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Review Article

Mechanisms of hemorrhagic cystitis

Subhash Haldar[1*], Christopher Dru[1*], Neil A Bhowmick[1,2]

[1]Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, CA; [2]Greater Los Angeles Veterans Administration, Los Angeles, CA. [*]Equal contributors.

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Abstract: The vast majority of cases of infectious cystitis are easily treated, and most patients have no long-term complications. However, hemorrhagic cystitis is a potentially deadly complication associated with pelvic radiation therapy, chemotherapy, and stem-cell transplant therapy. The focus of current understanding, and hence therapy, is directed toward urothelial cell death. However, the primary functional ramification of inflammatory bladder disease is the loss of compliance due to muscular expansion. Recent studies on smooth muscle response in models of bladder inflammation demonstrate a process of pyroptotic cell death that potentiates further muscle hyperplasia. These findings may support alternative interventions for subjects with hemorrhagic cystitis refractive to current therapy.

Keywords: Bladder inflammation, radiation cystitis, pyroptosis

Cystitis is the general term used to define any type of inflammation of the urinary bladder. Cystitis can be acute or chronic, and the severity can range from mild discomfort in the lower abdomen to severe life-threatening hemorrhage. There are several categories to describe the various etiologies of cystitis -- infection, radiation, chemical, mechanical, interstitial cystitis/chronic pelvic pain syndrome, as well as several conditions that masquerade as cystitis. But on an even broader level, cystitis can be classified as infectious versus non-infectious.

Mechanisms of bladder inflammation in people

The most common cause of cystitis is infection, specifically from bacteria, but also from several types of viruses and fungi. More than 50% of women will experience at least one urinary tract infection during her lifetime. Uropathogenic E. coli (UPEC) are the most common bacteria implicated in these infections. UPEC express type 1 pili and corresponding adhesin FimH on the surface of the cell wall that bind to mannose-coated proteins on the outermost layer of the urothelium, called umbrella cells [1, 2]. The UPEC replicate within the umbrella cells and eventually cause cell lysis, spilling more bacteria into the urine. Before the cells die, a signaling cascade initiated by toll-like receptor 4 (TLR-4) is activated to recruit polymorphonuclear leukocytes (PMNs) to combat the infection [3, 4]. Primary fungal cystitis is rare and tends to be associated with the treatment of bacterial cystitis; elimination of the commensal vaginal bacterial flora by antibiotics allows for uninhibited growth and proliferation of fungal species, in particular candida and lactobacillus. Viral cystitis, typically from adenovirus and BK virus, targets patients who are immunosuppressed. Examples of this patient population include HIV/AIDS patients, patients on immunosuppresent medications such as prednisone and chemotherapeutics, and patients with leukemia who have the inability to produce functional leukocytes. Patients with infectious cystitis typically complain of irritative voiding symptoms, dysuria, frequency, urgency, and suprapubic pain; only in rare instances is gross hemorrhage present.

The other broad class of cystitis is sterile, or non-infectious, cystitis. Etiologies include radiation and chemical irritation. Unlike the infection-induced counterpart, non-infectious cystitis tends to be more clinically severe and can

cause extreme pain, hematuria, and irritative voiding symptoms. Hemorrhagic cystitis is the severe clinical manifestation of radiation and chemical cystitis. Radiation-induced cystitis is associated with pelvic external beam radiation treatment for urologic and pelvic malignancy. There is no definitive time-frame that constitutes the "at risk" window for radiation-induced cystitis; patients can experience a wide-range of symptoms from mild pelvic pain to life-threatening hemorrhagic cystitis weeks, months, or years after treatment. To date, there is no way to anticipate which subset of radiation treatment patients will experience this potentially devastating complication. Chemical cystitis can be caused by many classes of medications but is most commonly caused by intravenous chemotherapeutic treatment for breast cancer and lymphoma with cyclophosphamide and ifosfamide. These medications have a corrosive liver metabolite called acrolein that is freely filtered by the kidneys to accumulate in the bladder. Acrolein causes a pyroptotic reaction in the bladder urothelium with ulceration and exposure of underlying muscularis mucosa and vasculature. Administration of Mesna (2-mercaptoethane sulfonate sodium) before initiation of cyclophosphamide can prevent hemorrhagic cystitis by binding to and neutralizing acrolein. However, the greatest source of acrolein is cigarette smoke. Mesna has no role in the treatment of hemorrhagic cystitis after its onset. Alternative treatment modalities of hemorrhagic cystitis include continuous bladder irrigation with evacuation of clots, instillation of alum or formalin, fulguration with electrocautery, hyperbaric oxygen therapy, and in extreme cases, cystectomy with urinary diversion.

