Systemic histone release disrupts plasmalemma and contributes to necrosis in acute pancreatitis

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ARTICLE INFO

Article history:
Received 24 June 2017
Received in revised form 1 October 2017
Accepted 6 October 2017
Available online 7 October 2017

Keywords:
Extracellular histones
Nucleosomes
Neutrophil extracellular traps
Acute pancreatitis
Pancreatic acinar cells
Necrosis

ABSTRACT

Background: Clinical and experimental acute pancreatitis feature histone release within the pancreas from innate immune cells and acinar cell necrosis. In this study, we aimed to detail the source of circulating histones and assess their role in the pathogenesis of acute pancreatitis.

Methods: Circulating nucleosomes were measured in patient plasma, taken within 24 and 48 h of onset of acute pancreatitis and correlated with clinical outcomes. Using caerulein hyperstimulation, circulating histones were measured in portal, systemic venous and systemic arterial circulation in mice, and the effects of systemic administration of histones in this model were assessed. The sites of actions of circulating histones were assessed by administration of FITC-labelled histones. The effects of histones on isolated pancreatic acinar cells were further assessed by measuring acinar cell death and calcium permeability in vitro.

Results: Cell-free histones were confirmed to be abundant in human acute pancreatitis and found to derive from pancreatitis-associated liver injury in a rodent model of the disease. Fluorescein isothiocyanate-labelled histones administered systemically targeted the pancreas and exacerbated injury in experimental acute pancreatitis. Histones induce charge- and concentration-dependent plasmalemma leakage and necrosis in isolated pancreatic acinar cells, independent of extracellular calcium.

Conclusion: We conclude that histones released systemically in acute pancreatitis concentrate within the inflamed pancreas and exacerbate injury. Circulating histones may provide meaningful biomarkers and targets for therapy in clinical acute pancreatitis.

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Introduction

Acute pancreatitis (AP) is one of the commonest gastrointestinal causes of hospital admission [1,2] with rising incidence and significant socio-economic cost [3,4]. Severe disease features persistent organ failure, often with profound pancreatic injury [5]. However, where pancreatic necrosis was once thought as causative of organ failure, it is accepted that necrosis occurs both with and without distal organ injury and it is the systemic insult that most contributes to mortality [6–8]. Experimental models demonstrate causal relationships between the innate immune system, pancreatic and systemic injury [9], suggestive of immune feedback exacerbating end-organ damage [10].

A potential mechanism by which the innate immune system exacerbates pancreatic injury is the generation and release of neutrophil extracellular traps (NETs). Neutrophils release NETs, a primary structural and functional component of which are histones [11–13], in a process termed NETosis [13]. Histones are nuclear chaperone proteins that are highly conserved across species [14].

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with microbicidal properties [15] and therefore considered an evolutionarily ancient component of the innate immune system. Histones furthermore act as damage-associated molecular patterns (DAMPs) via toll-like receptors, stimulating the NLRP3 inflammasome or inducing calcium influx into target cells by an unknown mechanism [16,17]. The ability to generate calcium influx into cells is of particular interest, as calcium overload is a critical pathway towards acinar cell necrosis in acute pancreatitis [18,19].

NETosis has recently been demonstrated to contribute to disease severity in an experimental model of acute pancreatitis [11,20]. Furthermore, the concentration of circulating histones correlates with severity of experimental pancreatitis [21] and has most recently been shown to be predictive of organ failure in human pancreatitis [22]. This is consistent with a hypothesis that histones are released either passively from necrotic pancreatic acinar cells [23] or actively through pro-inflammatory NETosis. In this work, we aim to determine the primary source of histones in circulation and detail the mechanisms by which they contribute to pancreatic acinar cell injury, better understanding of which will allow design of novel therapies.

Materials and methods

Patient samples

Patients with acute pancreatitis included in the National Institute of Health Research Liverpool Pancreas Biomedical Research Unit Acute Pancreatitis Biobank were selected at random and plasma samples obtained as approved by the regional ethics committee (REC 10/H1308/31). All adult (18–99 years of age) patients attending the Royal Liverpool University Hospital with a diagnosis of acute pancreatitis of any aetiology (amylase >450U/L, typical pain and/or pancreatic inflammation on cross-sectional imaging) were eligible for inclusion to the biobank. Patients who were unable to consent (e.g. unconscious), had a history of recurrent acute or chronic pancreatitis or a history of pancreatic surgery or malignancy were excluded. Samples were collected prospectively within 24 h of admission from consenting patients who had presented within 72 h of onset of pain, together with clinical data that allowed severity stratification according to the 2012 Revised Atlanta Classification [5] after discharge. All samples were processed within 30 min of blood sampling and stored at −80 °C. Collection, processing, storage, monitoring and usage of samples followed pre-defined standard operating procedures and Good Clinical Practice.

