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O-GlcNAc Mediated Glycosylation Down-Regulation in Mice With Cyclophosphamide Induced Cystitis

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Abstract

Purpose—Cyclophosphamide induced cystitis is an established model for the study of bladder injury and wound healing. Glycosylation is an important modification mechanism that regulates the structure and function of secreted proteins and growth factors from inflammation sites. We determined the effect of cyclophosphamide induced cystitis on *O*-GlcNAc mediated glycosylation in the bladder.

Materials and Methods—Cystitis in WT C57BL6 mice was induced with intraperitoneal cyclophosphamide. Retrieved bladders were analyzed using histology, immunohistochemistry, reverse transcriptase-polymerase chain reaction and Western blot for glycosylation associated factors.

Results—Acute bladder injury was seen up to 168 hours (7 days) after injection. Reverse transcriptase-polymerase chain reaction revealed down-regulation of *O*-GlcNAc transferase, a key enzyme in *O*-GlcNAc mediated glycosylation, at the 8, 48 and 168-hour time points. Also, the glycosidase menangioma expressed antigen 5 was up-regulated at similar time points. Western blot analysis revealed decreased glycosylated protein during cyclophosphamide induced inflammation.

Conclusions—To our knowledge we report the first study of alterations in *O*-GlcNAc mediated glycosylation activity in bladders with cyclophosphamide induced cystitis. Glycosylation may have a significant role in the bladder wound healing process. Future studies of the glycosylation signaling pathways in the bladder would assist in future potential therapy for bladder inflammatory disease and cancer by elucidating pathways that guide bladder development and wound healing.

Keywords

urinary bladder; inflammation; cystitis; glycosylation; intercellular signaling peptides and proteins

Inflammation is an integral component of the nonspecific immune response of vascular tissues reacting to harmful stimuli such as pathogens, injured cells and irritants.¹ Signs of inflammation include increased blood flow, vasodilatation, altered cellular metabolism and release of soluble mediators. Inflammatory disease often engenders numerous physiological and biochemical changes,² including glycosylation of secreted proteins or growth factors from inflammation sites.

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Glycosylation is a site specific saccharide linking process to produce glycans by enzymes co-translationally or post-translationally. As an important modification mechanism that regulates secreted protein structure and function, glycosylation can provide stability to proteins³ and promote adhesive properties used in cell-cell adhesion in the immune system.⁴ Changes in glycosylation patterns were noted in certain human diseases, such as inflammatory bowel disease,⁵ breast cancer⁶ and other inflammatory diseases. However, the molecular pathways guiding these processes are not well understood.

O-GlcNAc mediated glycosylation is the addition of N-acetylglucosamine (*O*-GlcNAc) to serine or threonine residues by the enzyme OGT.⁷ It is distinct from classic protein glycosylation in that 1) *O*-GlcNAc mediated glycosylation occurs in the cytoplasm and nucleoplasm, 2) no further elongation or modification occurs after *O*-GlcNAc mediated glycosylation and 3) the mechanism is similar to phosphorylation.⁷ While the OGT enzyme adds *O*-GlcNAc to cytoplasmic and nucleoproteins, the glycosidase MGEA5 removes it from the proteins, where these related modifications achieve dynamic balance.⁸

CYP has well described urological sequelae, including microhematuria and hemorrhagic cystitis.⁹ In laboratory animals CYP induced cystitis has been used extensively to investigate bladder inflammation and wound healing¹⁰ with demonstrated alterations in neurochemical¹¹ and electrophysiological¹² properties of bladder afferent neurons. These changes may be mediated by chemical mediators released from bladder inflammation sites.¹³ Also, CYP induced cystitis leads to down-regulation of bladder nerve growth factor and brain-derived neurotrophic factor expression while major pelvic ganglia expression was up-regulated.¹⁴

We investigated the role of *O*-GlcNAc mediated glycosylation in bladders with CYP induced cystitis since it appears to have a significant role in other inflammatory diseases. Our eventual goal is to elucidate the signaling pathways in the bladder during bladder development and wound healing.

