Over Expression of Stem Cell Homing Cytokines in Urogenital Organs Following Vaginal Distention

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Purpose: Vaginal delivery is a risk factor for stress urinary incontinence. Rat models of simulated childbirth demonstrated hypoxia of the urogenital organs as well as the development of stress urinary incontinence following vaginal distention. Stromal derived factor-1 and monocyte chemotactic protein-3 were identified as cytokines that are over expressed after myocardial ischemia and signal stem cell migration to ischemic sites in a rat cardiac model. Given the focal hypoxia observed with vaginal distention, we characterized stromal derived factor-1 and monocyte chemotactic protein-3 expression by pelvic organ tissues after vaginal distention.

Materials and Methods: A total of 16 female rats were randomized into 4 groups. Two groups underwent vaginal distention with harvest of pelvic tissues immediately or 24 hours after vaginal distention, a sham group underwent anesthesia only and a control group underwent no intervention. Reverse transcriptase-polymerase chain reaction was performed on RNA extracted from the urogenital organs.

Results: Monocyte chemotactic protein-3 expression in the urethra was increased 20 and 6-fold immediately and 24 hours after vaginal distention, respectively. Monocyte chemotactic protein-3 was 8 and 4-fold increased in the vagina after vaginal distention. There was no difference in monocyte chemotactic protein-3 expression in the rectum or bladder in any group. Stromal derived factor-1 was significantly under expressed immediately after vaginal distention in all tissues.

Conclusions: Monocyte chemotactic protein-3 is significantly over expressed in rat urethral and vaginal tissues immediately following vaginal distention with above normal but decreasing expression 24 hours later. The association between monocyte chemotactic protein-3 over expression and targeted stem cell migration is under investigation. Successful characterization and control of such a repair mechanism in the lower urinary tract would introduce the potential for novel nonoperative treatments and/or preventive measures for stress urinary incontinence.

Key Words: vagina; urinary incontinence, stress; cytokines; stem cells; rats, Sprague-Dawley

Vaginal delivery is a known risk factor for the development of SUI in women.[1] Rat models of childbirth demonstrate the development of SUI symptoms following VD, as evidenced by lowered leak point pressures on urodynamic testing.[2] Prior studies also revealed a relationship between VD and regional tissue hypoxia, suggesting that ischemic damage to the lower urinary tract may have a role in the development of SUI.[3] Interestingly leak point pressure measurements attain a nadir 4 days after vaginal distention in female rats and return to normal values by 2 weeks after VD, demonstrating the occurrence of an innate reparative process.[4]

A separate rat model of myocardial ischemia led to the identification of a subset of cytokines that are transiently over expressed in myocardial tissues after ischemic injury. Known as MCP-3 and SDF-1, these chemoattractants function as homing molecules that signal MSCs and hematopoietic stem cell migration to damage sites, thus, promoting subsequent tissue repair.[5–7]

Given the focal hypoxia and tissue damage observed after VD, we hypothesized that the same cytokines are over expressed after VD and may participate in the reparative pathways. We characterized SDF-1 and MCP-3 expression in the urogenital tissues of a rat model of vaginal delivery. After it is fully characterized this regenerative process could be supplemented to facilitate continence recovery after childbirth injury.

MATERIALS AND METHODS

After obtaining full institutional animal care and use committee approval 16 virgin female Sprague-Dawley rats weighing 175 to 200 gm were randomized into 4 groups of 4 each. The first group underwent 4-hour VD under anesthesia, followed by immediate tissue harvest and sacrifice. The

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second group also underwent VD but tissue procurement was delayed for 24 hours. The third group was a sham treated group that underwent 4 hours of anesthesia only without any vaginal procedure, followed by immediate tissue harvest. The final group served as unanesthetized controls.

