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Pathophysiological aspects of cyclophosphamide and ifosfamide induced hemorrhagic cystitis; implication of reactive oxygen and nitrogen species as well as PARP activation

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Abstract

Cyclophosphamide (CP) and ifosfamide (IF) are widely used antineoplastic agents, but their side-effect of hemorrhagic cystitis (HC) is still encountered as an important problem. Acrolein is the main molecule responsible of this side-effect and mesna (2-mercaptoethane sulfonate) is the commonly used preventive agent. Mesna binds acrolein and prevent its direct contact with uroepithelium. Current knowledge provides information about the pathophysiological mechanism of HC: several transcription factors and cytokines, free radicals and non-radical reactive molecules, as well as poly(adenosine diphosphate-ribose) polymerase (PARP) activation are now known to take part in its pathogenesis. There is no doubt that HC is an inflammatory process, including when caused by CP. Thus, many cytokines such as tumor necrosis factor (TNF) and the interleukin (IL) family and transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) also play a role in its pathogenesis. When these molecular factors are taken into account, pathogenesis of CP-induced bladder toxicity can be summarized in three steps: (1) acrolein rapidly enters into the uroepithelial cells; (2) it then activates intracellular reactive oxygen species and nitric oxide production (directly or through NF-κB and AP-1) leading to peroxynitrite production; (3) finally, the increased peroxynitrite level damages lipids (lipid peroxidation), proteins (protein oxidation) and DNA (strand breaks) leading to activation of PARP, a DNA repair enzyme. DNA damage causes PARP overactivation, resulting in the depletion of oxidized nicotinamide–adenine dinucleotide and adenosine triphosphate, and consequently in necrotic cell death. For more effective prevention against HC, all pathophysiological mechanisms must be taken into consideration.

Abbreviations: AP-1, activator protein-1; CAT, catalase; CP, cyclophosphamide; eNOS, endothelial nitric oxide synthase; EPCG, epigallocatechin 3-gallate; GSH, glutathione; GSH-Px, glutathione peroxidase; HC, hemorrhagic cystitis; IF, ifosfamide; IL-1, interleukin-1; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; mesna, 2-mercaptoethane sulfonate; NAD^+, nicotinamide–adenine dinucleotide; NF-κB, nuclear factor-κB; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide anion (radical); $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; PAF, platelet-activating factor; PARP, poly(adenosine diphosphate-ribose) polymerase; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosing factor alpha

Introduction

Cyclophosphamide (CP), an oxazaphosphorine alkylating agent introduced in 1958, is widely used in the treatment of solid tumors and B-cell malignant disease, such as lymphoma, myeloma, chronic lymphocytic leukemia and Waldenstrom macroglobulinemia. Furthermore, CP and ifosfamide (IF), a synthetic analogue of CP, have had an increasing role in the treatment of non-neoplastic diseases, such as thrombocytopenic purpura, rheumatoid arthritis, systemic lupus erythematosis, nephritic syndrome, and Wegener granulomatosis, and as a conditioner before bone marrow transplantation (Levine and Richie, 1989).

The first side-effects of CP were reported by Coggins and co-workers as early as 1960. The urological side-effects, a major limiting factor in its use, vary from transient irritative voiding symptoms, including urinary frequency, dysuria, urgency, suprapubic discomfort and strangury with microhematuria, to life-threatening hemorrhagic cystitis. Bladder fibrosis, necrosis, contracture, and vesicoureteral reflux also have been reported (Coggins et al., 1960).

Later, other oxazaphosphorine alkylating agents were found to have similar effects. In early series the incidence of HC during and after treatment was reported to be as high as 68%. Mortality from uncontrolled hemorrhage has been reported to be 4% and morbidity from severe hemorrhage is extremely high (Gray et al., 1986). Hemorrhage usually occurs during or immediately after treatment, whether with short-term high or long-term low dosages. When mesna (2-mercaptoethane sulfonate) is given as prophylaxis, the incidence is decreased to approximately 5%.

