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Increased cancer risk of augmentation cystoplasty: possible role for hyperosmolal microenvironment on DNA damage recognition

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Abstract

Patients who have had surgical bladder augmentation have an increased risk of bladder malignancy, though the mechanism for this increased risk is unknown. Hyperosmolal microenvironments such as the bladder may impair DNA damage signaling and repair; this effect may be more pronounced in tissues not normally exposed to such conditions. Comparing gastric and colon epithelial cell lines to transitional epithelial cell lines gradually adapted to an osmolality of 600mOsm/kg with either sodium chloride or urea, cell lines of gastrointestinal origin were inhibited in their ability to activate ATM and downstream effectors of DNA damage signaling and repair such as p53, Nbs1, replication protein A (RPA), and γ H2AX following the induction of DNA damage with etoposide. In contrast, bladder cell lines demonstrated a preserved ability to phosphorylate ATM and its effectors under conditions of hyperosmolal urea, and to a lesser extent with sodium chloride. The bladder cell lines' ability to respond to DNA damage under hyperosmolal conditions may be due in part to protective mechanisms such as the accumulation of intracellular organic osmolytes and the uroplakin-containing asymmetric unit membrane as found in transitional epithelial cells, but not in gastrointestinal cells. Failure of such protective adaptations in the tissues used for augmentation cystoplasties may place these tissues at increased risk for malignancy.

Keywords

Hyperosmolar microenvironment; DNA repair; carcinogenesis; bladder augmentation

1. Introduction

Microenvironmental factors often have profound effects on cellular metabolism, survival, and proliferation. Such microenvironmental effects may be highly dependent on the tissue origin of cells. Augmentation cystoplasties, bladder reconstruction procedures in which gastrointestinal tissue is used to repair the urinary bladder (reviewed in depth by Mitchell [1]), are an example of the tissue-specific microenvironment response. Such cystoplasties have an approximately 600-fold increased risk of malignancy in the anastomosed gastrointestinal tissue compared to the risk of bladder cancer in the general population [2]. Approximately forty

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

percent of these malignancies were reported to be transitional cell carcinomas, suggesting metaplasia in the enteric patch [3]. Experimental animal data [4–6] and surveillance biopsy data in humans [7] demonstrate that transitional metaplasia is frequently observed in the gastrointestinal segment of bladder augmentations, and this metaplasia may herald the onset of more severe dysplasia and malignancy. Recent analyses of biopsied enterovesicular anastomoses revealed mutations in p53 by restriction site mutation assay in 20% of patients, but found no mutations in the native bladder tissue [8], suggesting a tissue-specific response to the bladder microenvironment. The mechanism underlying the increased metaplasia, dysplasia, and malignancy of augmented bladders has been hypothesized to be due to microenvironmental factors including chronic bacterial colonization with the subsequent formation of nitrosamines, extremes of urinary pH, chronic trauma from urolithiasis, and the apposition of dissimilar tissues. The carcinogenic pathomechanisms stemming from exposure to these factors are not clear, and a role for the normal bladder microenvironment is equally as plausible.

The mammalian renal inner medulla and urinary bladder microenvironments are characterized by hyperosmolality that may reach 1200 mOsm/kg in maximally concentrated urine [9]. Both sodium chloride and urea produce this hyperosmolality in the renal inner medulla [10], while urea is primarily responsible for urinary hyperosmolality. To adapt to this osmotic pressure, inner medullary collecting duct cells [11,12] accumulate organic osmolytes such as betaine, myo-inositol, and taurine to balance the osmotic gradient; induce heat-shock proteins HSP70, HSP27, and HSP110 [13]; and activate signaling pathways vital to cell survival [14–18]. Transitional epithelial cells within the bladder also accumulate organic osmolytes [19], but in addition construct an apical barrier called the asymmetric unit membrane (AUM), which is made up of uroplakins forming a network of crystalline plaques [20]. This additional barrier creates a high transepithelial resistance and a low permeability to water and urea [21,22].