VD

The rats were anesthetized with a mixture of ketamine (100 mg/kg body weight intraperitoneally) and xylazine (10 mg/kg body weight intraperitoneally). Rats assigned to VD first underwent vaginal accommodation to a larger capacity using increasing sizes of urethral dilators (24Fr to 32Fr), as previously described.[2] A trimmed 10Fr Foley catheter was then inserted into the vagina and the balloon was inflated intravaginally to 3 ml, which was determined in preliminary experiments to be the capacity of a 200 gm rat vagina. A single 3-zero silk stitch was placed in the skin near the vagina to secure the catheter in place. After 4 hours the balloon was deflated, and the stitch and catheter were removed.

Tissue Dissection and Preparation

Pelvic dissection was performed in all rats. The urethra, anterior vaginal wall, bladder base and anterior rectal wall were immediately snap-frozen at −80C upon removal. Total RNA was isolated from the frozen tissues using an RNeasy® Mini RNA isolation kit according to manufacturer instructions. RNA concentration was determined using Ribogreen™ assay. Contaminating DNA was removed with DNA-free, DNase Treatment and Removal Reagent 1906 (Ambion®). Reverse transcription reaction was performed in 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. Input cDNA (80 to 100 ng) was used for TaqMan® quantitative real-time PCR using SYBR® Green PCR Master Mix. Amplification was targeted to SDF-1 and MCP-3 mRNA using forward and reverse primer pairs. The same amount of cDNA was used for SYBR Green TaqMan® PCR of GAPDH as a separate endogenous control. Two-step TaqMan PCR was performed at a 25 μl reaction volume using an ABI® Prism® 7700 sequence detection system. Cycle conditions were 50C hold for 2 minutes, 95C hold for 10 minutes, 40 cycles at 95C for 15 seconds and 60C for 1 minute. Critical threshold values for each sample were determined and data were analyzed according to the manufacturer protocol. Relative mRNA levels were determined by comparison to the GAPDH internal control.

Statistics

MCP-3 and SDF-1 levels measured in the control group of animals were first normalized to GAPDH. Cytokine expression from each treatment arm was then compared to values measured in the control group. Quantitative data are presented as the mean ± SE of the relative expression of MCP-3 and SDF-1 normalized to the mean of the control group. The Kruskal-Wallis test was performed using the logarithm of MCP-3 and SDF-1 expression level to the base 2. If any difference was significant, paired comparisons of the groups were performed using the Wilcoxon signed rank test to assess which groups differed. Given the small sample size of this study, p values for each test were determined using Monte Carlo simulation techniques. All comparisons assumed a significance level of 0.05.

RESULTS

MCP-3 expression in the urethra was significantly increased 20-fold immediately after vaginal distention compared to sham treated animals and controls ($p = 0.029$, fig. 1). At 24 hours after vaginal distention MCP-3 expression had decreased significantly to 6 times control values ($p = 0.026$) and it was no longer significantly different from that in the sham treated or control groups. There was no difference between relative expression in urethral or vaginal tissues in rats in the sham treated and control groups. Significant differences in urethral MCP-3 levels were also seen between the immediate VD and 24-hour VD groups compared to those in sham treated animals (0.029 and 0.027).

Relative MCP-3 expression in anterior vaginal wall tissues was 8 and 3-fold increased immediately and 24 hours after vaginal distention compared to the control group ($p = 0.028$ and 0.19, respectively). As in the urethra, relative MCP-3 expression was significantly increased compared to that in the sham treated group (0.029). Similar to data on the urethra, 24 hours after VD relative MCP-3 expression in the anterior vagina decreased, although in the vagina this difference was not significant.