Experimental animals

All animal studies were ethically reviewed and conducted as per the UK Animals (Scientific Procedures) Act 1986 under a project license approved by the UK Home Office (PPL 70/8109). Male C57BL/6 mice (age 8–10 weeks) were purchased from Charles River UK Ltd (Margate, Kent, UK), housed in a pathogen-free unit with 12h light–dark cycles and had free access to standard lab chow and water.

Reagents

Digitonin was from Calbiochem (Manchester, UK). BCA protein assay was from Thermo (Rockford, USA). Anti-histone H3 antibody was from Abcam (Rabbit monoclonal, 1:00 dilution; Abcam, Cambridge, UK). Calf-thymus histones, propidium iodide (PI), poly-glutamic acid (PGA), caerulein, acetic anhydride, protease inhibitors, phosphate-buffered saline (PBS) and other chemicals were from Sigma-Aldrich (Gillingham, UK) of highest quality available. Non-specific polyacetylation of histones was achieved by addition of a molar excess of acetic anhydride, similar to established protocols [24]. Histones were recovered by solvent evaporation in a fume cabinet and resuspended in PBS prior to use. For some experiments histones were conjugated with fluorescein isothiocyanate (FITC) and passed through an ion exchange column to remove excess dye according to established procedures [25].

In vitro assays

Murine acinar cells were freshly isolated as previously described [26–28]. Cell death assays were performed with minor modifications of previous protocols [29,30]. In brief, freshly isolated pancreatic acinar cells were suspended in PBS in the presence of PI (2 μl) with or without PGA (50 μg/ml) and seeded on a 96 well plate in a total volume of 200 μl per well. Signal was recorded every minute (Ex 540 nm/Em 620 nm) for 150 min using a BMG POLARstar Omega Microplate Reader (Imgen Technologies, New York, USA). Histones (50, 100 or 200 μg/ml) or digitonin (600 μM) was added after 30 min of establishing a stable baseline. Percentage cell death in each experimental well was calculated as a proportion of maximum fluorescence in digitonin wells. Confocal images of isolated acinar cells were taken using a Zeiss LSM 710 (ZEISS Microscopy, Cambridge, UK) inverted confocal microscope with a 40x objective.

Cellular Ca2+ measurements

Murine acinar cells were loaded and incubated with fura-2 (5 μM) as previously described [31]. Cells were visualised using a Till Photonics imaging system (Till Photonics GmbH, Germany), exciting at 340 and 380 nm (and in selected experiments at 360 nm) and emission collected using a 510 nm narrow band-pass filter. Data for each excitation wavelength as well as the ratio of 340 vs 380 excitation were collected.

In vivo experiments

Mild oedematous acute pancreatitis was induced by 4 hourly [21] and necrotizing acute pancreatitis by 12 hourly [21,32] intraperitoneal injections of caerulein (50 μg/kg). FITC-tagged histones (20 mg/kg) were administered via the tail vein immediately following the last caerulein injection. Animals were sacrificed 6 h following administration of the first caerulein injection. Organs were harvested, washed in PBS and briefly dried on sterile gauze. Organs were imaged using an IVIS Spectrum preclinical imaging system (Perkin Elmer, Waltham, MA, USA) utilizing the epifluorescence function collecting signal for 8 s. In selected experiments, calf-thymus histones (20 mg/kg) or PBS (200 μl) was administered in the same way as above. Animals were sacrificed and tissues harvested for further analysis 12 h after the first caerulein injection. For portal, central venous and arterial blood sampling animals received an overdose of pentobarbital, abdominal and thoracic cavities where opened and plasma samples taken into EDTA syringes containing 1%w/v heparin from portal vein, thoracic inferior vena cava and left ventricle. Histone quantification was performed by Western blot and densitometry performed in ImageJ [33].