The mechanism of acrolein-induced hemorrhagic cystitis from cyclophosphamide is complex and multimodal. Acrolein is a major component of cigarette smoke as well as charred meats. Acrolein can directly mechanically cleave proteins and break strands of DNA given that it has a reactive unsaturated aldehyde residue causing cell death [5]. Additionally, acrolein increases reactive oxygen species (ROS) in the urothelium by catalyzing the reaction of glutathione to glutathionylpropionaldehyde (GTPD). GTPD activates the NF-κB apoptotic pathway and interacts with several enzymes, most notably xanthine oxidase and aldehyde dehydrogenase, to form superoxide radicals such as peroxynitrite [6]. Peroxynitrite directly breaks DNA crosslinks which triggers upregulation of DNA damage repair genes and depletion of nicotinamide-adenine dinucleotide (NAD) and adenosine triphosphate (ATP), the energy sources of the cell [7]. This vicious cycle continues until all of the cell's energy sources are depleted and protein synthesis can no longer take place; at this point, the cell dies. As the acrolein destroys the urothelium, the underlying detrusor smooth muscle and blood vessels become exposed to urine, causing further cell death. But, as we later describe, the mechanism of cell death of the detrusor smooth muscle cells differ from that of the urothelium in animal models administered cyclophosphamide.

The mechanism of radiation cystitis is similar to that for acrolein-induced chemical cystitis in that the high-energy radiation causes single- and double-stranded DNA breaks leading to activation of DNA damage repair genes and apoptosis. In addition, radiation penetrates the deeper layers of the bladder muscle causing progressively worsening endarteritis [8]. This leads to a compromise in blood supply and delivery of nutrients to the tissue from the hypovascularity and hypocellularity [9]. The weakened blood vessels can survive months to years depending on the degree of injury which makes it very difficulty to predict if and when radiation cystitis will manifest.

Interstitial cystitis is unique in that it does not fit into the classic distinction of infectious or non-infectious cystitis. The etiology is not known at this time, but the most popular mechanisms include the "leaky epithelium" model and mast cell dysfunction. In the leaky epithelium model, it is proposed that irritants in the urine, such as caffeine or urokinase, are able to leak into the urothelium to cause chronic mild bladder inflammation [10]. This model also describes the destruction, or dysfunction, of the glycosaminoglycan (GAG) layer, the most superficial barrier between urine and bladder cells. An animal model mimicking the loss of the GAG layer involve the instilling of protamine sulfate [11]. The mast cell model hypothesizes that there is either a primary defect within the mast cells themselves causing histamine release or there is a stimulus causing chronic histamine release [12]. Overtime, the histamine causes sloughing of the superficial urothelium and chronic inflammation and pain.

Inflammasome and pyroptosis in immune cells

Inflammasomes are molecular platforms activated to defend against cellular infection or stress that promote the maturation of proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 to engage the innate immune system. The innate immune system not only monitors the presence of microbes but also possesses germline-encoded pattern recognition receptors (PRRs) that recognize aberrant signals produced by cells in response to pathogenic conditions [13, 14]. PRRs include Toll-like receptors (TLRs), nucleotide-binding domain leucine-rich repeat containing receptors (NLRs), RIG-I-like RNA helicases (RLHs), and C-type lectin receptors (CLRs). TLRs and CLRs are expressed on the cell surface or in endosomal compartments, while RLR are located in the cytosol [14-16]. Stimulation of these receptors results in activation of the NF-κβ, MAPK, Syk, and IRF-signaling pathways culminating in transcriptional induction and the secretion of a large number of cytokines, chemokines, and immunomodulatory factors [17, 18].