Osmotic stress has been found to compromise genomic integrity. Murine inner medullary collecting duct (mIMCD3) cells abruptly subjected to hypertonicity from sodium chloride developed numerous double strand breaks (DSBs) [23]. While acute exposure to hyperosmolal urea failed to induce DSBs [23], Zhang et al observed such treatment with urea led to single strand breaks (SSBs) by alkaline comet assay in mIMCD3 cells [24]. Dmitrieva et al posited that the DSBs associated with osmotic stress were due to dysregulation of the DNA damage response pathway, as Mre11 was exported from the nucleus and both Chk1 and H2AX failed to be phosphorylated when the cells were exposed to hyperosmolality [25]. H2AX failed to be phosphorylated *in vivo* in association with spontaneously occurring as well as ionizing radiation-induced DSBs in the hyperosmolal renal medulla but was appropriately phosphorylated in the iso-osmolal renal cortex of 129/SVE mice [26].

Ataxia-telangiectasia mutated (ATM) kinase, along with ataxia-telangiectasia Rad3-related (ATR) kinase and DNA-PKcs, serve as master controllers of the DNA damage response [27]. In the presence of DSBs, ATM autophosphorylates and dissociates from an inactive homodimer to an active monomer, which then phosphorylates and activates downstream targets of the DNA damage response pathway such as Nbs1, replication protein A (RPA), p53, chk2, and γ H2AX [28]. ATM also undergoes autophosphorylation following acute hyperosmolal stress from either sodium chloride or urea [29], but despite this autophosphorylation, it does not phosphorylate targets such as γ H2AX [25]. ATM has additionally been shown to be autophosphorylated following hypotonic stress, and H2AX is also not phosphorylated under these conditions [30]. ATM autophosphorylation was determined to be due to the modification of chromatin structure, as chloroquine had a similar effect. H2AX is also a substrate of DNA-PKcs, and the size of γ H2AX foci was found by Olive and colleagues to be increased in cells exposed to hypertonic media shortly after induction of DSBs in a DNA-PKcs dependent but

ATM-independent manner [31]. This increase was determined to be due to ineffectual non-homologous end-joining under hypertonic conditions.

Tissues chronically exposed to hyperosmolal conditions may not activate the DNA damage response despite ongoing intrinsically as well as extrinsically induced DNA damage, leading to the accumulation of mutations and increasing the risk for developing malignancy. We hypothesized that nonbladder tissues in augmentation cystoplasties are suboptimally adapted to such a hyperosmolal microenvironment and have an inadequate response to DNA damage, and that this disturbed equilibrium between damage and repair leads to the accumulation of mutations and eventually malignancy. To test this hypothesis, we characterized the identification and repair of double-strand breaks in cell lines derived from gastric (NCI-N87 and AGS), colonic (Caco2) and bladder (RT4 and KK47) epithelium gradually adapted to hyperosmolal conditions, and found that a hyperosmolal microenvironment impairs these processes in gastrointestinal but not bladder-derived cells. These findings suggest a tissue-specific effect of osmotic stress on DNA damage response.

2. Materials and Methods

2.1 Cell Lines and Tissue Culture Techniques

The well-differentiated NCI-N87 and moderately differentiated AGS gastric adenocarcinoma [32], well-differentiated Caco2 colon adenocarcinoma, and uroplakin-expressing [33] RT4 transitional cell carcinoma [34] cell lines were obtained from the American Type Culture Collection (Manassas, VA). The KK47 transitional cell carcinoma cell line derived from a well-differentiated Grade I TCC [35] was a generous gift of Dr. Seiji Naito from Kyushu University (Fukuoka, Japan). Cell lines were propagated in tissue culture using DMEM/F12 1:1 culture media (Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone), 0.5mM sodium pyruvate (Invitrogen), and 100 units/mL penicillin and 100µg/ml streptomycin (Gibco). To adapt cell lines to hyperosmolal conditions, the osmolality of the media was increased by 50 mOsm/kg every 48 hours by aspirating the media and replacing with fresh media adjusted to the corresponding osmolality by the addition of sterile-filtered 5M NaCl (Sigma) or 5M urea (Fluka). To remove isocyanates, a degradation product of urea, 20mL of 5M urea stock solution was exchanged with 1g of AG-501-X8 resin (Bio-Rad) in the method published by Zhang et al [24]. Osmotically adapted cells continue to proliferate, and were passaged at 90% confluency. Following adaptation, cells were maintained either at basal media osmolality (~300 mOsm/kg) or at the target osmolality (600 mOsm/kg) for 48 hours, following which the cells were exposed for 12 hours to 50µM etoposide (Sigma) or the DMSO vehicle (Sigma).

