Proteomic identification of interactions between histones and plasma proteins: implications for cytoprotection

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Abbreviations: HPPP, histone-precipitated plasma protein; **HS,** heparan sulphate; **LMWH,** low molecular weight heparin; **PPS,** pentosan polysulphate; **UFH,** unfractionated heparin

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Abstract

Extracellular histones released from cells during acute inflammation contribute to organ failure and death in a mouse model of sepsis, and histones are known to exert *in vitro* cytotoxicity in the absence of serum. Since addition of histones to serum and plasma is known to induce protein aggregation, we reasoned that plasma proteins may afford protection from cytotoxicity. We found that MODE-K mouse small intestinal epithelial cells were protected from histone induced toxicity in the presence of 10% foetal calf serum. Therefore, the main aim of this study was to identify histone-interacting plasma proteins that might be involved in cytoprotection. The precipitate formed following addition of calf thymus histones to human EDTA-plasma was characterised by shotgun proteomics, identifying a total of 36 protein subunits, including complement components, coagulation factors, protease inhibitors and apolipoproteins. The highly sulphated glycosaminoglycan heparin inhibited histone-induced plasma protein aggregation. Moreover, histones bound to heparin agarose were capable of pulling down plasma proteins from solution, indicating their effective crosslinking properties. It was particularly notable that inter-alpha-trypsin inhibitor was prominent among the histone-precipitated proteins, since it contains a chondroitin sulphate glycan chain, and suggests a potential role for this protein in histone sequestration during acute inflammation *in vivo*.

Introduction

Nuclear histones are small, highly basic proteins that play an indispensable role in organising DNA structure. Within the nucleosome, an octamer of histones (two H2A-H2B dimers and two H3-H4 dimers) form a core, around which 146 bp of DNA is wrapped. X-ray crystallography has elucidated the nucleosome core particle structure[1, 2], showing that core histone dimers take the form of interlocking "U" shaped molecules, each formed from a conserved motif of three alpha helices (α1, α2 and α3, joined by loops L1 and L2). Positively charged amino acids, primarily in the α1 helices and loops L1 and L2, interact with phosphate groups of the DNA backbone, whilst the α2 helices of dimeric histones interact at hydrophobic regions. Highly basic N-terminal histone tails extend outside the core nucleosome, and are the targets for modifications such as acetylation and methylation, which are important in controlling chromatin structure and gene transcription, as reviewed in[3].

The extracellular roles of histones are less well researched, but it is clear that histones have a number of extra-nuclear functions. For example, antibacterial neutrophil extracellular traps (NETs) have been described[4], which consist of a fibrous extracellular network extruded from neutrophils, and which is derived from granule contents, DNA and histones. In another example, histone H2A present in catfish skin mucus is cleaved at Ser19 by cathepsin D, which becomes activated following injury to the skin[5]. The resulting 19-amino acid peptide, termed parasin I, possesses potent antimicrobial activity, protecting catfish skin from opportunist infection following mucosal injury[6].

Histones have been shown to be present in the normal human circulation at low levels. Data generated by the Human Plasma Proteome Project indicated that histones (primarily H2A and H2B) were present in human plasma analysed by sensitive proteomic techniques[7]. Additional

proteomic studies have detected histone H2B in rat mesenteric lymph[8] and histone H4 in sheep gastric lymph[9]. Histones H2A, H2B and H4 have also been detected in bovine milk[10]. Extracellular histones, in the form of nucleosome fragments, are detectable in plasma[11], and are elevated in systemic inflammation and sepsis[12] as well as auto-immune conditions such as SLE[13], where anti-nucleosome antibodies are a recognised pathological feature.