The urotoxicity of these cytostatics is not based on a direct alkylating activity on the urinary system but rather on the formation of 4-hydroxy metabolites, in particular, renal excretion of acrolein, which is formed from hepatic microsomal enzymatic hydroxylation (Brock et al., 1981).

Toxicity of acrolein

Humans are exposed to acrolein in industrial, environmental, and therapeutic situations. Industrially, acrolein is mostly used as a herbicide. Environmentally, acrolein occurs naturally in foods and is formed during the combustion of organic materials. Thus, acrolein is found in all types of smoke including cigarette smoke. *In vivo*, acrolein is a metabolic product of CP and IF (Kehrer and Biswal, 2000).

In order to understand the pathophysiological mechanism of CP-induced HC, the question "How is acrolein toxic?" needs to be answered. Acrolein is the most reactive of the α,β-unsaturated aldehydes, and will rapidly bind to and deplete cellular nucleophiles such as glutathione. It can also react with some residues of proteins and with nucleophilic sites in DNA. However, this reactivity is the basis for the cytotoxicity evident in all cells exposed to high concentrations of acrolein, and monitoring of urinary acrolein concentration indicates that in humans who are admitted to hospital for treatment of solid tumors and hematological diseases it cannot reach such high concentrations (Takamoto et al., 2004). Thus, in case of CP-induced bladder damage, the toxicity of acrolein does not come from direct toxic effects.

At lower acrolein doses, other biological effects become evident. One of the most important features of acrolein is the ability to rapidly react at many cellular sites, for example, in depletion of cellular thiols or in gene activation, either directly or subsequent to effects of transcription factors such as nuclear factor-κB (NF-κB) (Horton et al., 1999) and activator protein-1 (AP-1) (Biswal et al., 2002). Furthermore, acrolein has also been identified as a product and also an initiator of lipid peroxidation (Adams and Klaidman, 1993). Alternately, or in addition, a more direct action of acrolein on various factors is possible. The direct alkylation of DNA by acrolein, while possible, seems unlikely at low doses, which would be expected to react with the abundant levels of cellular glutathione (GSH) or other nucleophiles prior

to reaching the nucleus. A lack of direct DNA damage is supported by some experimental work (Horton et al., 1997).

With CP or IF treatment, it seems possible that a high enough concentration of acrolein is present only in urine. Thus the toxicity of acrolein has generally been encountered in the urinary system. Further, mesna—the most trusted preventive agent—binds the acrolein in the bladder or the whole urinary system and does not allow it to get into the uroepithelium. If acrolein does enter the uroepithelium, it induces compounds such as reactive oxygen species. Is there any mechanism in the uroepithelium to resist acrolein? Given the uroepithelial action of acrolein, we investigated oxidative stress and the antioxidant status of the cells.

Free oxygen radicals and antioxidant defense mechanism

Reactive oxygen species (ROS) are constantly generated under physiological conditions as a consequence of aerobic metabolism. ROS include free radicals such as the superoxide ($O_2^{-\bullet}$) anion, hydroxyl radicals ($OH^{\bullet}$) and the non-radical molecule hydrogen peroxide (H_2O_2). These are particularly transient species due to their high chemical reactivity and can react with DNA, proteins, carbohydrates, and lipids in a destructive manner. The cell is endowed with an extensive antioxidant defense system to combat ROS, either directly by interception or indirectly through reversal of oxidative damage. When ROS overcome the defense systems of the cell and redox homeostasis is altered, the result is oxidative stress (Sies, 1997) (Figure 1).

Antioxidant defense mechanisms against ROS

The endogen antioxidant defense system functions to prevent oxidative damage directly by intercepting ROS before they can damage intracellular targets. It consists of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) (Sies, 1997). SOD destroys the free radical superoxide ($O_2^{-\bullet}$) by converting it to H_2O_2. The primary defense mechanisms against H_2O_2 are CAT and GSH-Px. CAT is one of the most efficient enzymes known and cannot be saturated by H_2O_2 at any concentration. GSH-Px acts through the glutathione redox cycle (Sies, 1999) (Figure 1).