2.2 Single Cell Electrophoresis (Neutral Comet Assay)

Following treatment with etoposide or DMSO vehicle, cells were harvested by aspiration of the media, rinsing the cells with ice-cold PBS, and detachment with trypsin-EDTA (Gibco). Detached cells were resuspended in ice-cold PBS, and quantitated by hemocytometer. Approximately 1.2×10^4 cells from each group were aliquoted into microcentrifuge tubes, and the volumes of cell suspension equalized with PBS. A molten solution of 1% low melting point agarose (Gibco) in PBS, boiled to dissolution and cooled to 42°C, was added to each cell suspension. Aliquots of the cell/agarose suspensions were layered onto etched glass slides. Gels were allowed to solidify at 4°C, then transferred to a lysis buffer (150 mM NaCl, 1 mM Tris, 18mM N-laurylsarcosine, 4mM EDTA) adjusted to pH 7.4 [36,37] for 3 minutes. Slides were rinsed in double distilled water (ddH₂O), placed in an electrophoresis apparatus (Owl Separation Systems) filled with TBE (47mM Tris borate, 0.04mM EDTA), and subjected to an electromagnetic field of 20V, 2–4mA for 10 minutes. Following electrophoresis, slides were stained in a 50µg/ml propidium iodide (Fluka) bath, rinsed, and covered with coverslips. Slides

were analyzed with a Zeiss Axiovert 200M inverted fluorescent microscope at 40x magnification within 48 hours of preparation. Resulting images were analyzed by CometScore 1.5 software (TriTek, Sumerduck, VA) and the extent of DNA damage was scored by the mean of 40 measurements of tail moment per single experiment. Mean tail moment of cells treated with etoposide were normalized to the mean tail moment of DMSO treated cells, and expressed as a fold-increase. Comet assays were performed in duplicate from each osmolal condition and treatment through four separate experiments. Comets were scored as apoptotic if the tail moment exceeded 20 based on the findings by Fairbairn et al [38], and the percent of apoptotic cells in each treatment group and osmolal condition were quantified in the four experimental data sets, and expressed as a mean percentage and standard error of the mean.

2.3 Protein Isolation and Western Blot Analysis

Cell cultures gradually adapted to either basal osmolality or hyperosmolality with urea or sodium chloride were seeded into dishes at a density of 1×10^6 cells/dish. Following treatment with etoposide or DMSO vehicle for 12 hours, the media was aspirated, and cells rinsed with ice cold PBS. Cells were then lysed with ice-cold RIPA buffer (50mM Tris HCl pH 8, 150mM NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors (10mM NaF, 1mM Na_3VO_4 , 1mM PMSF, 2 $\mu\text{g}/\text{ml}$ aprotinin, and 10 $\mu\text{g}/\text{ml}$ leupeptin) and harvested with a cell scraper. Cell suspensions were transferred to prechilled microcentrifuge tubes, shaken vigorously for 30 minutes at 4°C, and cellular debris was pelleted by centrifugation at 4°C for 20 minutes at $15,000 \times g$. Supernatants were aliquoted and stored at -80°C.

Protein concentration of these lysates was determined by bicinchoninic acid assay (BCA, Pierce) according to the manufacturer's instructions. Equal amounts of protein were loaded onto 12% or 15% polyacrylamide gels, or alternatively onto 3–8% Tris-Acetate gels (Invitrogen). Separated proteins were transferred to Immobilon-P PVDF membranes (Millipore), using an XCell SureLock MiniCell electrophoresis apparatus and XCell II Blot Module (Invitrogen). Membranes were blocked with 5% nonfat dry milk in TBS with 0.1% Tween 20 v/v (TBST), then blotted with the following primary antibodies: RPA32 (1:20,000, Neomarkers); RPA32 phospho-S4,S8 (1:10,000), p53 (1:5000, Bethyl Laboratories); Nbs1 (1:5000), Nbs1 phospho-S343 (1:10,000), ATM (1:4000, Novus Biologicals); ATM phospho-S1981 (1:2000, Rockland Immunologicals); p53 phospho-S15 (1:2000), cleaved caspase-3 (1:1000) and PARP (1:1000, Cell Signaling Technologies); procaspase-3 (1:5000, Santa Cruz Biotechnology); γH2AX (1:1000, Trevigen); and p21^{cip1} (1:1000, BD Pharmingen). Equal protein loading was confirmed by blotting for G3PDH (1:20,000–1:40,000; Trevigen). Membranes were then washed and incubated with a horseradish peroxidase (HRP)-linked anti-mouse or anti-rabbit IgG secondary antibody (GE Healthcare Biosciences), and protein bands detected by chemiluminescence with the Amersham ECL Plus kit (GE Healthcare Biosciences) and developed by autoradiography.