The presence of elevated levels of extracellular histones is associated with endothelial cytotoxicity, organ failure and death in mouse models of sepsis[14]. Several previous studies have demonstrated that histones are cytotoxic *in vitro*[15-17]. Crucially in these studies, *in vitro* histone cytotoxicity was demonstrated in serum-free or low-serum medium. In the presence of 10% FCS, Class et al.[18] found that histone H1 was cytotoxic to a range of leukaemia cell lines, but not to normal PBMC, fibroblasts or bone marrow cells. Rabbit urinary bladder epithelium was found to be sensitive to treatment with histones, which caused an increase in transepithelial permeability, followed by cytotoxicity[16]. In further studies, histone H4 was found to induce channel formation in phospholipid bilayers, suggesting a mechanism for histone-induced damage to the bladder epithelium[19].

In the present study, we aimed to investigate the interactions between histones and plasma proteins, in order to identify those interacting proteins that may confer cytoprotection, and also to make some predictions as to the significance of histone – plasma protein interactions *in vivo*. The *in vitro* interactions of histones with plasma proteins fibrinogen, fibronectin and alpha-2-macroglobulin have already been described in some detail[20-22]. However, there has not previously been a systematic proteomic analysis of histone – plasma protein interactions. Therefore, we have used shotgun proteomic analysis to study the composition of the precipitate formed between human plasma proteins and histones. This has confirmed previous data on fibrinogen and fibronectin, and provided novel data, which suggests that elevated levels of extracellular histones have important *in*

vivo consequences.

Materials and Methods

Materials

Calf thymus histones and heparin agarose were purchased from Sigma (Poole, Dorset, UK). MODE-K mouse small intestinal epithelial cells[23] were kindly supplied by Dr Julia-Stefanie Frick (Institute of Medical Microbiology and Hygiene, University of Tübingen, Germany). SDS-PAGE was carried out using mini-Protean apparatus (Bio-Rad, Hemel Hempstead, UK) according to manufacturer's protocols. Human EDTA plasma and serum samples were donated by consenting healthy volunteers (two male; two female; ages 26-46).

Histone induced cytotoxicity

The mouse intestinal epithelium cell line, MODE-K, was routinely cultured in DMEM (Invitrogen #22320022 supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin) plus 10% fetal calf serum (FCS). For experiments, MODE-K cells were seeded into 24-well tissue culture plates at $2x10^5$ cells /well in DMEM + 10% FCS and grown to confluence. Wells were washed in two changes of PBS, before being cultured for a further 24 hours in DMEM with and without 10% FCS and between 0 and 200 µg/ml of calf thymus histones. Cytotoxicity was measured using a commercial kit based on lactate dehydrogenase (LDH) release, as directed by the manufacturer (Roche Diagnostics Ltd, Burgess Hill, UK).

Incubations of histones with human plasma and serum

All incubations were carried out in PBS at room temperature (21°C) unless otherwise stated. Dilutions (1 in 10) of EDTA plasma and serum in 2x PBS were incubated for 1 hr with an equal volume of 1mg/ml calf thymus histones in H_2O (or H_2O). Following incubation, samples were

centrifuged (20,000g for 5 min at 15°C). Supernatants were reserved, and pellets dissolved in SDS-PAGE sample buffer lacking reducing agent. Samples were then analysed by SDS-PAGE (12%T) under reducing and non-reducing conditions. To test the effect of temperature, the above experiment was carried out using a plasma sample at 21°C and repeated in a warm room at 37°C. In titrations, histones (100µl of 0, 0.125, 0.25, 0.5 and 1mg/ml in PBS; pre-filtered 0.45µm) were added to 100µl of human EDTA plasma (diluted 1 in 10 in PBS and centrifuged at 20,000g for 5 min prior to incubation). Following 1 hour incubation at room temperature, turbidity was measured at 680 nm using a spectrophotometer.

Effects of sulphated glycosaminoglycans on histone-induced precipitation of plasma proteins

Samples of diluted human EDTA plasma (50µl of a 1 in 10 dilution in PBS) were mixed with 25µl of low molecular weight heparin (LMWH; at 10, 1, 0.1, 0.01, 0 mg/ml in PBS), then 25µl of 1mg/ml calf thymus histones in PBS was added. The mixtures were incubated at 21°C for 1 hr, then turbidity measured at 680 nm. The effects of pentosan polysulfate (PPS), unfractionated heparin (UFH), LMWH and heparan sulfate (HS) were compared by adding 25µl of a 0.1mg/ml solution of each in PBS (or PBS alone) to 50µl of human EDTA plasma (diluted 1 in 10), followed by 25µl of 1mg/ml histones. After 1 hour at 21°C, turbidity was measured at 680 nm.