Nitric oxide and the nitric-oxide synthase family

Nitric oxide (NO) is produced by a family of enzymes called nitric-oxide synthases (NOS). Constitutive expression of two NOS isoforms is responsible for a low basal level of NO synthesis in neural cells (nNOS) and in endothelial cells (eNOS). Induction of the inducible isoform (iNOS) by cytokines (TNF-α, interleukins) bacterial products (endotoxin) and chemical agents has been observed in virtually all cell types tested including macrophages, fibroblasts, chondrocytes, osteoclasts, and epithelial cells and results in the production of large amounts of NO (Moncada et al., 1991). Controversy arises from observations reporting both cytotoxic and cytoprotective effects of NO. In cases where NO was found to be cytotoxic, it was questioned whether NO exerted these effects directly or indirectly through the formation of more reactive species such as peroxynitrite ($ONOO^-$) (Szabo, 1996).

The activated "Devil Triangle" in the target cell

As both excess NO and excess $O_2^{-\bullet}$ decreases the bioavailability of $ONOO^-$, equimolar concentrations of the radicals are ideal for $ONOO^-$ formation. The $ONOO^-$ anion is in pH-dependent protonation equilibrium with peroxynitrous acid (ONOOH). Homolysis of ONOOH gives rise to formation of the highly reactive $OH^{\bullet}$ mediating molecular and tissue damage associated