There was no difference in relative MCP-3 expression in the anterior rectal wall in any group compared to that in controls, although relative MCP-3 expression immediately after VD was found to differ significantly from that in the sham treated group (0.031). Relative MCP-3 expression in

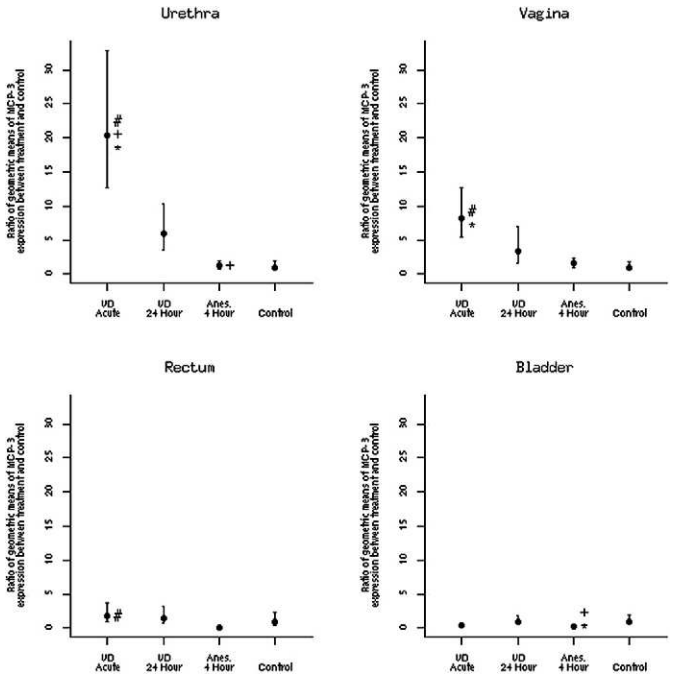

FIG. 1. Relative MCP-3 expression in urethra, vagina, rectum and bladder immediately after VD, 24 hours after VD, immediately after anesthesia (*Anes.*) only and in control animals. Error bars indicate mean ± SE. Asterisk indicates significant difference vs sham treated and control groups. Plus sign indicates significant difference vs 24-hour VD group. Pound sign indicates significant difference vs 4-hour anesthesia sham treated group.

the bladder was only found to differ in sham treated animals compared to that in rats 24 hours following VD and in controls (0.033 and 0.029, respectively).

In contrast to the cardiac model, SDF-1 over expression was not observed in any tissues following vaginal distention. Indeed, SDF-1 was found to be significantly under expressed in all tissues immediately after VD (fig. 2). In the vagina significant SDF-1 under expression continued to 24 hours after VD (0.029). In all tissues except the vagina relative SDF-1 expression immediately following VD and in sham treated animals it was decreased compared to 24 hours after VD. However, only in the rectum was SDF-1 expression immediately after VD significantly decreased compared to 24 hours after VD (0.029).

DISCUSSION

Stem cells are functionally responsible for the development and regeneration of tissues and organs.[8,9] They have gained much recent attention due to their unique properties of self-renewal and multilineage differentiation.[8,9] In the adult normally quiescent stem cells become activated following an episode of tissue damage, during which the body generates specific chemical signals that serve to direct cell migration and differentiation.[9]

In general smooth muscle in the bladder and urethra lacks the ability to regenerate. Current research in the treatment of urinary incontinence with stem cells focuses on pluripotent stem cells derived from other lineages, such as skeletal muscle or adipose tissue. Lee et al reported improvements in leak point pressure following periurethral injection of muscle derived stem cells in a rat model of SUI.[10]