Pancreatic myeloperoxidase activity

Pancreatic myeloperoxidase activity was determined as previously described [34] and protein concentration measured by a standard BCA protein assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, UK) as per the manufacturer's instructions.
Pancreatic histopathology

Pancreatic tissue was fixed in 10% formalin, embedded in paraffin and stained (haematoxylin and eosin). Histological scoring was performed on 10 random fields (x200) by two experienced, independent investigators blinded to the experimental groups. Scores (0–3) were given for each of oedema, inflammatory cell infiltration and acinar cell necrosis as described [32].

Statistical analysis

All analyses were performed in GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla, CA, USA). Data are presented as mean ± SEM. The differences between groups were compared using two-way ANOVA followed by Tukey’s multiple comparisons test. P value < 0.05 was considered significant.

Results

Histone concentration in circulation correlates with disease severity in patients.

In accordance with our hypothesis and in agreement with data published by our group using alternative methods [22], we demonstrated that circulating histone concentrations (measured as DNA-histone complexes) correlate with AP severity in plasma samples from 50 patients (mild and/or moderate, n = 36; severe, n = 14) taken on admission to hospital. Using the revised Atlanta classification (RAC) of AP [7], levels of circulating, cell-free nucleosomes were significantly higher in severe compared to mild/moderate disease (Fig. 1A, P < 0.0001), in keeping with what is known about experimental AP [1]. ROC curve analysis revealed 0.87 accuracy (Fig. 1B, P = 0.001) in discrimination between mild/moderate and severe AP on admission, highlighting the potential clinical utility of measuring circulating nucleosome levels in predicting severe AP.

Circulating histones derive primarily from the liver and concentrate in the pancreas in AP.

We postulated that the primary source of circulating histones in AP would be either necrotic pancreatic acinar cells or recruited innate immune cells within the pancreas, which have been shown to release histones as part of neutrophil extracellular trap complexes locally in the context of AP [3]. We induced necrotic AP by 12 hourly injections of caerulein, collected plasma from three different sites of each experimental animal: portal vein, thoracic inferior vena cava and left ventricle, and measured relative concentrations of histone H3 by Western blot (Fig. 1C). Histone H3 levels were significantly elevated in central venous blood (Fig. 1D) over both portal and arterial blood. Levels of alanine aminotransferase (ALT) signified liver injury and histological scoring confirmed their effects on cell integrity. A concentration-dependent increase of PI fluorescence was observed using histone concentrations relevant to experimental AP models [21] (0, 50, 100 and 200 µg/ml), with 200 µg/ml causing necrosis of almost all cells within 60 min (Fig. 1A). Histone-induced necrotic cell death pathway activation was inhibited by polyglutamic acid, a biologically inert, negatively charged polypeptide of similar molecular weight with high charge density (Fig. 1B). Poly-acetylation of histones with acetic anhydride had a similar effect (Fig. 1C). Application of FITC-labelled histones resulted in fluorescence exclusively at the acinar cell membrane persisting until membrane integrity was lost (Fig. 1D), consistent with binding of strongly positive histones to negatively charged membrane phospholipids [36].

Circulating histones exacerbate pancreatic necrosis in vivo

Given that levels of circulating histones correlate with disease severity and that histones accumulate in the inflamed pancreas in vivo, we hypothesised circulating histones could exacerbate pancreatic injury in AP, as seen in ischaemia-reperfusion liver injury [35]. We administered histones (20 mg/kg) intravenously via the tail veins of C56Bl6j mice following either 4 hourly injections of caerulein or saline and measured parameters of pancreatic injury 12 h after the first caerulein injection. Histones alone did not raise serum amylase levels or pancreatic myeloperoxidase activity beyond control, but administration of histones in the context of mild caerulein-induced AP increased pancreatic inflammation as measured by myeloperoxidase activity above caerulein alone (Fig. 2C and D). Similarly, histones markedly increased pancreatic inflammatory cell infiltration and acinar cell necrosis in caerulein-induced AP (Fig. 2E), confirmed by blinded histopathological scores (Fig. 2F–H).

Histone toxicity is dose- and charge-dependent

Freshly isolated murine pancreatic acinar cells were treated with histones in the presence of propidium iodide (PI) to interrogate their effects on cell integrity. A concentration-dependent increase of PI fluorescence was observed using histone concentrations relevant to experimental AP models [21] (0, 50, 100 and 200 µg/ml), with 200 µg/ml causing necrosis of almost all cells within 60 min (Fig. 3A). Histone-induced necrotic cell death pathway activation was inhibited by polyglutamic acid, a biologically inert, negatively charged polypeptide of similar molecular weight with high charge density (Fig. 3B). Poly-acetylation of histones with acetic anhydride had a similar effect (Fig. 3C). Application of FITC-labelled histones resulted in fluorescence exclusively at the acinar cell membrane persisting until membrane integrity was lost (Fig. 3D), consistent with binding of strongly positive histones to negatively charged membrane phospholipids [36].