2.4 Colocalization of DNA repair proteins by foci analysis

Cells adapted to basal and hyperosmolal conditions were seeded onto glass coverslips in 24 well plates at a density of 5×10^4 cells/well. Cells were allowed to attach to the coverslips for 48 hours at the respective osmolalities, and then treated for 12 hours with 50 μM etoposide or DMSO vehicle. Media was then aspirated from the wells, and cells were rinsed with ice-cold PBS, fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS, and permeabilized with 0.5% Triton X-100 (Sigma) in PBS. Cells were blocked with 15% FBS in PBS, then incubated with primary antibodies directed against RPA32 phospho-S4, S8 (1:10,000) and full-length RPA32 (1:1500) in PBS with 1% bovine serum albumin (BSA, Sigma) overnight at 4°C. The cells were rinsed with PBS, then incubated with anti-rabbit Alexa-Fluor 488 and anti-mouse Alexa-Fluor 594 (Molecular Probes) at 1:500 dilution made

in PBS with 1% BSA. Cells were then mounted with ProLong Gold with DAPI (Invitrogen) and coverslips applied to glass slides. Slides were analyzed with a Zeiss Axiovert 200M inverted fluorescent microscope with 40x and 100x oil-immersion objectives. The percentage of cells containing diffuse punctate foci out of a minimum of 100 cells counted was scored. These assays were performed in three separate experiments in both the gastric- and bladder-derived cells, and expressed as a mean percentage of cells containing foci.

2.5 Statistical Analyses

Mean comet tail moments of DMSO and etoposide-treated cells were compared by Student's paired t-test. In addition, the fold increase in mean comet tail moment following damage with etoposide was compared between different osmolal treatment groups using Student's t-test. Error was calculated as the standard error of the mean (SEM) from both comet assay and foci quantitation experiments.

3. Results

3.1 Failure to detect DNA damage under hyperosmolal conditions

To investigate DNA damage under hyperosmolal conditions, etoposide (a potent topoisomerase II inhibitor) was used to induce double strand DNA breaks. Whole cell lysates from RT4 bladder and NCI-N87 gastric derived cells damaged with etoposide were analyzed for the abundance and phosphorylation of DNA damage response pathway components. ATM was not autophosphorylated in either cell line gradually adapted to conditions of hyperosmolal urea or sodium chloride without the induction of DNA damage (Figures 1a and b). Gastric-derived NCI-N87 cells damaged with etoposide were able to autophosphorylate ATM only under basal osmolal conditions (Figure 1a). This failure to activate ATM under hyperosmolal conditions also resulted in the failure to phosphorylate p53 on serine 15 and H2AX on serine 139 (γ H2AX). Cells chronically adapted to hyperosmolal conditions also exhibited a decrease in p53 abundance, possibly potentiating the impact of osmolal stress on DNA damage detection. A downstream effector of activated p53, p21^{cip1}, was also found to have decreased expression under hyperosmolal conditions.

Bladder-derived RT4 cells autophosphorylated ATM in the presence of DNA damage under basal osmolal and hyperosmolal urea conditions, and to a lesser extent during sodium chloride-induced hyperosmolal conditions (Figure 1b). Increased abundance of p53 was demonstrated in these cells damaged with etoposide under all tested conditions, although phosphorylation of p53 on serine 15 did not appear to change with etoposide treatment. The expression of p21^{cip1} increased with etoposide treatment under iso-osmolal and hyperosmolal urea conditions, and was substantially increased under hyperosmolal sodium chloride conditions but did not increase further with etoposide treatment. Phosphorylation of γ H2AX was present in bladder cells damaged with etoposide under both iso-osmolal and hyperosmolal conditions.