The NLR family is a cytoplasmic PRR, and several types have been described to date: NLRP1, NLRP3, NLRP6, NLRP7, NLRP12 or NLRC4. NLRP1, NLRP3, and NLRC4 are all well-characterized and act as cytosolic sensors to regulate cytokine secretion and trigger the assembly of large proinflammatory caspase-1-activating complexes with adaptor molecule ASC (apoptosis associated speck-like protein containing a CARD) termed inflammasomes. The NLRP3 inflammasome is the most widely investigated of the all inflammasome identified [19-22]. NLRP3 is generally activated by exposure of whole pathogens or a number of structurally diverse PAMPs (pathogen associated molecular patterns), DAMPs (damage associated molecular patterns), and environmental irritants (Silica, asbestos, alum etc.) [23-27]. PAMPs are a diverse set of molecules carried by pathogens, such as bacterial endotoxin (or lipopolysaccharide) of gram-negative bacteria, and DAMPs are endogenous molecules indicative of cellular damage (extracellular ATP, Glucose etc.) [14, 28-36]. Several recent studies have described that mitochondria provide an ideal platform for assembly of the NLRP3 inflammasome complex. NLRP3 may be activated directly by mitochondria-derived effector molecules such as mitochondrial reactive oxygen species, mitochondrial oxidized DNA, and phospholipid cardiolipin [37-39]. NLRP3 is characterized by its N-terminal pyrin domain (PYD), which allow NLRP3 to interact with ASC adapter by PYD-PYD interaction and thus facilitating the recruitment of pro-caspase-1 to build inflammasome complex [19, 21, 40-42]. Caspase-1 is a cysteine protease that is synthesized as an inactive zymogen with potent cellular activities regulated by proteolytic activation. Maturation of caspase-1 within the inflammasome triggers maturation and secretion of the proinflammatory cytokines IL-1β, IL-18 and IL-33. Active IL-1β and IL-18 play a crucial role in adaptive immune response to favor Th17 differentiation [43-47]. These pyroptotic products also participate in host defense against extracellular bacteria and fungi, and are involved in autoimmunity. IL-18 acts as an inducer of IFN-γ production by Th1 cells, which in turn helps to restrict intracellular pathogens.

Recent evidence indicates that the NLRP3 inflammasome provides danger recognition platforms and drives the proinflammatory cytokine IL-1β and IL-18 in various disease conditions. Acute myocardial infarction (MI) and Kawasaki disease (KD) are the common inflammasome mediated cardiac diseases where active IL-1β is produced by the NLRP3-ASC-caspase1 axis; however, the molecular mechanism of NLRP3 activation is not completely understood [48-52]. Myocardial infarction is defined as myocardial cell death due to prolonged ischemia, followed by an intense inflammatory response which can result in cardiac failure. Increased activation of caspase-1 by inflammasome formation during acute myocardial infarction promotes cell death, adverse cardiac remodeling, and heart failure when examined in mice [53-55].

Expression of the NLRP3 inflammasome and its components play a crucial role in development of hepatocellular carcinoma (HCC), one of the most prevalent malignant tumors [56, 57]. Inflammation is the most common potential modulator of diabetic nephropathy, a leading cause of end-stage renal disease in adults. Activation of NLRP3 by mitochondrial reactive

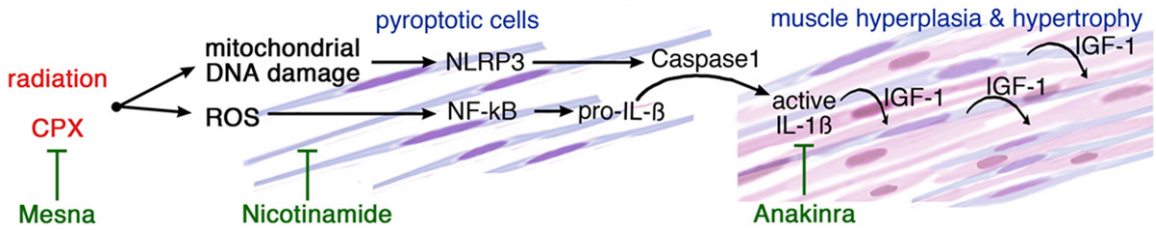

Figure 1. A model of inflammasome formation in urinary bladder muscle mediated by ROS production and oxidative damage in mitochondria. Activation of NLRP3 allows inflammasome complex formation and bioactivation of NF-κB mediated IL-1β expression via cleavage of caspase1. IL-1β secretion by a bladder smooth muscle cell undergoing pyroptotic cell death can support the proliferation of the remaining muscle cells by activation of any number of growth factors including IGF1. There are a few known inhibitors of the multiple steps process of bladder muscular expansion.

oxygen species (ROS) form glomerular inflammasomes causing nephropathy in diabetic mice whereas NLRP3 deficient mice are protected from diabetic nephropathy [58-60].

