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BASIC AND TRANSLATIONAL—PANCREAS

Intracellular Hmgb1 Inhibits Inflammatory Nucleosome Release and Limits Acute Pancreatitis in Mice

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BACKGROUND & AIMS: High mobility group box 1 (HMGB1) is an abundant protein that regulates chromosome architecture and also functions as a damage-associated molecular pattern molecule. Little is known about its intracellular roles in response to tissue injury or during subsequent local and systemic inflammatory responses. We investigated the function of Hmgb1 in mice after induction of acute pancreatitis. **METHODS:** We utilized a Cre/LoxP system to create mice with pancreas-specific disruption in *Hmbg1* (Pdx1-Cre; HMGB1$^{flox/flox}$ mice). Acute pancreatitis was induced in these mice (HMGB1$^{flox/flox}$ mice served as controls) after injection of L-arginine or cerulein. Pancreatic tissues and acinar cells were collected and analyzed by histologic, immunoblot, and immunohistochemical analyses. **RESULTS:** After injection of L-arginine or cerulein, Pdx1-Cre; HMGB1$^{flox/flox}$ mice developed acute pancreatitis more rapidly than controls, with increased mortality. Pancreatic tissues of these mice also had higher levels of serum amylase, acinar cell death, leukocyte infiltration, and interstitial edema than controls. Pancreatic tissues and acinar cells collected from the Pdx1-Cre; HMGB1$^{flox/flox}$ mice after L-arginine or cerulein injection demonstrated nuclear catastrophe with greater nucleosome release when compared with controls, along with increased phosphorylation/activation of RELA nuclear factor κB, degradation of inhibitor of κB, and phosphorylation of mitogen-activated protein kinase. Inhibitors of reactive oxygen species (N-acetyl-L-cysteine) blocked L-arginine–induced DNA damage, necrosis, apoptosis, release of nucleosomes, and activation of nuclear factor κB in pancreatic tissues and acinar cells from Pdx1-Cre; HMGB1$^{flox/flox}$ and control mice. Exogenous genomic DNA and recombinant histone H3 proteins significantly induced release of HMGB1 from mouse macrophages; administration of antibodies against H3 to mice reduced serum levels of HMGB1 and increased survival after L-arginine injection. **CONCLUSIONS:** In 2 mouse models of acute pancreatitis, intracellular HMGB1 appeared to prevent nuclear catastrophe and release of inflammatory nucleosomes to block inflammation. These findings indicate a role for the innate immune response in tissue damage.

Keywords: DNA Damage; Pancreatitis; Oxidative Stress; Nuclear Factor κB.

Innate immune cells orchestrate both the physiologic and pathologic inflammatory immune response after engagement by pattern-recognition receptors, including pathogen-associated molecular pattern or damage-associated molecular pattern (DAMPs) molecules.[1–3] Many DAMPs are derived from the nucleus, and are collectively termed *nuclear DAMPs* (nDAMPs). Examples of nDAMPs include high mobility group box 1 (HMGB1)[4,5] and components of the nucleosome (eg, DNA[6] and histones[7]). Within the nucleus, HMGB1 maintains chromosomal structure and regulates DNA damage responses.[8] Under a variety of stressful situations, however, HMGB1 translocates to the cytosol, where it sustains autophagy, and then is released into the extracellular space. There, it coordinates inflammation, immunity, and other local cellular processes.[9] Pathologic nDAMP release is increasingly being recognized as an etiology for a variety of human inflammatory diseases and represents an emergent target for therapy.[10,11] A better understanding of the intricate mechanisms underlying the release and response to nDAMPs will aid in this effort.

Abbreviations used in this paper: **AP, acute pancreatitis; HMGB1, high mobility group box 1; IκB, inhibitor of κB; IKK, inhibitor of κB kinase; IL, interleukin; IP, intraperitoneal; MPO, myeloperoxidase; NAC, N-acetyl-L-cysteine; nDAMP, nuclear damage-associated molecular pattern; NFκB, nuclear factor κB; pMAPK, phosphorylated mitogen-activated protein kinase; ROS, reactive oxygen species.**

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Acute pancreatitis (AP) is a poorly understood inflammatory disease, responsible for significant human morbidity and mortality each year worldwide.[12] In patients and animals with AP, serum levels of HMGB1 are significantly increased and positively correlate with the severity of the disease.[13–15] Inhibiting the release or cytokine activity of HMGB1 confers protection against experimental AP.[16–18] The precise role of HMGB1 during AP-induced tissue injury and subsequent local and systemic inflammation is poorly understood. Here, we show, in contrast to neutralization of HMGB1 in the serum, that conditional knockout of HMGB1 in the pancreas rendered mice dramatically more susceptible to experimental AP. Deficiency of endogenous pancreatic HMGB1 resulted in accelerated tissue injury and lethality. This enhanced severity was associated with increased nuclear catastrophe and nucleosome (histone and DNA) release and increased recruitment and activation of inflammatory cells. Interestingly, the subsequent activation of innate immune effectors was associated with increased HMGB1 release into the circulation. Neutralizing extracellular histone and/or HMGB1 conferred protection against AP in conditional pancreas-specific HMGB1 knockout mice. Therefore, intracellular nuclear HMGB1 serves as a previously underappreciated negative regulator of inflammation-limiting nuclear catastrophe after injury with resultant release of other nDAMPs. This work improves our understanding of the complex role of HMGB1 in protecting against cellular injury and subsequent activation of innate immune responses after sterile tissue damage.