MATERIALS AND METHODS

CYP Induced Cystitis in Murine Model

This study was performed in accordance with a protocol approved by the Children's Hospital Los Angeles institutional animal care and use committee. Adult WT mice with a body weight of 25 to 35 gm on a C57BL6 background were used in all experiments. All injections were done using 2% isoflurane anesthesia. CYP (150 mg/kg) was injected intraperitoneally in experimental animals. Control animals were injected intraperitoneally with saline (0.1 ml/100 gm). A total of 36 mice were sacrificed after CYP injection, including 3 at each of the 4, 8, 12, 24, 48 and 168-hour (7-day) time points after CYP injection. Bladders were harvested for analysis.

Histopathology and Immunohistochemistry

Bladder tissue samples were fixed overnight in 10% buffered formaldehyde, embedded in paraffin wax, sectioned at 3 μ and stained with hematoxylin and eosin. For PCNA staining the PCNA staining kit (Zymed®) was used according to the manufacturer protocol. PCNA positive cells were reacted with monoclonal anti-PCNA antibody. Counterstaining was done with hematoxylin.

Reverse Transcriptase-Polymerase Chain Reaction

To measure the transcript level in harvested bladders RT-PCR was done using primers for mouse OGT, MGEA5 and GAPDH genes. Oligonucleotide sequences for mouse OGT were

forward primer 5'-GTGCACTGTT CATGGATTAC ATCATC-3' and reverse primer 5'-TCCATTGTGT ATTGTTTGGT GTTG-3'. Oligonucleotide sequences for mouse MGEA5 were forward primer 5'-CTTCGTTGGA GCAGTCGGTA GCTCC-3' and reverse primer 5'-CAGTCTCAAT GTCTTCGTCA CTGC-3'. GAPDH served as the housekeeping gene. Primer sequences for mouse GAPDH were forward primer 5'-CATCAACGGGAAGCCCATCACCA-3' and reverse primer 5'-GGGCCTCTCTTGCTCAGTGTC-3'. ImageJ for PC 1.40 (<http://rsb.info.nih.gov/>) was used to measure and quantify band intensity. On transcriptional expression analysis $p < 0.05$ was considered statistically significant.

Western Blot Analysis

Harvested bladders were homogenized with a Power-MaxTM AHS200 in protein extraction buffer composed of 20 mM tris-HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid, 50 M leupeptin, 1 M pepstatin A, 10 M 3, 4-dichloroisocoumarin, 1 mM phenylmethylsulfonyl fluoride and 0.05% sodium dodecyl sulfate. The amount of total protein was measured with the NanoDropTM instrument. Protein aliquots (30 μ g) were loaded on 8% polyacrylamide gel, electrophoresed and transferred to polyvinylidene membrane (Bio-Rad®). The protein containing membrane was blocked for 1 hour with Western BreezeTM blocking solution and incubated overnight with the primary *O*-GlcNAc antibody (Affinity BioReagents, Golden, Colorado) (1:500 dilution). After washing the membrane was incubated with goat anti-mouse alkaline phosphatase labeled secondary antibody using 5% skim milk in tris buffered saline for 2 hours at room temperature. Proteins were visualized by an AmershamTM ECLTM detection system. B-actin served as an internal control.

RESULTS

Bladder Histological Changes After CYP Injection

An intact urothelial layer with normal caliber blood vessels and a lack of edema or infiltrate were seen in control bladders but extensive urothelial inflammation was noted in the bladder of CYP injected mice (fig. 1, A and B). Edema was observed in the lamina propria, bladder mucosa and submucosal layers with inflammatory cell accumulation. As in previous series, 15–18 CYP administration resulted in urothelial destruction and subsequent regeneration by proliferation activity of the remaining cells with the proliferation index gradually increasing from day 1 and peaking at day 7. CYP administration also induced increased urothelial cell proliferation and regeneration, as shown by increased PCNA nuclei staining in CYP induced bladders (fig. 1, C and D).