Disruption of plasmalemna is main mechanism of histone toxicity

As calcium overload is critical in pancreatic acinar cell injury and there is uncertainty as to the role and mechanism of intracellular Ca2+ changes in histone-mediated cellular injury [12], we measured intracellular Ca2+ changes in fura-2 loaded pancreatic acinar cells treated with histones. Two types of response were observed (Fig. 3E and F), sometimes in the same cell: fluorescence elevations at 340 nm excitation mirrored by opposing falls at 380 nm, indicative of true Ca2+ elevations at 340 nm excitation mirrored by opposing falls at 380 nm, indicative of true Ca2+ elevations (Fig. 3G). The signal recorded at 360 nm was stable until cell permeabilization occurred, when the signal dropped to a new baseline that indicated loss of dye through cell permeabilization. When repeated in Ca2+–free solution, similar falls were seen without preceding elevations, confirmed by recordings with excitation at 360 nm, close to the Ca2+–independent isosbestic point of fura-2 (Supp. Fig. 2A). The signal recorded at 360 nm was stable until cell permeabilization occurred, when the signal dropped to a new baseline (Supp. Fig. 2B). Higher histone concentrations led to greater and more rapid dye loss (Supp. Fig. 2C). Together, these data indicate that histones permeabilize cells non-specifically to small molecules in a Ca2+–independent manner. This was confirmed measuring PI signals in response to increasing concentrations of histones in Ca2+–free solution supplemented with Ca2+ chelator (0.5% EGTA) or supra-maximal extracellular Ca2+ (5 mM). There were no differences in histone-related cell permeability in any of these groups compared to standard buffer (1.2 mM Ca2+ HEPES buffer; Fig. 3G). This finding does not exclude the possibility that histones participate in Ca2+–dependent signalling pathways observed by others at...
Fig. 1. Circulating nucleosomes correlate with human AP severity and extracellular histones induce plasmalemma leakage and cell death. (A) Admission plasma nucleosome levels in patients with AP (median ± IQR; RAC mild/moderate n = 36; severe n = 13); (B) ROC curve of circulating nucleosomes and CRP in predicting severe disease; (C) Semi-quantitative Western Blot of histone H3 from plasma of mice with severe AP (12 hourly i.p. caerulein 50 μg/kg injections). The first three lanes contain recombinant histone H3, the next six are biological repeats. Matched portal venous, central venous and arterial samples are from the same animal; (D) Relative densities of histone H3 bands from murine plasma; (E) Plasma alanine aminotransferase levels in mice with severe AP and saline control; (F) Representative Haematoxylin/Eosin micrograph of livers of mice with severe AP and saline controls.
Fig. 2. Systemically administered histones aggregate within the inflamed pancreas to exacerbate necrosis. (A) Representative epifluorescent image of pancreas and spleen of animals with mild AP injected with FITC-labelled histones or saline 6 h following the first caerulein injection; (B) Maximum pancreatic fluorescence at 6 h in AP and control animals injected with FITC-histones or saline; (C) Serum amylase and (D) pancreatic myeloperoxidase 12 h following first caerulein injection; (E) Representative H&E image of pancreas (×400 magnification) and histopathological (F) edema, (G) inflammatory cell infiltration and (H) necrosis scores. All data mean ± SEM unless otherwise indicated from 3 to 6 independent experiments. *P < 0.05 vs saline alone or histones alone groups, **P < 0.01 vs all other groups, #P < 0.05 vs caerulein with saline group.
lower histone concentrations and in other cell types [37], however at the clinically relevant concentrations used in our experiment disruption of the plasmalemma appeared to be the predominant mechanism of toxicity.

**Discussion**

Mechanisms of innate immunity and inflammation contribute greatly to organ injury and mortality in acute pancreatitis and this work advances understanding of some of the pathways involved, summarised in Fig. 4. We demonstrate correlation between circulating histone concentration and severity of AP, in agreement with data from experimental models [21] and patients [38] alike. We furthermore demonstrate an early rise in histone concentration within 24 h of disease onset in patients with AP.