Materials and Methods

Reagents

The antibodies to p-RELA, RELA (also known as p65), phosphorylated inhibitor of κB (IκB), IκB, IκB kinase (IKK)α, IKKβ, IKKγ, p100, RELB, phosphorylated mitogen-activated protein kinase (pMAPK) 14, MAPK14 (also known as p38), pMAPK8, MAPK8 (also known as JNK), pMAPK1, MAPK1 (also known as extracellular signal-regulated kinase), H3, H4, γ-H2AX, cleaved caspase 3, cleaved poly(ADP-ribose) polymerase, and actin were obtained from Cell Signaling Technology (Danvers, MA). The antibody to HMGB1 came from Novus (Littleton, CO). The antibody to 4-hydroxy-2-nonenal came from Alpha Diagnostic (San Antonio, TX). Mouse genomic DNA and DNase I came from New England Biolabs (Ipswich, MA). Recombinant calf thymus histone H3 came from Roche (Madison, WI). Recombinant HMGB1 proteins were generated as described previously.[19] Contaminating endotoxin was removed from protein by Triton X-114 extraction. Mouse HMGB1 neutralizing antibody (IgG2B) was generated as described previously.[20] Rabbit H3 neutralizing antibody came from Abcam (Cambridge, MA). Control mouse IgG2B and rabbit IgG came from R&D Systems (Minneapolis, MN). Unless otherwise stated, all other reagents were purchased from Sigma (St Louis, MO).

Mouse Strains

The protocol for animal use was reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Pancreatic specific HMGB1 knockout mice were prepared and bred in our laboratory by crossing floxed HMGB1 (*HMGB1$^{flox/flox}$*) and *Pdx1-Cre* transgenic mice. In the floxed mice, exons 2 and 3 of HMGB1 gene were flanked by 2 lox/p sites that enable the recombination of the HMGB1 loci in the presence of cre recombinase (Supplementary Figure 1*A*). *Pdx1-Cre* transgenic mice on C57BL/6 background were obtained from the MMHCC/NCI Mouse Repository. As both mouse strains were on the B6 background, all progeny used in our study were generated with B6 mice with pure genetic backgrounds. Specifically, F1 offspring of an initial *Pdx1-Cre* $\times$ *HMGB1$^{flox/flox}$* intercross mating were genotyped to confirm the presence of both the *Pdx1-Cre* and floxed HMGB1 alleles by standard polymerase chain reaction. To generate mice homozygous for the floxed HMGB1 allele, F1 generation mice were backcrossed with the *HMGB1$^{flox/flox}$* line (Supplementary Figure 1*B*). *Pdx1-Cre*; *HMGB1$^{flox/flox}$* (termed *CH mice*) offspring were identified by genotyping for the presence of both the *Pdx1-Cre* and floxed HMGB1 alleles and the absence of the wild-type HMGB1 allele. Recombination/deletion of the HMGB1 gene in pancreatic cells was confirmed by genotyping analysis (Supplementary Figure 1*C*). The presence of the *Pdx1-Cre* transgene was detected by polymerase chain reaction amplification with primers 5′-CTGGACTACATCTTGAGTTGC-3′ and 5′-GGTGTACGGTCAGTAAATTTG-3′ ; the identification of floxed (700 bp) and wild-type (635 bp) HMGB1 with 5′-TGATGCGAACACGGCGTGCTCTA′ and 5′-GCACAAAGAATGCA TATGAGGAC-3′. In parallel, HMGB1 level in pancreatic tissue or extracts from the pancreatic acinar cells of CH mice was assayed by immunofluorescent staining (Supplementary Figure 1*D*) and Western blot (Supplementary Figure 1*E*), respectively.

Experimental Animal Models of AP

For L-arginine-induced pancreatitis, a sterile solution of L-arginine hydrochloride (8%; Sigma) was prepared in normal saline and the pH was adjusted to 7.0. Mice received 2 hourly intraperitoneal (IP) injections of L-arginine (4 g/kg), and controls were administered saline IP as a control as described previously.[21] For cerulein-induced pancreatitis, mice received 7 hourly IP injections of 50 μg/kg cerulein (Sigma) in sterile saline, while controls were given saline as described previously.[22] Animals were sacrificed at the indicated time by CO_2 asphyxia, and a blood sample and tissue were collected. Blood samples were collected in heparinized syringes and centrifuged at 10,000*g* for 10 minutes at 4°C. After centrifugation, the plasma was aspirated and used for measurement of amylase, lactate dehydrogenase, nucleosomes, HMGB1, and other cytokines by enzyme-linked immunosorbent assay. Tissue samples were collected, snap frozen in liquid nitrogen, and stored at −80°C for analysis of myeloperoxidase (MPO) activity. Formalin-fixed pancreas samples were processed, and 5-μm thick paraffin sections were stained with H&E for histological analysis.

Full Methods and any associated references are available in the Supplementary Material.

Results

Pancreas-Derived HMGB1 Protects Against Experimental AP

Because global HMGB1 knockout mice die shortly after birth,[23] we generated transgenic mice with conditional