Plasma protein binding properties of histones bound to heparin-agarose

A suspension of heparin covalently linked to 4% beaded agarose (Sigma; 900µg Type 1 heparin per ml packed gel) was mixed well in the bottle, then 100µl of slurry dispensed into spin columns (VectaSpin Micro, 10µm, Whatman International Ltd., Maidstone, UK). Columns were given a pulse spin then washed with 500µl of PBS followed by a pulse spin. Calf thymus histones (200µl of 4mg/ml in PBS) or PBS were added and incubated for 5 min at room temperature. The columns were then pulsed and washed 4 times with 375µl of PBS per wash. Columns were then incubated with 100µl of human EDTA plasma (diluted 1:1 with PBS) and incubated for 40 min at room

temperature. Columns were then pulsed and the filtrate collected. The columns were then washed a further 4 times with 375µl PBS. Finally, columns were incubated with 200µl of reducing SDS-PAGE sample buffer for 30 min at 37°C, then pulsed and the filtrate collected. Collected filtrates were compared to the original plasma sample by SDS-PAGE.

Shotgun proteomic analysis of histone-induced precipitate of plasma proteins

The precipitates formed by incubating 150µl of four diluted (1 in 10 with PBS) human EDTA plasma samples with 150µl of 1mg/ml calf thymus histones for 1 hr at room temperature in PBS were collected by centrifugation, dissolved in 100µl of reducing SDS-PAGE sample buffer and heated (95°C) for 5 min. The solubilised precipitates (10µl) were separated by SDS-PAGE (12% T) and stained with Coomassie Brilliant Blue (Imperial Protein Stain, Perbio, Cramlington, UK). The stained gel lane was excised and then sliced horizontally from top to bottom to yield a series of ~25 equal gel slices of 2.5mm deep. Each of the resulting 25 gel slices was then subjected to standard in-gel destaining, reduction, alkylation and trypsinolysis procedures[24].

LC-ESI-MS/MS

Samples were transferred to HPLC sample vials and stored at +4^{o}C until required for liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) analysis. Liquid chromatography was performed using an Ultimate 3000 nano-HPLC system (Dionex UK) comprising a WPS-3000 well-plate micro auto sampler, a FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micropump and an SRD-3600 solvent rack controlled by Chromeleon chromatography software (Dionex UK). A micro-pump flow rate of 246µl/min^{-1} was used in combination with a cap-flow splitter cartridge, affording a $^{1}/_{82}$ flow split and a final flow rate of 3µl/min^{-1} through a 5cm x 200µm ID monolithic reversed phase column (Dionex UK) maintained at 50°C. Samples of 4µl were applied to the column by direct injection. Peptides were eluted by the application of a 15min linear gradient from 8-45% solvent B

(80% acetonitrile, 0.1% ($^{v}/_{v}$) formic acid) and directed through a 3nl UV detector flow cell. LC was interfaced directly with a 3-D high capacity ion trap mass spectrometer (Esquire HCTplus^{TM}, Bruker Daltonics) via a low-volume (50µl/min^{-1} maximum) stainless steel nebuliser (Agilent Technologies, cat. no.G1946-20260) and ESI. Parameters for tandem MS analysis were set as previously described[25].

