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Expression of Monocyte Chemotactic Protein-3 Following Simulated Birth Trauma in a Murine Model of Obesity

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Abstract

Objective—To determine the effect of obesity on simulated birth trauma in leptin-deficient obese mice as measured by relative MCP-3 expression.

Materials and Methods—A total of 25 wild-type and 25 obese C57BL/6 virgin female mice underwent 1 hour of vaginal distension (VD), sham VD or anesthesia without VD. Pelvic organ tissues were then harvested either immediately or 24-hours post VD and subsequent real-time PCR analysis was performed.

Results—Urethral MCP-3 levels in wild-type mice were elevated from baseline at 0-hours with a return to baseline at 24-hours in both VD and sham VD groups. In obese mice, there was a 6-fold elevation in MCP-3 levels at 0-hours after sham VD versus control ($P < 0.05$), which then returned to baseline levels at 24-hours. After undergoing VD, MCP-3 levels increased to 6-fold baseline values ($P = 0.002$) at 0-hours, with continued elevation in MCP-3 levels to 15 times control levels ($P = 0.0003$) at 24-hours.

Conclusions—MCP-3 is significantly over expressed in the urethral tissues of both wild-type and obese mice immediately after any urethral manipulation. At 24-hours, the MCP-3 expression patterns become divergent between VD and sham VD in obese mice. With a greater degree of trauma, MCP-3 continued to rise at 24-hours, suggesting that the underlying obesity resulted in alterations in response to tissue injury paralleling the degree of injury. Such associations warrant further investigation into the role of MCP-3 as a chemokine for stem cell migration with implications for subsequent tissue repair mechanisms after birth trauma.

Keywords

stress urinary incontinence; obesity; cytokines; stem cells; vaginal distension

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INTRODUCTION

Among the plethora of medical conditions affected by obesity are those of pelvic floor disorders, including urinary incontinence (UI). There is evidence from a number of epidemiologic studies to support the causal relationship between obesity and UI.¹⁻⁶ Another well-established risk factor for the development of stress urinary incontinence (SUI) is vaginal delivery, reflected by the 30% prevalence of SUI in postpartum women.⁷ What is unique and intriguing about this process is that birth trauma-related SUI recovers in the majority of women within one year after the time of injury, only to recur within the next decade and progress thereafter.⁷ A number of studies have also demonstrated that an increased body mass index during pregnancy and at the time of vaginal delivery increases a woman's risk of SUI development.⁸⁻¹⁰

The vaginal distension (VD) model of SUI simulates birth-related trauma to the pelvic organs experienced with the passage of the fetal head through the birth canal in women.¹¹ VD in the mouse results in measurable dysfunction to the continence mechanism. Upon urodynamic evaluation, leak point pressure (LPP) decreases to a nadir level four days following VD and recovers by 20 days.¹² The reversibility of SUI after VD in murine models mimics the clinical scenario of SUI recovery following birth trauma in women.

In the investigation of recovery mechanisms, our group has observed that expression of monocyte chemoattractant protein-3 (MCP-3) is dramatically increased in the urethra of rats after VD.¹³ MCP-3 is a mesenchymal stem cell (MSC) homing cytokine, which plays a key role in post-ischemia recovery.¹⁴ Moreover, we have demonstrated selective homing of intravenously infused MSCs to the urethra, following VD. Migration of MSCs was associated with an accelerated functional recovery of urethral function suggesting that promotion of stem cell mobilization may have a positive effect on SUI recovery following VD.¹⁵

The use of VD injury to simulate birth trauma and subsequent SUI has been mechanistically validated in mice by demonstrating significantly lowered LPPs and histologic evidence of reduced density of pelvic nerves after VD.¹⁶ However, whether the biochemical results of pelvic organ MCP-3 expression after VD injury in rat models can be extrapolated to mice is at present unclear. Therefore, the specific aims of our study were three-fold. First, we sought to characterize relative MCP-3 expression in the urogenital tissues of a mouse model of simulated birth trauma. Secondly, we sought to characterize relative MCP-3 expression in obese mice compared with normal weight controls by repeating the experiments in a leptin gene knockout of the same mouse strain (leading to an almost three-fold increase in weight at the same age). Finally, we sought to determine if the relative amount of MCP-3 expression in the serum had any correlation to levels in the harvested urogenital tissues.