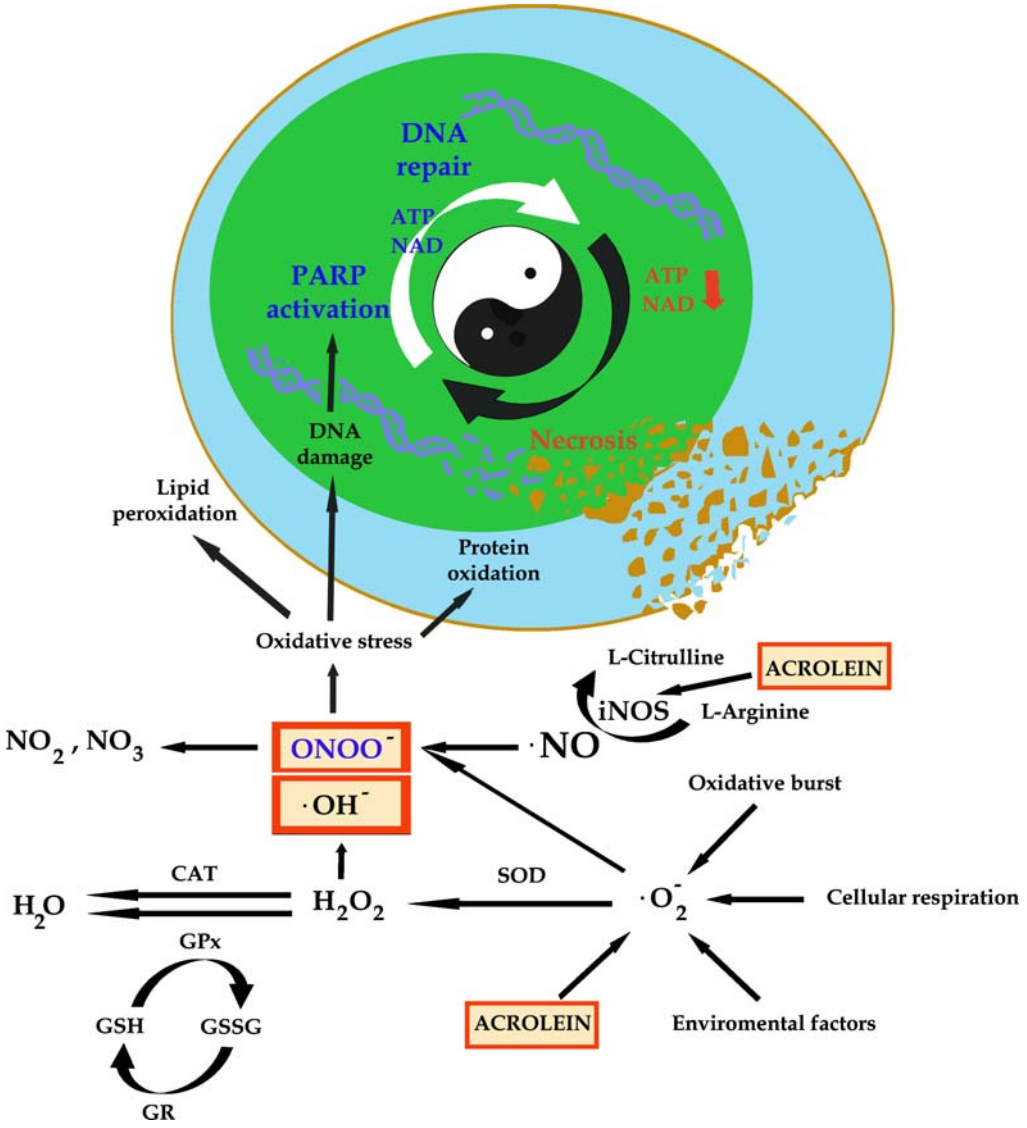

Figure 1. The activated "Devil Triangle" (NO–$O_2^{-\bullet}$–$ONOO^-$) leading to permanent cellular damage. Under normal circumstances, oxidants and antioxidant defense mechanisms are in redox homeostasis. Additional oxidants may alter the equilibrium. Note that SOD is first in enzymatic scavenging; if SOD does not work, neither GPx nor CAT will scavenge. Once acrolein has entered the uroepithelial cells, both ROS production and iNOS activation increase. Excess NO can outcompete SOD for $O_2^{-\bullet}$, resulting peroxynitrite formation. Once produced, peroxynitrite can cause lipid peroxidation, protein oxidation, and DNA damage. DNA damage then causes PARP activation, leading to cellular energy crisis.

with $ONOO^-$ production (Radi et al., 2001). $ONOO^-$ is formed when NO and $O_2^{-\bullet}$ react in a near diffusion-limited reaction. The most powerful cellular antioxidant system protecting against the harmful effects of $O_2^{-\bullet}$ is represented by SOD. However, it has been shown that NO efficiently competes with SOD for $O_2^{-\bullet}$ (Figure 1). Beckman et al. have therefore proposed that under conditions of increased NO production NO can outcompete SOD for $O_2^{-\bullet}$, resulting

in $ONOO^-$ formation (Beckman and Koppenol, 1996).

How is peroxynitrite harmful?

$ONOO^-$ is not a radical but is a stronger oxidant than its precursor radicals. It can directly react with target biomolecules via one or two-electron oxidations. Higher concentrations and the uncontrolled generation of $ONOO^-$ may result in unwanted oxidation and consecutive destruction of host cellular constituents. $ONOO^-$ may oxidize and covalently modify all major types of biomolecules. One of the most important mechanisms of cellular injury is a $ONOO^-$-dependent increase in DNA strand breakage, which triggers the activation of poly(adenosine diphosphate-ribose) polymerase (PARP), a DNA repair enzyme. DNA damage causes PARP overactivation, resulting in the depletion of oxidized nicotinamide–adenine dinucleotide (NAD^+) and adenosine triphosphate (ATP), and consequently in necrotic cell death (Virag and Szabo, 2002).