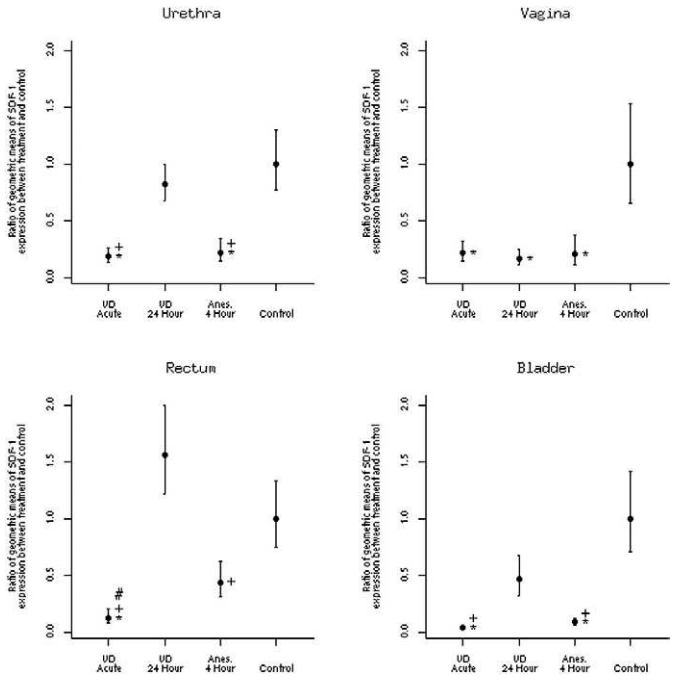

FIG. 2. Relative SDF-1 expression in rat urethra, vagina, rectum and bladder immediately after VD, 24 hours after VD, immediately after anesthesia (*Anes.*) only and in control animals. Error bars indicate mean ± SE. Asterisk indicates significant difference vs controls. Plus sign indicates significant difference vs 24-hour VD group. Pound sign indicates significant difference vs 4-hour anesthesia sham treated group.

Likewise Yiou et al injected muscle precursor cells into previously damaged rat urethral sphincters, resulting in regeneration of myotubes and some restoration of sphincter function.[11] Strasser et al noted subjective, anatomical and functional improvement after transurethral muscle derived stem cell injection in 5 female patients with SUI.[9] Additionally, processed lipoaspirate cells isolated from human adipose tissue were shown to have potential for smooth muscle differentiation in vivo after injection into the urethra of rats.[12]

While these studies focused on muscle or adipose derived stem cells,[9–12] Feil et al recently reported successful myogenic differentiation of bone marrow or MSCs in vitro.[13] After injection of these differentiated MSCs into the bladder neck of rats successful integration and spread of these cells into host tissue was observed at 4 months. Although these studies provide promise for the application of autologous cell transplantation and tissue engineering, the field remains fraught with difficulties, including long-term survival of transplanted cells, the possibility of introducing pathogenic agents and the theoretical risk of tumor formation.[9,10,14]

To our knowledge experimental data relating to the actual migration and homing of autologous stem cells to sites of urothelial injury are lacking. Despite the great interest generated by MSCs the molecular signals that regulate MSC trafficking and homing to injured tissues are not fully understood. MSC homing is being investigated by researchers in cardiology and neurology who seek to elucidate the specific molecular pathways that are up-regulated immediately after myocardial infarction and stroke, respectively.[6,15]

SDF-1 has been identified as a homing molecule for hematopoietic stem cells and lymphocytes to various organ systems, including myocardium after myocardial infarction, brain after stroke and synovial tissues in the setting of rheumatoid arthritis.[6,15,16] Recent studies showed that SDF-1 levels are related to the degree of hypoxia, such that hypoxic gradients in tissues directly correlate with SDF-1 gene expression.[17] It is only recently that MCP-3 was identified as another MSC chemoattractant.[7] Stem cell homing to the myocardium as well as improvement in cardiac function were demonstrated following transplantation of SDF-1 and MCP-3 expressing fibroblasts into the heart of rats subjected to ischemic cardiac damage.[5,7]

The concept of ischemic injury as a contributing factor to the development of SUI was recently suggested by experiments in rat models of vaginal delivery, in which VD resulted in measurable dysfunction to the continence mechanism.[2,3,4,18] Following VD histological studies also demonstrated extensive disruption and thinning of skeletal muscle associated with the external urethral sphincter.[2] In addition, decreased blood flow and hypoxic damage to the bladder, urethra and vagina were also observed after VD.[3] Interestingly dysfunction appears to be transient, suggesting the action of an innate reparative process.[4] Based on these findings we determined whether similar stem cell homing pathways are up-regulated following vaginal distention.