MATERIALS AND METHODS

After obtaining approval of the Institutional Animal Care and Use Committee, a total of 25 wild-type (wt) and 25 obese (ob/ob) C57BL/6 virgin female mice were randomized into one of the following groups (5 mice per group): VD+0hr harvest, VD+24hr harvest, sham VD

+0hr harvest, sham VD+24hr harvest, and control anesthesia. After the assigned procedure, pelvic tissues were harvested immediately in the VD+0hr, sham VD+0hr, and control anesthesia groups. Tissues were harvested 24 hours post procedure in both the VD+24hr and sham VD+24hr groups. All mice were then sacrificed. Tissues were immediately immersed in RNeasyTM RNA Stabilization Reagent (QIAGEN) for subsequent RNA extraction and RT-PCR of relative MCP-3 expression. All mice were between 9 and 11 weeks old at the time of tissue harvest, with wt mice weighing 16.8 to 19.5 grams and ob/ob mice weighing 46.7 to 56.6 grams.

For VD, all mice were anesthetized with a mixture of intraperitoneal ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). Vaginal accommodation was achieved with sequential insertion and removal of increasing sizes of urethral dilators (8F to 16F) after lubrication with Surgilube (Fougera, Melville, NY). A modified 6F Foley catheter (Mentor, Tempe, AZ) was then lubricated and inserted into the vagina. The balloon was then carefully inflated with 0.3 mL of sterile water, as this amount of vaginal distension had previously been determined in preliminary experimental mouse models.¹⁶ We then secured the catheter in place using a single stitch of 5-zero silk placed through the skin at the level of the vaginal introitus. After a period of 1 hour had elapsed, the balloon was deflated and the catheter was removed. In the mice that underwent sham VD, all previous steps were duplicated except for inflation of the balloon.

Pelvic dissection was performed in all mice with attention given to minimal tissue handling. The urethra, vagina, bladder and rectum were harvested from all mice. Total RNA was extracted from tissues using RNeasyTM Mini Kit according to manufacturer instructions. RNA concentration was subsequently determined by using NanoDrop[®] ND-1000 spectrophotometry. The reverse transcription reaction was performed using High Capacity cDNA Reverse Transcription kit (Applied BiosystemsTM, Valencia, California) in a 50 µl of reaction volume with 1 µg of total RNA. Then 40 ng of cDNA was used for TaqMan[®] quantitative real time PCR by using Applied BioSystems TaqMan Assay. Quantitative real-time PCR was performed on the tissue for the expression of MCP-3. Amplification was targeted to MCP-3 and the same amount of cDNA was used for GAPDH to be tested as a separate endogenous control. RNA specimens were analyzed in triplicate using primer sets for both GAPDH and MCP-3. Two-step TaqMan[®] PCR was performed at a 25 µl reaction volume using the ABI Prism[®] sequence detection system with Standard 7500 Mode. Cycle conditions were 50°C hold for 2 minutes, 95°C hold for 10 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Critical threshold cycle values for genes of interest were normalized to GAPDH and were used to calculate the relative quantity of mRNA expression. MCP-3 serum analysis was performed using the Mouse MCP-3 Instant ELISA (Bender MedSystems, Vienna, Austria). Whole blood samples were obtained from mice via tail vein. Samples were centrifuged at 6000rpm for 10 minutes at 4°C. Serum layer was extracted and stored at -20°C. Prior to analysis, serum samples were slowly warmed to room temperature. 100µl of distilled water was added to all sample wells, as well as standard wells (to produce a concentration gradient of 1,000, 500, 250, 125, 63, 32, and 16 pg/ml) and blank wells. 50µl of serum was added to sample wells with all samples run in duplicate. The 96-well plate was incubated at room temperature for 3 hours with gentle shaking. All wells were then washed four times with wash buffer (provided in kit). Then 100µl of substrate

solution was added to all wells, and plate was incubated at room temperature for 10 minutes. Stop solution (100 μ l; provided in kit) was added to all wells once the highest standard reached a dark blue color. The plate was read on a Victor2 V Multi-label Counter (Perkin Elmer, Waltham, MA) at 450nm.