CYP Induced Cystitis

OGT down-regulation—To investigate whether glycosylation has a role in CYP induced bladder inflammation the expression of OGT, a key *O*-GlcNAc mediated glycosylation enzyme, was monitored by RT-PCR in bladder specimens. At the 8, 48 and 168-hour time points after CYP injection, corresponding to the acute, intermediate and late inflammation periods, respectively, a modest decrease was noted in OGT expression (fig. 2). After 8 hours *OGT* transcription was decreased by 25%, while 48 and 168 hours after CYP injection *OGT* transcription was decreased by 26% and 49%, respectively ($p < 0.05$). Significant changes were not seen at the 4, 12 or 24-hour time point. These findings indicate that CYP induced inflammation leads to down-regulation of *O*-GlcNAc mediated glycosylation activity in the bladder.

MGEA5 expression up-regulation—Expression of MGEA5, a glycosidase that removes N-acetylglucosamine, was also monitored by RT-PCR in bladder specimens. At the 8, 48 and 168-hour time points after CYP injection, corresponding to the acute, intermediate and late

inflammation periods, respectively, increased MGEA5 transcriptional expression was noted (fig. 3). After 8 hours MGEA5 transcription was increased by 39%, while 12 and 48 hours after CYP injection MGEA5 transcription increased by 28% and 18%, respectively ($p < 0.05$). No changes were seen at the 168-hour time point. These findings further indicate that CYP induced inflammation leads to down-regulation of *O*-GlcNAc mediated glycosylation activity in the bladder.

Decreased glycosylated protein—Western blot analysis of CYP induced bladders revealed decreased glycosylated protein levels at the 8, 48 and 168-hour time points after CYP injection (fig. 4). After 8 hours glycosylated protein levels decreased by 41%, while the 48 and 168-hour time points showed a 35% and 18% decrease in glycosylated protein, respectively. This provides further evidence of early down-regulation of *O*-GlcNAc mediated glycosylation activity during CYP induced cystitis.

DISCUSSION

CYP induced cystitis has long been used as an in situ model for studying bladder injury and wound healing processes, and for inflammatory bladder diseases, including interstitial cystitis. However, to our knowledge no previous series has shown the role of glycosylation and its molecular mechanisms during bladder inflammation.

Glycosylation produces ample, diverse, regulated cellular glycans that are associated with essential cellular processes with accumulating evidence showing that glycosylation helps govern cellular physiology and can contribute to the formation of human disease.³ Intracellular modification of proteins by OGT involves the addition of an *O*-GlcNAc molecule¹⁹ and this process is counter regulated by MGEA5. Since *O*-GlcNAc modification competes with phosphorylation by protein kinases at similar sites, glycosylation is essential, in that it regulates important signaling pathways where phosphorylation has a key role, such as for cancer and inflammation. *O*-GlcNAc modification and the corresponding enzymes also appear to have a central role in the immune response with the activation of T and B lymphocytes.¹⁹

Defects in *O*-GlcNAc modification were previously noted to be involved in numerous human diseases, such as diabetes, Alzheimer's disease and cancer. In regard to diabetes in pancreatic β cells *O*-GlcNAc has a key role in the regulation of insulin signaling with OGT reversibly modifying the serine/threonine kinase AKT, PI3K and insulin receptor substrate 1. Recruitment of OGT to the plasma membrane prevents AKT phosphorylation and effectively shuts down insulin signaling.²⁰ *O*-GlcNAc glycosylation of the clathrin assembly proteins AP-3 and AP-180 is decreased in neurons in patients with Alzheimer's disease, indicating that decreased *O*-GlcNAc glycosylation is essential for the loss of synaptic vesicle recycling.²¹ Using a variation of the hexaminidase activity assay *O*-GlcNAcase and lysosomal hexaminidase activities were increased in breast tumor tissue compared to that in matched normal tissue.²² These examples highlight the widespread role of *O*-GlcNAc glycosylation in normal cellular function and its contribution to disease when altered.