The neutral comet assay was used to test whether the magnitude of DNA damage was similar between osmolal groups, to ensure that hyperosmolal conditions did not influence the interaction between etoposide and topoisomerase II. Under iso-osmolal conditions, gastric and transitional epithelial cells exposed to etoposide demonstrated 4.7-fold and 3.3 fold increases, respectively, in comet tail moment compared to cells treated with the DMSO vehicle alone ($p < 0.05$) (Figure 1c). Cells damaged following adaptation to hyperosmolal conditions demonstrated similar increases in comet tail moment (3.0-fold for NaCl-adapted and 4.0-fold for urea-adapted gastric cells; 4.7-fold for NaCl-adapted and 4.9-fold for urea adapted bladder cells, $p < 0.05$). Comparison of the fold increase in comet tail moment between the basal and hyperosmolal groups showed no statistical difference, verifying the efficacy of etoposide to induce DNA damage under both iso-osmolal and hyperosmolal conditions.

3.2 Inhibition of DNA repair mechanisms under hyperosmolal stress

The activation of specific DNA repair proteins was characterized using western blot analyses of whole cell lysates from gastric, colon, and bladder-derived cells damaged with etoposide under iso-osmolal and hyperosmolal conditions. Nbs1, a member of the Mre11-Rad50-Nbs1 (MRN) complex vital to non-homologous end-joining (NHEJ) as well as homologous recombination (HR) [39], was phosphorylated on serine 343 following damage with etoposide under basal conditions in the NCI-N87 and AGS gastric cell lines, the colon derived Caco2 cell line, and the RT4 and KK47 bladder-derived cell lines (Figure 2 and Figure 3). However, NCI-N87 and to a lesser extent AGS gastric-derived cells adapted to hyperosmolal sodium chloride and urea were inhibited in their activation of Nbs1 following induction of DNA damage (Figure 2a and Figure 3a). Caco2 colon-derived cells also exhibited a modest inhibition of Nbs1 phosphorylation (Figure 3c) under hyperosmolal conditions. Bladder-derived cells adapted to identical conditions retained their ability to robustly activate Nbs1 following damage, though the response with sodium chloride was slightly suppressed in both the RT4 and KK47 cell lines (Figure 2b and 3b).

Replication protein A (RPA) participates in DNA replication as well as repair [40]. Hyperphosphorylation of the 32kDa subunit of RPA (RPA32) on serines 4 and 8 serves as a molecular switch, biasing its utilization from DNA replication to repair [41]. RPA32 was hyperphosphorylated in lysates from gastrointestinal and bladder-derived cells damaged under basal osmolal conditions (Figure 2 and Figure 3). Following gradual adaptation to hyperosmolal sodium chloride or urea, both NCI-N87 and AGS gastric cells and colon-derived cells exposed to etoposide attenuated their ability to hyperphosphorylate RPA32 (Figure 2a, Figure 3a and 3c). Bladder-derived cells gradually adapted to hyperosmolal urea, and to a lesser extent sodium chloride, continued to demonstrate the ability to hyperphosphorylate RPA32 following the induction of DNA damage (Figure 2b and Figure 3b). Abundance of total cellular Nbs1 and RPA32 was similar in all cell lines under both basal and hyperosmolal conditions (Figure 2 and Figure 3).

3.3 RPA32 repair foci under hyperosmolal conditions

Gastric- and bladder-derived cells were examined by immunofluorescence for the formation of RPA32 DNA repair foci. A majority (54.1%) of NCI-N87 gastric cells damaged with etoposide under iso-osmolal conditions contained diffuse punctate foci (Figure 4) consisting of hyperphosphorylated RPA32 (colabeled with antibodies directed against total and hyperphosphorylated RPA32). In contrast, 5.9% and 2.8% of cells damaged following adaptation to hyperosmolal sodium chloride and urea, respectively, contained hyperphosphorylated foci.

Under iso-osmolal conditions, a majority of RT4 bladder cells (63.2%) damaged with etoposide were found to contain hyperphosphorylated RPA32 foci (Figure 5). Cells adapted to hyperosmolal sodium chloride were able to form RPA32 repair foci following damage with etoposide, albeit somewhat less prominently than under basal osmolal conditions (19.7%). Cells gradually adapted to hyperosmolal urea and damaged with etoposide formed mature RPA32 foci similar to iso-osmolal controls (54.7%). Negative controls performed in the absence of primary antibody had no non-specific staining identified.