Data Analysis

For overall comparisons of age and weight among the ob/ob and wt mice, the Student's t-test was used. MCP-3 levels measured in the control group of animals were first normalized to GAPDH. Cytokine expression from each treatment arm was then compared with values measured in each control group. The relative expression of MCP-3 from the urethra, bladder, vagina, rectum, and serum are described as mean $\pm$ SE. The Kruskal-Wallis test was performed to evaluate differences in relative expression of MCP-3 among the five groups. If any significant difference in relative expression of MCP-3 occurred, paired comparisons between the groups were tested with the Wilcoxon rank-sum test. Differences in the relative expression of MCP-3 between 0h and 24h were performed using the paired Student's t-test. Given that weight was different between groups, we also adjusted for weight for all pair-wise comparisons with the use of linear regression models. All comparisons assumed a significance level of $P < 0.05$. Data were analyzed using S-Plus 7.0 (Insightful, WA).

RESULTS

When comparing the general characteristics of age (in weeks) and weight (in grams) among the two groups of mice, we found no statistically significant difference in the mean age (10.4), however, the mean $\pm$ SD weight was significantly higher in the ob/ob cohort (51.9 ± 3.3) compared with the wt cohort (18.1 ± 0.9) ($P < 0.00001$, Table). We found no statistically significant differences in age and weight within the two groups when stratified by treatment type and time point analyzed (data not shown).

Urethral MCP-3 levels in wt mice were elevated from baseline by a factor of three immediately after VD with a return to baseline at 24 hours post VD ($P = 0.03$, fig.1). There was no significant difference in relative urethral MCP-3 expression at either time point as compared with controls in the sham VD group. MCP-3 expression in the bladder was significantly increased 2-fold immediately following VD and sham VD ($P = 0.04$), only to return to baseline values in both treatment groups by 24 hours. MCP-3 expression in the vagina increased to almost 4 times control values, both immediately and 24 hours post VD ($P = 0.009$). There was no difference in the relative expression of MCP-3 in the rectum as compared with controls. Although the relative expression of MCP-3 in the serum of wt mice was elevated immediately after VD or sham, these levels did not reach statistical significance when compared with control.

MCP-3 levels in the urethra of ob/ob mice were significantly increased 6-fold immediately after VD and sham VD compared with controls ($P = 0.001$, fig. 2). While MCP-3 expression returned to baseline control levels at 24 hours post sham VD, they continued to rise to 15-fold the control levels at 24 hours post VD ($P = 0.002$, fig. 3). In the bladder, MCP-3 levels increased 9-fold and 6-fold immediately post VD and sham VD, respectively ($P = 0.002$ and

$P = 0.004$, fig. 2). The relative expression of bladder MCP-3 at 24 hours decreased and were not significantly different from control levels in both the VD and sham VD groups. MCP-3 levels in the vagina increased 40-fold as compared with controls immediately post VD and sham VD ($P = 0.003$ and $P = 0.009$). While the relative expression of MCP-3 at 24 hours decreased to levels that were not significantly different than controls in the sham VD group, MCP-3 levels remained significantly elevated to 10 times control values in the VD group ($P = 0.02$). Although MCP-3 expression in the rectum was elevated at 24 hours post VD, this was not statistically significantly different than control levels. Similarly, while the serum concentration immediately after VD and sham VD were elevated in obese animals as compared to control, statistical significance was not achieved.