DNA single-strand breakage is an obligatory trigger for the activation of PARP. $ONOO^-$ and $OH^\bullet$ are the key pathophysiologically relevant triggers of DNA single-strand breakage (Schraufstatter et al., 1988). Moreover, nitroxyl anion, a reactive molecule derived from nitric oxide, is a potent activator of DNA single-strand breakage and PARP activation *in vitro* (Schraufstatter et al., 1988; Virag and Szabo, 2002). Subsequent studies clarified that the actual trigger of DNA single-strand breakage is $ONOO^-$, rather than NO (Szabo et al., 1996). The identification of $ONOO^-$ as an important mediator of the cellular damage in various forms of inflammation stimulated significant interest in the role of the PARP-related suicide pathway in various pathophysiological conditions. Endogenous production of $ONOO^-$ and other oxidants has been shown to lead to DNA single-strand breakage and PARP activation (Szabo, 2003).

NF-κB and cytokines involved in bladder toxicity

NF-κB is a member of the Rel protein family and resides in the cytoplasm. This factor is normally bound to a member of the family of inhibitory proteins known as inhibitor-κB (I-κB) (May and Gosh, 1997). The exposure of cells to NF-κB activators, including ROS and cytokines (e.g. TNF-α, IL-1), degrades I-κB. Activated NF-κB then is translocated to the nucleus where it is an important mediator of transcription events associated with a variety of stress conditions. The pro-inflammatory cytokine TNF-α plays an important role in diverse cellular events such as septic shock, obesity, diabetes, cardiovascular events, cancer, induction of other cytokines, cell proliferation, differentiation, necrosis, and apoptosis (Liu, 2005). In response to TNF, transcription factors such as NF-κB are activated in most types of cells and, in some cases, apoptosis or necrosis may also be induced.

Cells are often under genotoxic stress induced by both endogenous (e.g., ROS) and exogenous sources (e.g., ultraviolet radiation, ionizing radiation, DNA damaging chemicals, and acrolein). The cellular response to genotoxic stress includes damage sensing, activation of different signaling pathways, and biological consequences such as cell cycle arrest and apoptosis. Transcription factors such as NF-κB have been suggested to play critical roles in mediating cellular responses to genotoxic responses (Canman and Kastan, 1996). These transcription factors elicit various biological responses by inducing expression of their target genes. Because activation of NF-κB can have anti-apoptotic or pro-apoptotic effects, the engagement of these two pathways may be key cellular responses that modulate the outcome of cells exposed to radiation and genotoxic chemicals.

In most types of cells, inactive NF-κB is sequestered in the cytoplasm through its interaction with the inhibitory proteins. In response to various stimuli, such as TNF-α and IL-1, inhibitory

proteins of NF-κB release NF-κB and allow its translocation into the nucleus and the subsequent activation of its target genes. In case of CP-induced HC, acrolein itself, cytokines, and ROS may lead to NF-κB activation and intensification of the harmful effects of acrolein.

Possible mechanisms of CP-induced bladder damage

The first step in the pathogenesis of CP-induced bladder damage is the entry of acrolein into the uroepithelium. Then the cascade is activated as suggested below and summarized in Table 1.

First, acrolein rapidly enters into the uroepithelial cells. Second, it activates intracellular ROS and NO production (directly or through NF-κB and AP-1), leading to $ONOO^-$ production. Third, the increased $ONOO^-$ level damages lipids (lipid peroxidation), proteins (protein oxidation), and DNA (strand breaks), leading to PARP activation. Figure 2 demonstrates the proposed mechanism of acrolein-induced HC in detail.

Table 1. The proposed mechanism of acrolein-induced hemorrhagic cystitis

1. Acrolein enters rapidly into the uroepithelium because of its chemical nature.
   a. Acrolein causes increased ROS production in the bladder epithelium.
   b. Acrolein causes both directly and/or indirectly iNOS induction leading to NO overproduction.
   c. Acrolein induces some intracellular transcription factors such as NF-κB and AP-1.
   d. Activated NF-κB and AP-1 cause cytokine (TNF-α, IL-1β) gene expression, iNOS induction, and again ROS production. Thus, the production of harmful molecules (cytokines, ROS, NO) increases dramatically.
   e. Cytokines leave the uroepithelium and spread to other uroepithelial cells, detrussor smooth muscle, and bloodstream.
2. ROS and NO form peroxynitrite in both uroepithelium and detrussor smooth muscle.
3. Peroxynitrite attacks cellular macromolecules (lipids, proteins, and DNA) and causes damage.
4. Cellular and tissue integrity are broken and damage appears as edema, hemorrhage, and ulceration.