3.4 Apoptosis in response to DNA damage under hyperosmolal conditions

Suppression of the amount and activation of p53 in the NCI-N87 gastric cell line following adaptation to hyperosmolality (Figure 1a) raised the possibility that there could be dysregulation of apoptosis. At basal osmolality, gastric cell lysates had measurable levels of the incompletely processed p20 and mature p17 cleavage products of caspase-3, a key effector of apoptosis activated by both the intrinsic and extrinsic pathways, as well as cleavage of poly-

ADP ribose polymerase (PARP), a substrate of active caspase-3. This cleavage was substantially increased by exposure to etoposide (Figure 6a). Gastric cells gradually adapted to hyperosmolal sodium chloride and urea demonstrated increased cleavage of caspase-3 and PARP with DMSO vehicle treatment, indicating that osmotic stress alone may lead to apoptosis in these cells. Supporting their inability to detect DNA damage under osmotic stress, gastric cells failed to increase cleavage of caspase-3 or PARP above vehicle-treated levels following etoposide-mediated damage under conditions of hyperosmolal urea and sodium chloride.

Cleavage of caspase-3 and PARP was not demonstrated in RT4 bladder-derived cells treated with the DMSO vehicle under iso-osmolal or hyperosmolal conditions. In contrast to the gastric-derived cell line, bladder cells demonstrated increased cleavage of both caspase-3 and PARP following exposure to etoposide under both iso-osmolal conditions and following gradual adaptation to hyperosmolal sodium chloride and urea (Figure 6b).

In contrast, quantitation of cells in later stages of apoptosis revealed that both gastric and bladder cell lines demonstrated increased apoptotic activation when exposed to 50 μ M etoposide under both iso-osmolal and hyperosmolal conditions. As shown in Figure 6c, the late apoptotic cell population quantified by this method was between 2.8% and 15.1% of cells, suggesting that the late apoptotic response was modest compared to early apoptotic events such as activation of the caspase cascade.

4. Discussion

Children with complex urogenital anomalies are often treated by surgical augmentation of their bladders with gastrointestinal tissue to allow for a larger, more compliant urinary reservoir. Augmented bladders have a significantly increased risk of developing malignancies [2]. Mechanisms underlying this propensity towards carcinogenesis are poorly understood, though certain abnormal microenvironmental factors have been postulated. We have explored the possibility that normal microenvironmental factors may contribute to malignancy.

The mammalian urinary bladder is a hyperosmolal microenvironment. Transitional epithelium withstands this environment by the production and transport of organic osmolytes, as well as the asymmetric unit membrane consisting of uroplakins. As depicted in the model in Figure 7, gastrointestinal tissues placed into this environment lack such robust protective mechanisms and thus may sustain greater osmotic stress from urinary solutes, disrupting normal cellular processes such as the repair of DNA damage and leading to mutagenesis. Accumulation of mutations has been identified in these tissues [8], suggesting that susceptibility to mutations may underlie their risk of malignant transformation. Cells from the gastrointestinal tract also exhibit a higher index of mitosis than that of transitional epithelium [42], and in the bladder microenvironment greater DNA replication in the face of suppressed damage recognition and repair may pose an added risk of mutagenesis and resultant carcinogenesis.

To assess if gastrointestinal tissues placed in a bladder environment have an altered response to DNA damage, we compared the response to DSBs of gastric and colon adenocarcinoma cell lines with that of bladder transitional cell carcinoma lines under both iso-osmolal and hyperosmolal conditions. All cell lines demonstrated robust DNA damage recognition and repair under iso-osmolal conditions, indicating that the cell lines were sufficient in this respect. In contrast to bladder cells, however, gastrointestinal cells were inhibited in the activation of ATM and its downstream targets (Figure 1a, Figure 2a, Figure 3a, and 3c) following adaptation to osmotic stress. DNA-PKcs exhibits redundancy with ATM in the phosphorylation of H2AX following DNA damage, suggesting that the failure of gastric cells to phosphorylate H2AX in response to DNA damage following gradual adaptation to hyperosmolal conditions may be due to the inhibition of both PI3K related kinases. Activation of DNA-PKcs by phosphorylation

on serine 2609 in response to DNA damage is dependent on ATM [43], possibly explaining the failure of this redundancy under chronic osmotic stress. The ATM-dependent signaling and repair of DSBs appears to be specific to the chromatin state of the cell [44], although we did not detect qualitative differences in the heterochromatin vs. euchromatin content by DAPI staining in either the gastric or bladder cell lines adapted to hyperosmolar conditions when compared to iso-osmolar conditions (data not shown).