COMMENT

The rat model of myocardial ischemia led to the identification of a subset of cytokines, namely MCP-3 and stromal derived factor-1, that become transiently elevated in response to ischemia and signal hematopoietic and mesenchymal stem cells to the injured site where they facilitate healing and improve functional recovery.¹⁷ The development of SUI in murine models of vaginal delivery utilizing VD suggests ischemic injury as a contributing factor as well.¹⁸

Stem cells have recently garnered attention because of their exceptional properties of self-renewal and tissue regeneration.¹⁹ Following an episode of tissue damage, these normally quiescent cells become activated and are directed toward the injured site through specific chemical signals. Chemoattractants (like MCP-3) function as homing molecules that direct stem cell differentiation and migration and thus promote subsequent tissue repair.^{14,17} Through the understanding of these repair mechanisms there exists the possibility for manipulation of these pathways and the subsequent development of a potential novel treatment modality for SUI. Indeed, the era of stem cell mediated forms of treatment for UI is well underway.^{20,21}

The process of cellular injury and recovery presupposes an innate reparative mechanism that is triggered by tissue damage. This sequence of events mirrors the human condition whereby birth trauma related SUI recovers in the majority of women within one year from the time of initial injury.⁷ We propose that the clinical development of SUI after birth trauma is intimately related to an imbalance of injury and recovery processes. We hypothesize that obesity at the time of vaginal delivery impacts both the degree of injury and the recovery from birth related trauma. Thus, the findings of both an exaggerated and sustained expression of MCP-3 post VD in ob/ob versus wt mice support the hypothesis that an imbalance exists in the homeostasis of cellular injury and recovery after vaginal trauma. This may help to explain the clinical situation that exists among obese women who develop SUI after spontaneous vaginal delivery and identify a novel molecular target for possible therapeutic intervention.

In wt mice, we found vaginal (4 times control), urethral (3 times control) and bladder (2 times control) expression of MCP-3 to be significantly elevated immediately after VD, with all levels returning to baseline values not significantly different from controls at 24 hours

post VD. These results are similar to two previous studies by Woo et al,^{22,23} who demonstrated a noticeable up-regulation of MCP-3 in the rat urethra and vagina immediately after VD. However, in contrast to our findings, Woo et al did not observe up-regulation of MCP-3 in the bladder specimens.^{22,23} Indeed, Damaser et al¹⁸ observed marked hypoxia of the bladder both during and after VD injury, which are consistent with our findings of increased bladder expression of MCP-3 immediately after VD. Damaser's group suggested that the hypoxia experienced in the bladder after VD was most likely secondary to over distension from obstruction of the catheter balloon during VD. This is in contrast to urethral and vaginal hypoxia, which resulted from direct vascular occlusion during VD.

The lack of differences in rectal MCP-3 expression after VD injury in our study is consistent with the findings of Woo et al,^{22,23} but is in contrast to the findings of Wood et al¹³ who observed a 21-fold increase in relative MCP-3 expression in the rectum immediately after VD injury. It should be noted, however, that in the Wood study this difference was only seen after 4 hours of sustained VD injury, as there was no relative difference in MCP-3 expression after 1 hour of VD as compared to controls. This finding could be explained by the fact that the rectum is the only organ evaluated that repeatedly undergoes a relatively large physiologic stretch to accommodate solids on a daily basis and therefore would require a more robust and sustained injury to elicit a significant biochemical response. The relative differences in both size and structure of the pelvic floor that exists between rats and mice may also account for the differences seen in the relative expression of MCP-3. Nonetheless, these experiments suggest a possible role of MCP-3 in rectal healing after vaginal delivery.

As previously noted, in ob/ob mice urethral MCP-3 levels increased by a factor of six as compared with baseline immediately following both VD and sham VD. We hypothesize that the injury sustained through vaginal accommodation was enough to elicit a significant (albeit transient) response to tissue injury resulting in the increased expression of MCP-3. In the wt group undergoing VD, urethral expression of MCP-3 was significantly elevated immediately post VD and returned to baseline levels at 24 hours. At 24 hours, the urethral MCP-3 expression patterns become divergent between VD and sham. While urethral MCP-3 levels in the ob/ob mice that underwent sham VD returned to baseline, those undergoing VD had continued elevation in relative MCP-3 expression to 15 times control levels at 24 hours post VD. As stated previously, the development of SUI in murine models of vaginal delivery utilizing VD suggests ischemic injury as a contributing factor. We hypothesize that the redundant blood supply of the bladder and vagina (as opposed to the urethral blood supply) may ultimately decrease the hypoxic injury that is sustained relative to the urethra. This may help to explain the differences in MCP-3 expression found in the bladder and vagina as opposed to the urethra. With a greater degree of trauma (VD versus sham), MCP-3 continued to rise at 24 hours, suggesting that underlying obesity resulted in alterations in response to tissue injury paralleling the degree of injury. This finding corroborates epidemiological evidence associating obesity with an increased risk of SUI. This mandates further functional evaluation of obese mice following VD trauma and is the focus of research that is currently underway.