Current understanding led us to hypothesize that the source of histones in circulation was a combination of pancreatic cellular necrosis and intra-pancreatic extracellular trap release by invading innate immune cells [11]. This would mean the highest measurable histone concentration in any given animal with acute pancreatitis should be the first common venous drainage channel – the portal vein. We clearly demonstrate peak histone concentrations in the post-hepatic vena cava, concluding that the liver is the predominant source of histones in circulation. While we further demonstrate elevations in ALT and structural hepatocyte damage in poorly oxygenated zone 3, it is worth noting that the role of ALT in
human acute pancreatitis is less clear, as the two commonest causes of pancreatitis (gallstones and ethanol) can independently affect ALT levels. Apoptosis of lymphocytes [39] in systemic circulation has also been postulated as primary cause of the rise in circulating histones in AP21 or sepsis [40]. The relatively low histone concentrations in arterial blood, however, adds further support to the liver as primary source in our model and indeed allows us to hypothesize that the pulmonary circulation acts as a filter for circulating histones. It is possible that rather than hepatocyte injury, resident Kupffer cells or peritoneal macrophages, recruited to the liver in inflammation [41,42], contribute to the release of histones in response to portal vein DAMPs as previously shown in vitro [43]. This interpretation would be supported by data showing reduced liver injury in experimental AP following Kupffer cell depletion [44] as well as reduced lung injury seen in AP with Kupffer cell inhibition [45]. As one of the earlier descriptions of the role of hepatic NETs was to limit systemic spread of microorganisms in sepsis [46] and bacterial translocation resulting from intestinal barrier failure is a hallmark of human and experimental acute pancreatitis [47,48], portal sepsis may be the principle determinant of hepatic histone release. This hypothesis would provide a mechanistic link between pancreatitis severity and hepatic NET/histone release, and a potential explanation how pancreatic infection could contribute to disease severity [49]. The use of only a single experimental model of acute pancreatitis is an obvious limitation when making conclusions about the source of histones in acute pancreatitis. Nevertheless, we have previously demonstrated similar patterns of extracellular histones in the systemic circulation using several experimental models [21] as well as in pancreatitis patients [22].

Irrespective of the primary source of circulating histones, we needed to investigate the effect of systemic administration of histones on the pancreas. Our data confirms that histones administered via the tail vein of a mouse can not only concentrate within the inflamed pancreas, but exacerbate organ injury. Histones have been demonstrated to adhere to membranes of many cell types as well as artificial bilayers and a previous report on organ distribution of systemically administered FITC-labelled histones demonstrated targeting of the lung [25]. That study, however, used more than double the histone concentration (45 mg/kg) in the context of an experimental sepsis model, supporting our findings that histones only concentrate within an inflamed pancreas. Extravasation of net-positively charged histones is likely facilitated by interaction with and exposure to the extracellular matrix and net-negatively charge proteoglycans such as heparan sulfate. Given that histones themselves exert antimicrobial activity and that infection of necrosis significantly increases mortality in necrotizing pancreatitis [49–51], this mechanism may even offer some survival benefit at the cost of exacerbating disease in the short term.

Our in vitro work documents the charge dependent interaction between histones and pancreatic acinar cells and the predominant mechanism of acinar cell death seen in our experimental set-up using disease-relevant histone concentrations [21] is membrane disruption. Histones and histone fragments have been shown to form pore-like structures within lipid bilayers [52], which would explain our observation of increased membrane conductance to calcium and small molecular weight dyes in the context of cell death independent of extracellular calcium concentrations. Previous work documents enhanced histone-membrane interactions through negatively charged surface molecules [53] such as phosphatidylserine [36], suggesting histones may preferentially bind apoptotic cells and that neutralizing charge on extracellular histones could be a potential therapeutic strategy.

Collectively, these data demonstrate circulating histones are important early mediators of AP severity and implicate the liver as the primary source in circulation; circulating histones concentrate within the inflamed pancreas and actively contribute to pancreatic acinar cell necrosis by disruption of the plasmalemma in a charge- and dose-dependent manner. Strategies to detect sharp rises of circulating nucleosomes and detoxify histones may prove effective in detecting and/or preventing severe AP.

**Author contributions**

R.S. and P.S. conceived and designed the study; R.S., A.V.T. and D.N.C. supervised the study; R.S, P.S. and A.V.T. obtained funding,