Increased ROS production in the bladder epithelium and smooth muscle

Several studies have investigated whether scavenging of ROS with antioxidants may ameliorate HC symptoms. Ternatin, a flavonoid, is popular in Brazilian folk medicine and is known to exhibit antioxidant properties. Vieira et al. showed that in CP- or IF-induced HC, substitution of the last two doses of mesna by ternatin was as effective in preventing HC as the classical protocol using three doses of mesna (Vieira et al., 2004). Other flavonoids such as quercetin and epigallocatechin 3-gallate (EGCG), also have protective effects against CP-induced bladder damage (Ozcan et al., 2005). Several antioxidants such as α-tocopherol (Yildirim et al., 2004), β-carotene (Sadir et al., 2006) and melatonin (Sener et al., 2004; Topal et al., 2005) have similar effects on cystitis symptoms. It was also shown that the antioxidants glutathione and amifostine prevented IF- and acrolein-induced HC (Batista et al., 2007).

iNOS induction leading to NO overproduction

Souza-Filho et al. first reported that NO is involved in the inflammatory events leading to HC (Souza-Filho et al., 1997). The authors found that NOS inhibitors dose-dependently inhibited the CP-induced increase in plasma protein extravasation and bladder wet weight. NOS inhibition significantly reduced the mucosal damage, hemorrhage, edema, and leukocyte infiltration in the bladders of CP-treated rats. CP markedly increased iNOS activity in the bladder with a time course similar to that of the histopathological alterations observed. Several experimental studies performed in our laboratory have also shown that NO produced by iNOS was involved in CP-induced HC (Korkmaz et al., 2003; Oter et al., 2004). Furthermore, platelet-activating factor (PAF) was found to be one of the inflammatory mediators contributing to the activation of