Database Mining

Deconvoluted MS/MS data was submitted to an in-house MASCOT server and mined against the NCBInr database using "*homo sapiens*" as a taxonomical parameter. The presentation and interpretation of MS/MS data was performed in accordance with published guidelines[26]. To this end, fixed and variable modifications selected were carbamidomethyl (C) and oxidation (M) respectively and mass tolerance values for MS and MS/MS were set at 1.5Da and 0.5Da respectively. Molecular weight search (MOWSE) scores attained for individual protein identifications were inspected manually and considered significant only if a) two peptides were matched for each protein, and b) each peptide contained an unbroken "*b*" or "*y*" ion series of a minimum of four amino acid residues. For each individual plasma sample, a non-redundant list of identified proteins was obtained, and a master list was generated, which consisted of proteins identified in at least three out of the four samples. This list was termed the Histone-Precipitated Plasma Protein (HPPP) list.

Results

Serum protects MODE-K cells from histone-induced cytotoxicity

Mouse small intestinal MODE-K cells grown in the presence of 10% FCS were protected from histone-induced toxicity up to a concentration of 200µg/ml (Figure 1). In the absence of serum, concentrations of 25 µg/ml or above of calf thymus histones caused significant ($P<0.01$)

cytotoxicity.

Histones precipitate plasma and serum proteins

Addition of calf thymus histones to human plasma and serum samples resulted in the appearance of turbidity due to protein aggregation. The precipitates formed by incubating histones for 1 hour at 21°C with human EDTA plasma and serum were separated by centrifugation and analysed by SDS-PAGE (Figure 2A). This showed that certain proteins were quantitatively depleted from plasma under the conditions used, including a band at approximately 72 kDa, which was not present in serum. This band was excised from the gel and identified as prothrombin by peptide mass fingerprinting (Mascot score: 134; 29 of 56 peptides matched; 50% sequence coverage; accession no: P00734). The precipitates formed at 37°C and room temperature (21°C) were essentially identical (Figure 2B). Titration of calf thymus histones into human plasma resulted in a concentration dependent increase in turbidity, as measured at 680 nm (Figure 2C).

Heparin and other sulphated glycosaminoglycans inhibit histone-induced precipitation of plasma proteins

Histones are known to bind to heparin with high avidity[27]. Therefore, we investigated whether low molecular weight heparin (LMWH; ave. MW 3,000 Da) could preferentially bind to histones and thus prevent plasma protein precipitation. As expected, LMWH exhibited a concentration dependent inhibition of plasma protein precipitation by histones (Figure 2D). Furthermore, the sulphated glycosaminoglycans pentosan polysulfate (PPS), unfractionated heparin (UFH), LMWH and heparan sulfate (HS), each at 25µg/ml final concentration, all significantly inhibited histone-induced plasma protein turbidity to varying degrees (Figure 2E).

Histones immobilised on heparin-agarose bind human plasma proteins

In order to test whether histones are capable of acting as bridging molecules, binding to two or

more binding partners at the same time, we used heparin-agarose beads. Beads treated with either histones or PBS were washed, then incubated with human plasma at physiological pH and salt concentration. The unbound material was collected, then the beads were washed extensively before the bound proteins were eluted using SDS-reducing buffer. Although some plasma proteins bound to unmodified heparin-agarose, histone-charged heparin agarose was found to bind more protein from plasma, and a different subset of proteins (Figure 3A). The proteins eluted from histone-charged heparin-agarose gave a similar pattern, when analysed by SDS-PAGE, to the proteins precipitated from plasma by addition of histones (Figure 3B). This shows that histones are capable of bridging heparin and certain plasma proteins, and suggests that the precipitate formed following addition of histones to plasma is due to the bridging property of histones.

Proteomic analysis of proteins precipitated from human plasma by histones

The precipitates obtained by adding calf thymus histones to four samples of diluted human EDTA plasma were separated by SDS-PAGE and subjected to shotgun proteomic analysis. Proteins identified definitively in at least three out of the four samples were included on the final list of histone-precipitated plasma proteins (HPPP), which comprised a total of 36 unique protein subunits (Table 1). Lipoprotein-associated proteins, complement-related proteins, proteinase inhibitors and blood coagulation-related proteins were well represented (Figure 4).

