned to baseline 7 days later. CD191 is a known receptor for CCL7, which was noted in our study and previously¹⁰ to be up-regulated with VD in rats. CD191 may have a lower expression threshold and, thus, rat parturition alone may provide sufficient injury.

The time points chosen for this study were based on data that we and others reported. Salcedo et al found that similar chemokines were up-regulated in the anal sphincter up to 10 days after nerve crush in rats, a localized injury associated with VD.¹⁸ Given the hypothesized effects of pregnancy to increase the expression of chemokines and receptors to

facilitate repair, we anticipated an increase in expression beyond 24 hours. We observed persistent and delayed urethral responses in CD193 and CXCR4 expression but the effect required VD in virgin rats. It is also possible that parturition alone has a fleeting effect on chemokine and receptor expression that could not be determined in the current series, given the interval between time points.

This study focused on urethral expression because, as shown in multiple studies, VD critically injures the urethra and EUS, which are particularly vulnerable to damage.¹⁹ This correlates with the results of clinical anatomical and electrophysiological studies.^{20,21} Furthermore, studies by several groups demonstrated that homing cytokines are most significantly up-regulated in the urethra of rats after VD.^{8–10} Despite no effect of parturition on chemokine and/or receptor expression in the urethra, the possibility of differential expression in other organs cannot be ignored. Pelvic organs other than the urethra undergo injury after VD and show changes in chemokine and chemokine receptor expression.^{8–10} Furthermore, homing cytokines function by creating a concentration gradient and our study did not assess levels in systemic circulation, eg serum.

A number of models have been used to simulate human childbirth injury in rats. They generally include mechanical injury with or without surgically altered hormone levels. Targeted injury to the urethral sphincter (transabdominal urethrolisis or electrocauterization), ligamentous attachments (pubourethral ligament transection) and nerve (pudendal nerve crush or transection) have been described.^{22–26} Ovariectomy, which is done to mimic hormonal changes in women as they age, is potentially useful combined with VD but alone it did not induce functional deficits in the urethra of virgin rats.²⁷ VD is performed by inflating a Foley balloon with 3 ml saline for 4 hours. Refinements have been suggested, such as inflating the balloon to a target pressure, using various distention intervals and hanging the catheter from a countertop to simulate the second stage of labor.^{27–29}

Although pregnancy and delivery can decrease leak point pressure in rodents and they are clearly involved in the pathophysiology of SUI in humans, isolated pregnancy and delivery are generally not used to induce experimental SUI in rodents since they do not inflict severe enough injury.^{3,11} Nevertheless, our data reveal that pregnancy and parturition have an effect on the expression of bioactive factors, ie HIF-1 α and VEGF, that respond to hypoxia, likely by altering tissue distensibility before VD. Furthermore, we noted an isolated effect of pregnancy and parturition on CD191 expression. These data provide the motivation to further assess other systemic and/or local factors that may prime or protect the pelvic tissues before parturition.

A further study limitation is that we used gene expression as the primary outcome without correlative histological or functional testing to determine the ultimate effect of pregnancy and parturition on rats after VD. Prior reports indicated that pregnancy and parturition alone have a slight but significant effect on sphincter histology and functional outcomes, such as incontinence by the sneeze test and leak point pressure testing.²⁷ Adding such outcomes to our study would have likely repeated this observation and provided correlative but not causative data. Based on our study, which identifies a possible role of VEGF, HIF-1 α and

CD191 in the inflammatory and/or repair process after parturition in rats after VD, studies can be designed to characterize these effects using histological and functional outcomes.

The application of stem cell homing provides an attractive option for clinical use. However, therapy in humans will likely be initiated after vaginal delivery injury. Therefore, novel methods to increase stem cell homing are currently being investigated to harness this potential axis. A strategy to deliver CXCL12 plasmid via nonviral vectors is being evaluated in a phase I study in humans with ischemic heart failure ([clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01082094) Identifier NCT01082094). Given the focality of injury and relative accessibility of the EUS in women, similar direct delivery long after childbirth may help home and retain endogenous and therapeutically delivered stem cells. Novel hydrogels are also being developed that may be applied to provide sustained release of homing cytokines.³⁰ Biogels, scaffolds and nonviral vector plasmids that increase the duration of homing cytokines in the urethra and EUS may enable multiple doses of systemically injected stem cells to home to the area, increasing the number of stem cells and the interval to maximize repair.