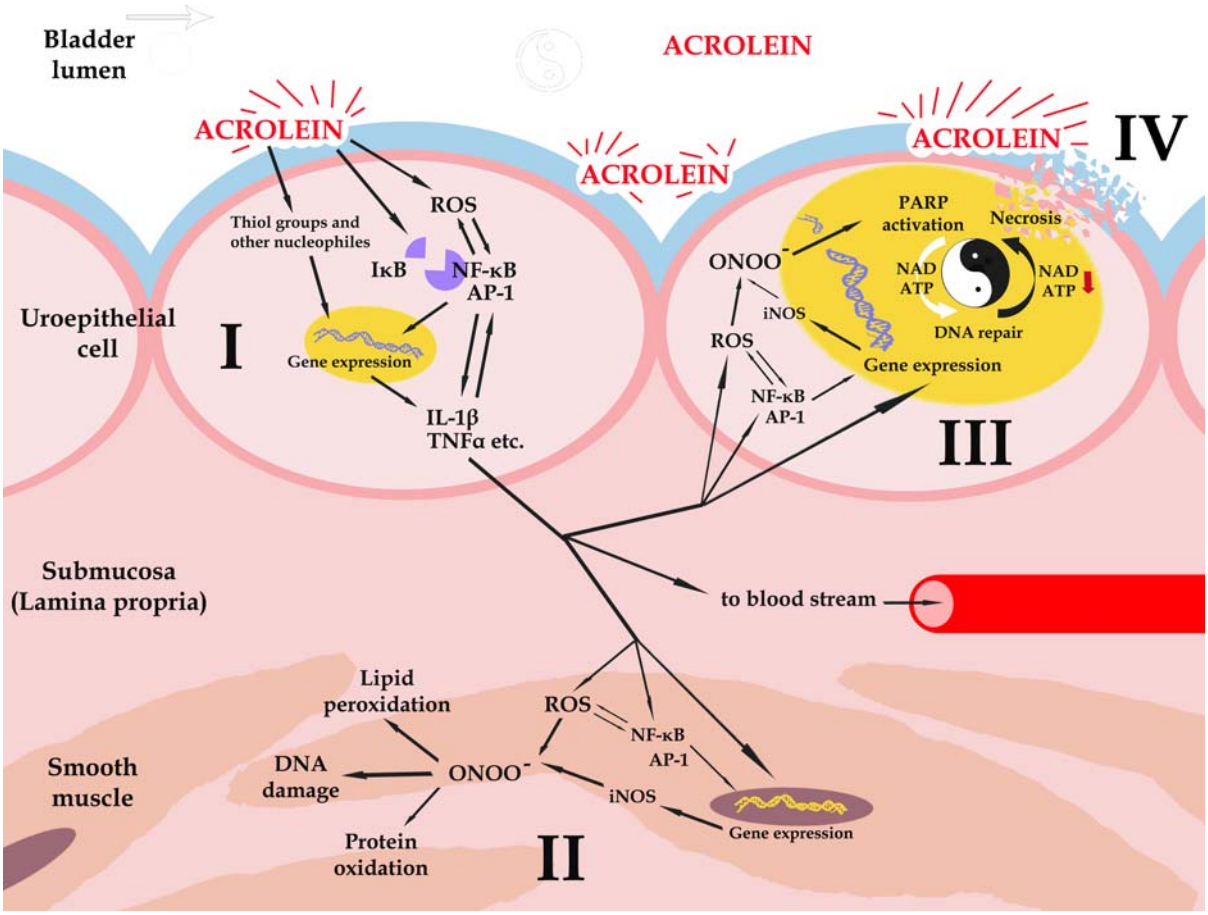

Figure 2. The overall mechanism regarding acrolein-induced HC pathogenesis. (I) Acrolein enters the uroepithelium and causes ROS production, iNOS induction, and activation of transcription factors (e.g. NF-κB and AP-1). Activated NF-κB and AP-1 cause cytokine (TNF-α, IL-1β) gene expression, iNOS induction and again ROS production. (II–III) Cytokine produced spreads out into other uroepithelial cells, the bloodstream, and detrusor smooth muscle. ROS and NO form peroxynitrite in both uroepithelium and detrusor, leading to lipid peroxidation, protein oxidation, and DNA damage. DNA damage causes PARP activation and energy crisis and eventually cellular necrosis. (IV) During necrotic cell death, the cellular content is released into the tissue, exposing neighboring cells to potentially harmful attack by intracellular proteases and other released factors.

the L-arginine–NO pathway (Souza-Filho et al., 1997). Besides PAF, other inflammatory mediators such as TNF-α and IL-1 were shown to mediate the production of NO (through iNOS induction) involved in the pathogenesis of IF- and CP-induced HC (Gomes et al., 1995; Ribeiro et al., 2002). The induction of iNOS in the urothelium appeared to depend on production of the cytokines IL-1β and TNF-α since antiserum against these cytokines reduced the inflammatory events as well as the expression of iNOS in the urothelium. This finding is supported by the fact that pentoxifylline (IL-1β inhibitor) and thalidomide (TNF-α inhibitor) reduced inflammatory events induced in the bladder by IF administration (Gomes et al., 1995). In cases where NO was found cytotoxic (e.g. CP-induced HC), it was questioned whether NO exerted its cytotoxic effects directly or indirectly through the formation of more reactive species such as $ONOO^-$.