MCP-3 was significantly over expressed in urethral and vaginal tissues immediately following VD compared to that in sham treated and control animals. MCP-3 levels were found to be decreasing and yet they still increased from baseline 24 hours after VD, although this difference was no longer significant. These findings are consistent with studies

in urogenital organs during and after VD, which demonstrate significant decreases in regional blood flow to the urethra and vagina.[3] The marked up-regulation of MCP-3 in the urethra after VD is of particular interest because it suggests the initiation of an injury-repair pathway similar to that described after myocardial infarction, although the damage may not be as severe.[6]

The lack of significant MCP-3 up-regulation in the bladder and rectum is perhaps surprising since Damaser et al observed marked hypoxia of the bladder during and after VD.[3] To our knowledge the rectum has not been studied with respect to ischemia or dysfunction due to VD. Damaser et al suggested that bladder hypoxia from VD was a result of over distention due to the inability to void during VD, whereas urethral and vaginal hypoxia resulted from direct occlusion of blood vessels supplying the organs during VD. Therefore, it is possible that a different reparative process occurs in the bladder and rectum than in the urethra and vagina. Further hypothesis driven studies may better define the process of injury and repair in the bowel and bladder after childbirth.

Peak SDF-1 expression in myocardial tissues is observed on day 1 of myocardial infarction, decreasing thereafter.[19] Peak SDF-1 expression by astrocytes following acute stroke was found to occur at 2 to 3 days.[16] In contrast, SDF-1 was not found to be over expressed after VD in any pelvic organ tissues at any time point. More unexpected was the finding of significant under expression in all tissues immediately following VD. A possible explanation of these findings is suggested by a study of dermal fibroblasts known to constitutively express SDF-1.[17] Fedyk et al observed that SDF-1 expression is largely down-regulated by inflammation and SDF-1 expression in cell culture is potently inhibited by activated macrophages via interleukin-1α and tumor necrosis factor-α.[20] Future studies will be designed to investigate the relationship between interleukin-1α, tumor necrosis factor-α and SDF-1 after VD in the urogenital organs.

The final unexpected result was significantly decreased SDF-1 expression in all tissues except the rectum in the anesthetized, sham treated group. This finding is difficult to explain in light of the fact that no invasive procedures were performed in conjunction with anesthesia. The effect in SDF-1 was observed in the majority of organs examined, which argues against a spurious outcome. To our knowledge there are no current reports documenting the specific effects of intraperitoneal ketamine/xylazine on SDF-1 expression, although it is plausible that an inflammatory process is generated during intraperitoneal injection. If this theory is valid, investigation of longer postoperative times would demonstrate amelioration of this effect.

The current data are provoking but preliminary and they clearly require further investigation. While real-time PCR allowed the quantification of MCP-3 over expression, future studies will include Western blot analysis to identify and quantify the MCP-3 protein product as well as identify other cytokines that may contribute to the tissue repair process. Our current studies focus on whether increased MCP-3 levels in the urethral tissues ultimately result in MSC homing, subsequent stem cell engraftment and functional improvement in urinary continence after VD. Potential clinical applications include the augmentation of innate repair processes activated after the childbirth trauma and the prevention of SUI secondary to vaginal delivery. This may be accomplished via localized infusion of cytokines, such as MCP-3, or by the administration of exogenous stem cells targeted to sites of known cytokine up-regulation, such as the urethral sphincter.

CONCLUSIONS

This study demonstrated significant over expression of the stem cell homing molecule MCP-3 in rat urethral and vaginal tissues immediately following birth injury simulation with above normal but decreasing expression 24 hours later. In contrast, SDF-1 was under expressed in all urogenital organs after VD. Successful characterization and control of the repair mechanism in urogenital organs after childbirth injury would introduce the potential for novel nonoperative treatments and/or preventive measures for SUI.

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Abbreviations and Acronyms

GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase
MCP-3	=	monocyte chemotactic protein-3
MSC	=	mesenchymal stem cell
PCR	=	polymerase chain reaction
SDF-1	=	stromal derived factor-1
SUI	=	stress urinary incontinence
VD	=	vaginal distension

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