Failure to activate the DNA damage response translated into an abrogated formation of RPA32 repair foci (Figure 4). This differential response suggests that gastrointestinal tissues may be less likely to identify mutagenic insults if they are subjected to microenvironments to which such tissues are not normally suited. Acute exposure to hyperosmolar stress has been reported to affect the DNA damage response in a species and cell type-dependent manner [45,46]. The enteric patch transitional cell metaplasia seen in augmented bladders in both animal models [4,5] and humans [7] may represent an attempt of the tissue to adapt to the hyperosmolar microenvironment in order to facilitate vital cellular activities like DNA repair. An intriguing possibility of the differential tissue susceptibility to DNA damage, mutation, and ultimately development of cancer is a bystander effect exerted on undamaged cells by adjacent damaged cells (reviewed in [47]) or even by intestinal commensal bacterial flora colonizing the augmented bladder [48]. Such microenvironmental effects require further investigation.

If DNA damage is not recognized and repaired, then the damage would accumulate and induce apoptosis if the damage reached a critical threshold. This DNA damage-associated apoptosis is mediated in large part by p53 augmenting the extrinsic pathway by activating the FasL death receptor and the intrinsic pathway through induction of PUMA and Bax (reviewed in reference [49]). Failure of cells with multiple acquired genotoxic insults to initiate the apoptotic cascade may lead to mutations in key cell regulatory genes and result in uncontrolled proliferation and malignancy. Although the NCI-N87 gastric cell line used in these experiments has both a point mutation [50] and truncation mutation [51] in p53, the cells activated apoptosis in response to etoposide-induced DNA damage under conditions of iso-osmolality. The gastric cells adapted to hyperosmolar sodium chloride and urea exhibited an increase in apoptosis compared to iso-osmolar conditions, but failed to increase this activity further when subjected to etoposide-induced DNA damage. This osmolar stress-induced apoptosis may be activated by other mediators than p53, such as the γ isoform of GADD45 [52] and the α and β isoforms of p38 MAPK [53]. Failure to phosphorylate p53 on serine 15 following DNA damage under hyperosmolar conditions was also found in AGS gastric cells which contain wild-type p53 [54], suggesting the effect of hyperosmolality upon p53 signaling was not due to the mutations carried by the NCI-N87 cell line.

Bladder epithelial cells did not demonstrate increased apoptosis indicated by cleavage of caspase-3 and PARP following adaptation to hyperosmolar sodium chloride or urea. This finding would suggest that transitional epithelial cells may be more osmotolerant than gastric cells, perhaps similar to the resistance to apoptosis as seen by mIMCD3 and MDCK cells gradually adapted to hyperosmolar conditions [16,26,55]. In addition, the bladder cells were able to activate apoptosis as measured by caspase-3 and PARP cleavage when damaged with etoposide. The induction of apoptosis occurred under both iso-osmolar and hyperosmolar conditions, although the activation of apoptosis was somewhat attenuated under hyperosmolar conditions.

The findings presented here suggest that gastrointestinal epithelial cells in an augmentation cystoplasty may be unable to effectively identify and repair DNA damage in the hyperosmolar bladder microenvironment, possibly due to a lack of osmoprotective mechanisms. In turn, these cells may fail to undergo apoptosis at a critical threshold of DNA damage. Transitional epithelium from the native bladder may have a greater tolerance for extremes of osmolality,

and may therefore more readily activate ATM/ATR and their downstream effectors when DNA damage is incurred. If the microenvironmental impact of hyperosmolality on gastrointestinal epithelium is validated in additional model systems, therapeutic interventions aimed at reducing urinary osmolality through the judicious use of diuretic therapy or increased fluid intake to restore the normal DNA damage response and repair mechanisms may be warranted. Improved recognition and repair of acquired DNA damage in the gastrointestinal tissues of bladder augmentations may prevent the accumulation of mutations and perhaps reduce the risk of malignancy in these patients.

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Figure 1.

Gastric-derived cells are unable to recognize DNA damage under hyperosmolar conditions. Lysates were generated from NCI-N87 gastric and RT4 bladder epithelial cell lines adapted to hyperosmolar conditions and exposed to etoposide or the DMSO vehicle. A.) Western blot of gastric cell lysates against ATM and downstream components of the DNA damage signaling pathway. (Multiple bands are present at >350kDa, pATM identified by white arrows.) B.) Western blot of bladder cell lysates. C.) Neutral comet assays of gastric (dark grey bars) and bladder-derived cells (light grey bars) exposed to etoposide (ETOP) or the DMSO vehicle under both iso-osmolar and hyperosmolar conditions. DNA damage in response to etoposide is expressed as a mean fold increase in comet tail moment over that of the DMSO treated group

for each osmolal condition. Error bars represent standard error of the mean (SEM) from four separate experiments.