Discussion

This study has shown for the first time, those human plasma proteins which have a high affinity for binding histones, and by acting as effective histone scavengers, may be responsible for protecting cells from histone cytotoxicity. To identify interacting proteins, we took advantage of the fact that when high concentrations of histones are added to plasma, they induce precipitation of a subset of plasma proteins. We also demonstrated that histones bound to immobilised heparin still possess the

ability to bind plasma proteins. The action of histones in these experiments can be rationalised in terms of their acting as bridging or crosslinking molecules capable of interacting with two or more binding partners simultaneously. Determining the precise mode of interaction of individual histones with individual plasma proteins is beyond the scope of the present study, but the general concept of histones acting as bridging molecules is consistent with the observed results. In effect, when present at high concentrations, histones generate insoluble co-polymers by interaction with plasma proteins.

One of the major histone-interacting plasma proteins was fibrinogen, which is known to precipitate following the addition of histones[20]. An electron microscopic study showed that histone – fibrinogen co-precipitates took the form of globules, clumps or rudimentary fibrils, dependent on the histone fractions used[28]. Gonias *et al.* found that histone H3 caused a concentration dependent increase in fibrinogen precipitation[20]. They demonstrated that histone H3 bound to fibrinogen fragment D but did not cause precipitation. Since intact fibrinogen contains two equivalents of fragment D, this suggests that this bi-functionality is necessary for fibrinogen to participate in the formation of a histone co-precipitate.

Prothrombin was a prominent member of the list of histone-precipitated proteins, being selectively depleted from plasma. This indicates a very strong affinity for histones. During processing, prothrombin undergoes a vitamin K dependent gamma carboxylation of ten Glu residues near the N-terminus of the molecule. This produces a highly acidic region, which participates in binding to negatively charged phospholipids *via* divalent calcium ion bridges[29]. It seems likely that this region in particular would be highly attracted to the strongly basic regions of histones. It is possible that the very strong interactions of both prothrombin and fibrinogen with histones has a biological significance in terms of coagulation and thrombosis at regions of tissue damage with associated histone release. In this respect, it was notable that complement components were also highly represented among the histone precipitated proteins. Component C3 appeared particularly abundant,

and C4a, C1r, C1s and C4bp alpha were also detected. More detailed work will be required to determine whether this represents simple protein deposition, or whether it has any relevance to complement activation. It has been suggested that histone interactions with complement C4A may be important in the development of anti-histone antibodies in drug-induced systemic lupus erythematosus (SLE)[30].

The strong affinity of histones for heparin and other sulphated glycosaminoglycans is well known[31]. In this regard, it is interesting to note that inter alpha trypsin inhibitor (IαI) is present on the HPPP list. This high molecular weight protease inhibitor consists of a variable number of heavy chains and bikunin (alpha-1-microglobulin), linked by a chondroitin sulphate chain[32]. IαI synthesised during inflammation has been shown to exhibit an increased glycosaminoglycan chain length[33]. It is possible that the acidic sulphated glycosaminoglycan chain of IαI contributes to its affinity for histones, thereby playing an important role in extracellular histone sequestration *in vivo*. Importantly, IαI levels are negatively correlated with survival in patients with severe sepsis[34], and administration of exogenous human IαI was effective in reducing mortality in a rat model of sepsis[35].

Unexpectedly, a significant proportion of apolipoproteins and other lipoprotein-associated proteins were detected in the precipitate. These included apoB, apoE, apoA-I, apoA-IV, apoC-III and serum paraoxonase. These results suggest that a mixture of lipoprotein classes was precipitated, possibly due to the interaction of basic histone residues with negatively charged phospholipids in the outer coat of the lipoproteins. Notably, histones demonstrate a particularly strong affinity for anionic phospholipids such as phosphatidylserine and cardiolipin [36]. Anionic phospholipids are known to comprise approximately 3% of rat chylomicron phospholipids[37], and may therefore contribute to the observed binding of histones to lipoproteins. The formation of LDL aggregates in the presence of histones was previously described by Skrzydlewski[38], who hypothesised in the 1970s that

histone-induced aggregation of LDL may contribute to atherosclerosis[39]. Although this hypothesis has not been considered further since that time, we suggest that it is worthy of re-examination in the light of these results and the established association between chronic inflammation and atherosclerosis[40].