Table 2. Drugs used experimentally in acrolein-induced cystitis

Drug	Action	Main outcome	References
Ternatin			Vieira et al. (2004)
Glutathione	Antioxidant	Histological improvement	Sener et al. (2004); Batista et al. (2007)
Amifostine			Batista et al. (2007); Kanat et al. (2006)
α-Tocopherol and β-carotene	Antioxidant	Decreased oxidative stress, histological improvement	Sadir et al. (2006); Yildirim et al. (2005)
Catechin and quercetin			Ozcan et al. (2005)
Melatonin	Antioxidant, iNOS inhibitor, peroxynitrite scavenger	Decreased iNOS activation, decreased GSH depletion, histological improvement	Sener et al. (2004); Topal et al. (2005); Sadir et al. (2006)
Thalidomide and pentoxyfilline	TNF-α and IL-1β inhibitors	iNOS inhibition, histological improvement	Gomes et al. (1995); Ribeiro et al. (2002)
L-NAME	Non-selective NOS inhibitor	NOS inhibition, histological improvement	Souza-Filho et al. (1997); Korkmaz et al. (2003)
S-Methylthiourea			Korkmaz et al. (2003); Oter et al. (2004)
Aminoguanidine	Selective iNOS inhibitor	iNOS inhibition, histological improvement	Korkmaz et al. (2005)
1400W			Korkmaz et al., unpublished
Ebselen	Peroxynitrite scavenger	Histological improvement	Korkmaz et al. (2005)
3-Aminobenzamide	PARP inhibitor	Histological improvement	Korkmaz et al., unpublished

Peroxynitrite formation

A preliminary study in our laboratory showed that $ONOO^-$ may contribute to the pathogenesis of bladder damage caused by CP (Korkmaz et al., 2005). In this work, acrolein was not blocked with mesna nor was iNOS inhibited with a NOS inhibitor; only ebselen was used to scavenge the $ONOO^-$ produced. The results were promising and bladder damage was clearly decreased. The results of this study suggest that scavenging of $ONOO^-$ and inhibition of iNOS have similar protective effects. Thus, $ONOO^-$ may also be involved in bladder damage caused by CP.

Macromolecular (lipids, proteins, and DNA) damage leading to cellular necrosis

Increased malondialdehyde (MDA) levels, an indicator of lipid peroxidation, have been observed in several studies (Korkmaz et al., 2005; Sener et al., 2004; Topal et al., 2005). This increase indicates that lipid peroxidation is present in damaged bladder tissue. Both ROS and $ONOO^-$ may cause lipid peroxidation and scavenging them could lead to decreased MDA levels in bladder tissue. Melatonin is known as an antioxidant but it also has iNOS-inhibitory and $ONOO^-$-scavenging properties. Recently, Topal et al. has shown that melatonin may ameliorate bladder damage and decrease MDA levels, possibly through scavenging of ROS and $ONOO^-$ and inhibition of iNOS activity in bladder tissue (Topal et al., 2005). Sener et al. also showed that melatonin was capable of reducing IF-induced nephrotic and bladder toxicity (Sener et al., 2004). In this work, melatonin acted as antioxidant and anti-inflammatory and enhanced cell ATPase activity. Furthermore, PARP activation caused by DNA damage also involves CP-induced HC leading to cellular necrosis (unpublished data).

Table 2 summarizes the outcome of experimental studies using several drugs against acrolein cystitis.

Conclusions

Acrolein is the main compound responsible for CP- and IF-induced cystitis and mesna is the agent commonly used to protect against this side-effect. Nevertheless, our current knowledge led us to seek more information about the pathophysiological mechanism of HC in detail: many cytokines, free radicals and non-radical reactive molecules, as well as PARP activation, are now known to take part in the pathogenesis of CP- and IF-induced HC. In addition, there is no doubt that HC is an inflammatory process, including when caused by CP or IF. Thus, many cytokines play a role in its pathogenesis, such as the TNF and IL families. Cytokines may trigger activation of transcription factors such as NF-κB and AP-1, leading to further events associated with a variety of stress conditions. Thus, we suggest that for more effective protection against CP- or IF-cystitis, all pathophysiological mechanisms must be taken into consideration. Possible alternative preventive methods may be discussed in a separate article.

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