We present what is to our knowledge the first report of alterations in *O*-GlcNAc mediated glycosylation activity in bladders with CYP induced cystitis. *O*-GlcNAc mediated glycosylation activity was down-regulated in CYP induced bladder inflammation and MGEA5 glycosidase activity was up-regulated. Decreased *O*-GlcNAc mediated glycosylated protein levels were identified during CYP induced cystitis. These results suggest that *O*-GlcNAc mediated glycosylation may have a significant role in bladder inflammation.

Pathways governing cellular and tissue changes in bladder development and wound healing are not completely understood. Further knowledge of these processes would help advance our

understanding of bladder disease and lead to the development of novel treatment modalities. For example, for inflammatory bladder diseases such as interstitial cystitis, *O*-GlcNAc glycosylation up-regulation would potentially lead to inhibition of the inflammation response, as in the endoluminal carotid artery injury model in rats.²³ The anti-inflammatory response seen with *O*-GlcNAc glycosylation up-regulation in arteries may also have applicability in the bladder.

Bladder inflammation is associated with wound healing, including that associated with tissue engineering. Inflammation often leads to dysregulation of endogenous and essential cellular processes such as glycosylation, as in cancer and inflammatory disease. For instance, select cancer cells may show glycans at different levels or different structures of glycans than those in normal cells.²⁴ Hence, alterations in glycosylation are likely to occur in the bladder healing process and may also be a potential hallmark of human disease. While many cancer or inflammation therapies currently target the cell cycles of DNA replication and cytoskeletal formation, more recent cancer therapies appear to target signaling pathways in which kinases, phosphatases and glycosylation enzymes have significant roles.

Future studies will be directed toward further elucidation of the glycosylation signaling pathways involved in the bladder inflammatory response. With knowledge of these pathways further advances in the diagnosis and treatment of bladder cancer and bladder inflammatory disease such as interstitial cystitis as well as improvements in bladder tissue engineering efforts may be possible.

CONCLUSIONS

To our knowledge we present the first report of alterations in *O*-GlcNAc mediated glycosylation activity in bladders with CYP induced cystitis. Glycosylation may have a key role in the bladder wound healing process. Future studies elucidating glycosylation signaling pathways in the bladder would assist in future potential therapies for bladder inflammatory diseases and cancer by elucidating the pathways that guide bladder development and wound healing.

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Abbreviations and Acronyms

CYP	cyclophosphamide
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
MGEA5	meningioma expressed antigen 5
<i>O</i> -GlcNAc	O-linked N-acetylglucosamine
OGT	<i>O</i> -GlcNAc transferase
PCNA	proliferating cell nuclear antigen
RT-PCR	reverse transcriptase-polymerase chain reaction

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Figure 1.

Bladder histopathology during CYP induced cystitis. *A*, normal layers in control bladder wall. H & E, reduced from $\times 10$. *B*, in CYP treated bladder at 168 hours (7 days) note extensive edema with inflammation and urothelial sloughing. H & E, reduced from $\times 40$. *C*, in control bladder immunostaining was seen only on normal urothelial basal layer. PCNA staining, reduced from $\times 100$. *D*, in CYP treated bladder at 168 hours (7 days) staining revealed proliferation throughout bladder wall. PCNA staining, reduced from $\times 40$.

Figure 2.

RT-PCR of OGT expression at 1,035 bp in bladder 4, 8, 12, 24, 48 and 168 hours (h) after CYP injection in 3 preparations per time point. GAPDH at 499 bp served as internal control (CTL). Asterisk indicates that for transcriptional expression statistical significance was considered at $p < 0.05$.

Figure 3.

RT-PCR of MGEA5 expression at 1,032 bp in bladder 4, 8, 12, 24, 48 and 168 hours (*h*) after CYP injection in 3 preparations per time point. GAPDH at 499 bp served as internal control (*CTL*). Asterisk indicates that for transcriptional expression statistical significance was considered at $p < 0.05$.

Figure 4. Western blot analysis of glycosylated proteins from homogenized bladders after CYP injection at 8, 48 and 168-hour (*h*) time points. *A*, single protein. *CTL*, control. *B*, multiple proteins. *C*, B-actin served as loading control.