Finally, it is worth noting that plasma proteins may also play an important cytoprotective role at mucosal epithelial surfaces. Normal human airway secretions, as sampled by induced sputum, have been found to contain histone H4, among other cellular components, as well as mucins and plasma proteins[41]. Necrosis of inflammatory cells present in bronchial mucus will result in the liberation of potentially toxic cellular remnants, such as histones. The exudation of bulk plasma proteins into the airways, which occurs readily in association with inflammation[42], is likely to contribute to protection of the epithelium from histones originating from necrotic cells. In cystic fibrosis, excessive inflammation leads to clogging of airway mucus with the remnants of necrotic neutrophils. The presence of F-actin and denatured DNA derived from these cells is known to contribute to the increased viscosity of cystic fibrosis mucus[43]. Clearly, large quantities of histones must also be present in cystic fibrosis mucus, with the capability of forming crosslinks and influencing mucus viscosity. Indeed, histone H1 has been shown to crosslink actin and DNA to form bundles, which are readily dispersed using anionic poly-amino acids[44]. The reported efficacy of anionic poly-amino acids and heparin in reducing the viscosity of cystic fibrosis sputum[44, 45] may therefore be in part due to their effect on reducing the crosslinking effects of histones in mucus.

In conclusion, we have demonstrated that serum and plasma proteins can contribute to cytoprotection from the toxic effects of extracellular histones and we have identified the most abundant participating plasma proteins. The nature of the interactions between histones and plasma proteins leads us to the conclusion that histones are potent bridging molecules. Inflammatory

conditions leading to the presence of elevated levels of extracellular histones may therefore have important *in vivo* consequences, in terms of thrombosis, complement activation and lipoprotein aggregation, which merit further detailed study.

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Table 1

Accession	Gene Name	Description	Mowse[a]	Peptides matched[a]	Example peptides
Lipoprotein associated					
P02647	APOA1	Apolipoprotein A-I	720	17	-.DEPPQSPWDR.V; K.VSFLSALEEYTK.K
P06727	APOA4	Apolipoprotein A-IV	637	15	R.TQVNTQAEQLR.R; R.SLAPYAQDTQEK.L
P04114	APOB	Apolipoprotein B-100	1344	60	K.LDVTTSIGR.R; R.NNALDFVTK.S
P02656	APOC3	Apolipoprotein C-III	223	4	R.GWVTDGFSSLK.D; K.DALSSVQESQVAQQAR.G
P02649	APOE	Apolipoprotein E	487	11	R.AKLEEQAQQIR.L; K.VQAAVGTSAAPVPSDNH.-
P10909	CLU	Clusterin (apolipoprotein J)	338	14	R.ASSIIDELFQDR.F; R.EILSVDCSTNNPSQAK.L
P80108	GPLD1	Phosphatidylinositol-glycan-specific phospholipase D	106	6	R.IADVTSGLIGGEDGR.V; K.VAFLTVTLHQGGATR.V
P27169	PON1	Serum paraoxonase/arylesterase 1	412	8	R.VVAEGFDFANGINISPDGK.Y; K.ILLMDLNEEDPTVLELGITGSK.F + Oxidation (M)
Proteinase inhibitors					
P01009	SERPINA1	Alpha-1-antitrypsin	227	6	K.SVLGQLGITK.V; K.LSITGTYDLK.S
P01023	A2M	Alpha-2-macroglobulin	442	16	K.VTGEGCVYLQTSLK.Y; R.TEVSSNHVLIYLDK.V
P19827	ITIH1	inter alpha-trypsin inhibitor heavy chain H1	985	13	K.LDAQASFLPK.E; K.AAISGENAGLVR.A
P19823	ITIH2	inter alpha-trypsin inhibitor heavy chain H2	627	10	K.FYNQVSTPLLR.N; K.IQPSGGTNINEALLR.A
Q06033	ITIH3	inter alpha-trypsin inhibitor heavy chain H3	230	9	R.DYIFGNYIER.L; K.VTFELTYEELLK.R
P02760	AMBP	Protein AMBP (contains alpha-1-microglobulin; bikunin)	216	3	R.TVAACNLPIVR.G; R.AFIQLWAFDAVK.G
Complement associated					
P04003	C4BPA	C4b-binding protein alpha chain	478	11	K.EDVYVVGTVLR.Y; K.LSLEIEQLELQR.D
P00736	C1R	Complement C1r subcomponent	406	9	R.FCGQLGSPLGNPPGK.K; R.ESEQGVYTCTAQGIWK.N
P09871	C1S	Complement C1s subcomponent	359	7	R.TNFDNDIALVR.L; K.SNALDIIFQTDLTGQK.K