Using ELISA quantification, serum MCP-3 levels were obtained in both the wt and ob/ob mice after VD and sham VD. The serum MCP-3 level experiment was intended as a pilot

project to observe the serum changes in relation to pelvic organ MCP-3 expression. Although serum concentrations 24 hours after VD were elevated compared with control, statistical significance was not noted when evaluating correlations between relative MCP-3 measurements at each location (urethra, vagina, bladder and rectum) and serum MCP-3 concentration. This finding might be explained by the fact that MCP-3 is produced locally and the tissue levels, although elevated as compared with control, are diluted as they pass through the entire blood volume.

CONCLUSIONS

Our study demonstrated significant over expression of the stem cell homing factor MCP-3 in mouse urethral, bladder and vaginal tissues immediately following simulated birth trauma in both wild type and obese mouse strains. The relative increase in the expression of MCP-3 was associated with increasing vaginal trauma, such that VD caused a more robust and durable response to cellular injury than did sham VD. In the obese mouse strain, urethral MCP-3 continued to rise at 24 hours, as compared to wild type, suggesting that underlying obesity resulted in alterations in response to tissue injury. This finding corroborates epidemiological evidence associating obesity with an increased risk of SUI. Such associations warrant further studies to investigate the role of MCP-3 as a chemokine for attraction of stem cell migration and resultant tissue repair following birth trauma.

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Figure 1. Relative MCP-3 expression in urethra, bladder, vagina, rectum and serum of wild-type mice: immediately and 24-hours after VD, immediately and 24-hours after sham and in control animals. Error bars indicate mean $\pm$ SE. Asterisk indicates significant difference versus control group.

Figure 2. Relative MCP-3 expression in urethra, bladder, vagina, rectum and serum of obese mice: immediately and 24-hours after VD, immediately and 24-hours after sham and in control animals. Error bars indicate mean $\pm$ SE. Asterisk indicates significant difference versus control group.

Figure 3.
Temporal relative expression of MCP-3 in obese and wild-type mice after VD and sham VD.

Table

General characteristics of age and weight among wild-type and obese mice.

	Mean Age in weeks (SD)	Mean Weight in grams (SD)
Wild-type	10.2 (0.3)	18.1 (0.9)
Obese	10.2 (0.4)	51.9 (3.3)
	$P^* = 0.9$	$P^* < 0.00001$
Wild-type (wt)		
VD+0hr	10.3 (0.5)	17.6 (0.8)
VD+24hr	10.1 (0.2)	18.4 (1.0)
Sham VD+0hr	10.2 (0.4)	18.2 (0.8)
Sham VD+24hr	10.1 (0.2)	18.3 (1.2)
Control	10.3 (0.3)	18.1 (0.8)
	$P^{**} = 0.4$	$P^{**} = 0.6$
Obese (ob / ob)		
VD+0hr	10.3 (0.5)	52.6 (4.0)
VD+24hr	10.1 (0.2)	50.6 (3.1)
Sham VD+0hr	10.2 (0.4)	50.2 (3.5)
Sham VD+24hr	10.1 (0.2)	52.0 (3.2)
Control	10.4 (0.4)	53.8 (1.8)
	$P^{**} = 0.7$	$P^{**} = 0.4$

* P values evaluate the differences in age and weight between wild-type and obese mice.

** P values evaluate the differences in age and weight among the five different treatment groups for wild-type and obese mice.