Pyroptosis and bladder fibroblasts

Painful bladder syndrome, hemorrhagic cystitis and other inflammatory bladder diseases generally occur after bone marrow or stem cell transplantation [61, 62], pelvic radiation therapy [63, 64], administration of alkylating chemotherapeutics [5, 65], or as a result of viral or bacterial infection [65, 66]. The increasing frequency of bone marrow or allogenic stem cell transplantation in the pediatric population has also been implicated in pathogenesis of hemorrhagic cystitis. It is evident that pro-apoptotic signaling molecules phospho-p53 (Ser 15), Bad, Bax, cleaved caspase-3, Fas and cleaved caspase-8 are upregulated in the urothelium of interstitial cystitis/painful bladder syndrome subjects [67, 68]. Cyclophosphamide induced hemorrhagic cystitis in mouse models causes apoptosis and necrosis of urothelial cells [5, 69, 70]. Inflamed bladder detrusor smooth muscle cells undergo autophagy following exposure to cyclophosphamide or macrophage migration inhibitory factors (MIF) [71, 72]. In bladder smooth muscle cultures, acrolein, a metabolite of cyclophosphamide, similarly induced cell death by necrosis, apoptosis, and autophagy mainly through elevation of ROS scavengers [69, 72-75]. Acrolein activates caspase-3 to trigger an apoptotic signal; however, other anti-apoptotic signaling molecules such as P-AKT and Mcl1 were not downregulated in detrusor muscle cell death. Acrolein induced senescence in bladder muscle cells as measured by p16 and p21 protein expression, and upregulated autophagic signaling proteins, Beclin-1 and LC3-II.

Pyroptosis was found to be one of the main causes of detrusor cell hyperplasia and death in the hemorrhagic cystitis model. In a mouse model of cyclophosphamide treatment as well as *in vitro* detrusor smooth muscle cell inflammation using acrolein and radiation potentiated pyroptosis signaling molecules. Acrolein is responsible for production of ROS associated with mitochondrial damage, and resultant ATP production is well reported [74, 76, 77]. Acrolein is capable of perpetuating oxidative stress by both inducing and bolstering lipid peroxidation and ROS generation; both have been linked to various pathologic diseases [78-80]. Cell exposure to acrolein leads to mitochondrial damage, denoted by the loss of mitochondrial transmembrane potential. The mitochondrial DNA is also at risk of oxidative damage because it sits on the inner mitochondrial membrane in close proximity to the electron transport chain. The levels of oxidized bases in mtDNA are two to three times higher than in nuclear DNA [81, 82]. Mice treated with cyclophosphamide resulted in oxidative mitochondrial DNA damage by ROS production as evidence by significant up regulation of mitochondrial 8-OXO-dG level. The oxidized form of mitochondrial DNA has the capability to bind and activate the inflammasome complex component, NLRP3, as stated before by Shimada et al, 2012 [38]. Recent evidence also indicates mitochondria as a key player in NLRP3 inflammasome signaling [39, 83]. Activated NLRP3 thereby stimulates the aggregation of the NLRP3-associated speck-like protein (ASC)-Caspase-1, enabling

the proteolytic maturation of caspase-1. As activated NF-kβ induces pro-IL-1β expression in the cytosol, active Caspase-1, in turn, cleaves pro-IL-1β, producing mature IL-1β [84-86] (**Figure 1**).

It is now apparent that epigenetic abnormalities, in particular altered DNA methylation, play a crucial role in the development of chronic inflammatory diseases [87-89]. The DNA methylation profile of inflammed bladders in humans demonstrated that more than 50% of bladder inflammatory diseases are associated with significant global methylation, as determined by 5-methyl cytosine staining. Oxidative damage causes replication and transcriptional dysfunction of mitochondrial DNA. This results in a decline in mitochondrial function which, in turn leads to enhanced ROS production and further damage to mitochondrial DNA, as evidence by guanine oxidative product: 8-oxo-dG. DNA methylation is one of the main epigenetic modifications in mammals, and abnormal methylation of the CpG islands located in the promoter region of the genes leads to transcriptional silencing [90-92]. The mitochondrial DNA damage repair enzyme, *OGG1* is significantly silenced in acrolein treated bladder muscle cells associated with more mitochondrial DNA damage and formation of oxidized mitochondrial DNA trigger activation of NLRP3 inflammasome. The *OGG1* protein is the main DNA glycosylase for the repair of 8-oxodG lesions in DNA [93, 94]. However, global methylation also silenced three tested base excision repair genes *Neil1*, *Neil2,* and *Parp1*, as well as a two homologous recombination repair gene, *Rad50*, *Rad54* after six hours of acrolein treatment. This was further substantiated with reactivation of these genes when cultured in the presence of DNA de-methylating agent, 5aza-DC (Decitabine, 5-Aza-2'-deoxycytidine). When cultured smooth muscle detrusor cells were pretreated with nicotinamide, an over-the-counter health supplement, pyroptosis was reversed and the detrusor cells did not die. Nicotinamide treatment resulted in re-expression of some of the above-mentioned DNA damage repair enzymes. Nicotinamide inhibits DNA methylase activity competitively with respect to S-adenosyl methionine. IL-1β produced by the epigenetic imprinted bladder pyroptotic damaged cells stimulated proliferation of neighboring smooth muscle detrusor cells. IL-1β secreted from the damaged bladder cells activated its downstream growth factors IGF-1 in the remaining detrusor cells (**Figure 1**). These cells did not become hyperplastic when the mice were pretreated with IL-1β antagonist, Anakinra.