CONCLUSIONS

Our study demonstrates that VD alone increases the expression of various chemokines and chemokine receptors involved in stem cell homing and the tissue injury response. However, pregnancy and parturition limit the HIF-1 α and VEGF response in VD rats. Pregnancy and parturition alter CD191 expression independently of VD. Therefore, pregnancy and parturition affect the urethral expression of factors implicated in the tissue response to inflammation and repair.

Acknowledgments

Study received Cleveland Clinic institutional animal care and use committee approval.

Supported by Cleveland Clinic Research Programs Council Grant NIH RO1 HD38679, the Department of Veterans Affairs Rehabilitation R and D Service, and the AUA Foundation Herbert Brendler, MD Summer Medical Student Fellowship Program.

Abbreviations and Acronyms

CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CXCL	chemokine (C-X-C motif) ligand
CXCR	C-X-C chemokine receptor
EUS	external urethral sphincter
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HIF-1α	hypoxia-inducible factor-1 α
HSC	hematopoietic stem cell

IL-8	interleukin-8
MSC	mesenchymal stem cell
RT-PCR	real-time polymerase chain reaction
SUI	stress urinary incontinence
VD	vaginal distention
VEGF	vascular endothelial growth factor

APPENDIX

Chemokines and chemokine receptors

Chemokines	Chemokine Receptors (known ligands)
CXCL12, formerly stromal derived factor-1 (SDF-1)	CXCR4 (CXCL12)
HIF-1 α	CD191, formerly CCR1 (MIP [macrophage inflammatory protein]-1 α , RANTES [regulated on activation normal T expressed and secreted protein], CCL7)
CCL7, formerly MCP (monocyte chemoattractant protein)-3	CD192, formerly CCR2 (MCP-1, CCL7, MCP-5)
VEGF	CD193, formerly CCR3 (eotaxin, RANTES, MCP-2, CCL7, MIP-1a, MIP-1b)
IL-8	CD195, formerly CCR5 (MIP-1a, MIP-1b, RANTES)

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Figure 1.

Mean $\pm$ SEM relative urethral expression of CCL7 (A), HIF-1 α (B) and VEGF (C) in 5 or 6 rats per group at each time point, normalized to GAPDH endogenous control and to virgin (V)-VD status at immediate time point. P, parous. Asterisk indicates significantly different vs similar parity group and opposite injury group at same time point. α indicates significantly different vs corresponding parity and injury group at immediate time point. Plus sign indicates significantly different vs similar injury group and opposite parity group at same time point.

Figure 2.

Mean $\pm$ SEM relative urethral expression of CD191 (A) and CD195 (B) in 5 or 6 rats per group at each time point, normalized to GAPDH endogenous control and to virgin (V)-VD status at immediate time point. P, parous. Plus sign indicates significantly different vs similar injury group and opposite parity group at same time point. α indicates significantly different vs corresponding parity and injury group at immediate time point. Asterisk indicates significantly different vs similar parity group and opposite injury group at same time point.

Figure 3.

Mean $\pm$ SEM relative urethral expression of CD193 (A) and CXCR4 (B) in 5 or 6 rats per group at each time point, normalized to GAPDH endogenous control and to virgin (V)-VD status at immediate time point. P, parous. Asterisk indicates significantly different vs similar parity group and opposite injury group at same time point. α indicates significantly different vs corresponding parity and injury group at immediate time point.

Figure 4. Mean $\pm$ SEM relative urethral expression of CXCL12 (A), IL-8 (B) and CD192 (C) in 5 or 6 rats per group at each time point, normalized to GAPDH endogenous control and to virgin (V)-VD status at immediate time point. P, parous. α indicates significantly different vs corresponding parity and injury group at immediate time point.