Figure 2.

Gastric cells are unable to activate DNA repair mechanisms under hyperosmolar conditions.

A.) Western blot of lysates of NCI-N87 gastric epithelial cells adapted to hyperosmolar conditions and exposed to etoposide or the DMSO vehicle, probing against components of HR (Nbs1, RPA32) and NHEJ (Nbs1) pathways. B.) Western blot of RT4 bladder cell lysates.

Figure 3.

Inhibition of Nbs1 and RPA32 activation in response to DNA damage by hyperosmolar conditions occurs in other gastrointestinal cells, but not bladder cell lines. A.) Western blot of lysates of AGS gastric-derived epithelial cells adapted to hyperosmolar conditions and exposed to etoposide or the DMSO vehicle, probing against components of HR (Nbs1, RPA32) and NHEJ (Nbs1) pathways. B.) Nbs1 and RPA32 western blots of KK47 bladder-derived cell lysates. C.) Nbs1 and RPA32 western blots of Caco2 colon-derived cell lysates.

Figure 4. Gastric epithelial cells are unable to form repair foci following DNA damage under hyperosmolar conditions. Immunofluorescence of NCI-N87 gastric-derived cells adapted to hyperosmolar conditions and exposed to etoposide (ETOP) or the DMSO vehicle. A.) Representative images of gastric-derived cells exposed to etoposide (ETOP) or the DMSO vehicle under iso-osmolar and hyperosmolar conditions. Cells are colabeled with antibodies directed towards hyperphosphorylated RPA32 (left panels, green), total RPA32 (middle panels, red), and merged images (right panels, yellow) B.) Quantitation of RPA32 repair foci in gastric-derived cells. A minimum of 100 cells was assessed for the presence of diffuse punctate foci.

The mean percentage of gastric cells containing hyperphosphorylated RPA32 foci for three separate experiments $\pm$ SEM is shown.

Figure 5.

Bladder epithelial cells form repair foci following DNA damage under both iso-osmolal and hyperosmolal conditions. Immunofluorescence of RT4 bladder-derived cells adapted to hyperosmolal conditions and exposed to etoposide (ETOP) or the DMSO vehicle. A.) Representative images of cells colabeled with antibodies directed towards hyperphosphorylated RPA32 (left panels, green), total RPA32 (middle panels, red), and merged images (right panels, yellow). B.) Quantitation of RPA32 repair foci in bladder-derived cells. A minimum of 100 cells was assessed for the presence of diffuse punctate foci. The mean percentage of bladder cells containing hyperphosphorylated RPA32 foci for three separate experiments $\pm$ SEM is shown.

Figure 6.

Gastric epithelial cells demonstrate activation of apoptosis under hyperosmolar conditions, but do not increase this activation further following DNA damage. Whole cell lysates were generated from NCI-N87 gastric and RT4 bladder-derived epithelial cell lines adapted to hyperosmolar conditions and exposed to etoposide or the DMSO vehicle. A.) Western blot of gastric cell lysates against caspase-3 (procaspase-3 and cleaved caspase 3) and PARP. Arrowheads identify uncleaved (116kDa) and the large cleaved fragment (89kDa) of PARP, as well as the incompletely (p20) and completely (p17) processed cleavage products of caspase-3. B.) Western blot of bladder cell lysates. C.) Quantitation of late apoptotic NCI-N87 gastric cells and RT4 bladder cells by neutral comet assay. Percentage of cells with comet tail

moment > 20 indicating massive DNA fragmentation (late apoptosis) is expressed as the mean of four separate experiments $\pm$ SEM. (Note the maximal vertical axis value of only 40%.)

Figure 7.

Model of carcinogenesis in augmentation cystoplasty. Gastrointestinal tissues, due to the lack of osmoprotective mechanisms, are unable to identify and repair DNA damage in the hyperosmolar microenvironment of the urinary bladder and thus accumulate mutations. This mutagenesis, in concert with other influences such as the pro-inflammatory state of chronic colonization with enteric flora, and the presence of growth factors producing a higher mitotic rate, may lead to uncontrolled proliferation of these tissues and carcinogenesis.