Means of limiting disease progression

Many cases of cystitis are self-limited, and there are no long-term consequences from the transient inflammation. This is the case for the vast majority of patients with infectious cystitis that are successfully treated with antibiotics.

The treatment of chemical cystitis from chemotherapeutics focuses on prevention and then expectant management of hemorrhagic cystitis. All patients who are to undergo infusions of cyclophosphamide or ifosphamide therapy receive pre- and post-treatment oral or intravenous 2-mercaptoethane sulfonate sodium (mesna). Mesna is filtered by the kidneys and excreted into the urine where it can directly bind and neutralize acrolein. This is accomplished by the sulfhydryl group of mesna binding to the vinyl group of acrolein. The inert compound is then rid from the body during normal voiding.

Current management of hemorrhagic cystitis caused by radiation or chemotherapeutics involves numerous interventions with degrees of efficacy. Commonly, manual irrigation of clots and continuous bladder irrigation by urinary catheterization is administered. The bladder irrigant, usually normal saline, reduces bleeding by removing urokinase, an anticoagulant substance secreted into the urine by the kidney. Additionally, discontinuation of any systemic anticoagulation is typically advised. For treatment of refractory hemorrhagic cystitis, there are several oral and intravesical agents that can be utilized. Aminocaproic acid is an oral agent that inhibits plasmin to prevent clot lysis. Intravesical agents include aluminum potassium sulfate, silver nitrate, formalin, or phenol. These agents act by causing chemical corrosion of the bladder urothelium and coagulate the tissue to stop the bleeding. There is an increasing role for hyperbaric oxygen therapy to treat refractory hemorrhagic cystitis. Oxygen therapy reduces bleeding by causing vasoconstriction, enhancing angiogenesis and granulation tissue formation, and lastly by optimizing immune function at the cellular level [95]. There are several surgical options for the treatment of refractory hemorrhagic cystitis. Cystoscopy and fulguration with electrocautery can treat

the bleeding. As a last resort once all other measures have failed, some patients require cystectomy with urinary diversion.

There are several recent clinical trials for the treatment of hemorrhagic cystitis. In 2003, NCT01561352 attempted to treat refractory hemorrhagic cystitis with activated recombinant human factor VII. The results did not demonstrate an advantage when compared to other treatment modalities. NCT01659723 is an ongoing randomized control trial to treat and determine the long-term effects on the bladder mucosa after hyperbaric oxygen therapy to treat refractory hemorrhagic cystitis. The results are not available at this time. NCT01295645 is an ongoing trial to test the efficacy of cidofovir in the treatment of BK virus-induced hemorrhagic cystitis. They estimate completion in 2018. This is currently an FDA-approved treatment for this particular disease. NCT02174536 is an ongoing randomized control trial to evaluate the efficacy of using placenta-derived decidual stromal cells for the treatment of hemorrhagic cystitis. There are currently no results at this time. The use of 5-azaDC, nicotinamide, or Anakinra discussed above could constitute further pre-clinical tests for possibility of clinical application. As all three of these agents are currently in common clinical use, implementation for hemorrhagic cystitis is feasible.

Address correspondence to: Dr. Neil A Bhowmick, Department of Medicine, Cedars-Sinai Medical Center, 8750 Beverly Blvd., Atrium 103, Los Angeles, CA 90048. Tel: 310-423-5992; Fax: 310-423-8543; E-mail: bhowmickn@cshs.org

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