P01024	C3	Complement C3	1579	62	R.QPSSAFAAFVK.R; K.TIYTPGSTVLYR.I
P0C0L4	C4A	Complement C4-A	324	15	K.VLSLAQEQVGGSPEK.L; R.STQDTVIALDALSAYWIASHTTEER.G
Blood coagulation					
P02671	FGA	Fibrinogen alpha chain	1106	36	K.GLIDEVNQDFTNR.I; K.DSHSLTTNIMEILR.G + Oxidation (M)
P02675	FGB	Fibrinogen beta chain	1313	35	K.HGTDDGVVWMNWK.G; K.DNENVVNEYSSELEK.H
P02679	FGG	Fibrinogen gamma chain	1029	30	K.YEASILTHDSSIR.Y; R.YLQEIYNSNNQK.I
P00734	F2	Prothrombin	841	18	R.ELLESYIDGR.I; R.ETAASLLQAGYK.G
P01042	KNG1	Kininogen-1	99	5	R.QVVAGLNFR.I; K.YNSQNQSNNQFVLYR.I
Immunoglobulins					
P01876	IGHA1	Ig alpha-1 chain C region	177	7	K.TFTCTAAYPESK.T; R.QEPSQGTTTFAVTSILR.V
P01859	IGHG2	Ig gamma-2 chain C region	311	8	K.NQVSLTCLVK.G; K.GPSVFPLAPSSK.S
P01834	IGKC	Ig kappa chain C region	391	8	K.DSTYSLSSTLTLSK.A; R.FSGSGSGTDFTLTISR.L
P01842	IGLC1	Ig lambda chain C regions	219	4	R.SYSCQVTHEGSTVEK.T; K.YAASSYLSLTPEQWK.S
P01871	IGHM	Ig mu chain C region	297	7	K.VSVFVPPR.D; K.YAATSQVLLPSK.D
Cell adhesion					
P02751	FN1	Fibronectin-1	978	21	R.GATYNIIVEALK.D; R.DLQFVEVTDVK.V
P07996	THBS1	Thrombospondin-1	169	7	R.TIVTTLQDSIR.K; K.GGVNDNFQGVLQNVR.F
P04004	VTN	Vitronectin	375	9	R.FEDGVLDPDYPR.N; R.DVWGIEGPIDAAFTR.I
Transport					
P02768	ALB	Serum albumin	1310	35	K.FQNALLVR.Y; K.LVNEVTEFAK.T
P00450	CP	Ceruloplasmin	474	24	K.GAYPLSIEPIGVR.F; K.DIASGLIGPLIICK.K
P02787	TF	Serotransferrin	234	5	K.SASDLTWDNLK.G; K.EDPQTFYYAVAVVK.K
P02766	TTR	Transthyretin	153	5	R.GSPAINVAVHVFR.K; K.AADDTWEPFASGK.T

[a)]Maximum Mascot MOWSE score and number of peptides matched for the protein of interest in any single gel slice

Table 1. List of plasma proteins precipitated by histones. Calf thymus histones were added to four human EDTA-plasma samples, and precipitates were collected by centrifugation, separated by SDS-PAGE and analysed by shotgun proteomics, as described in Materials & Methods. A full list of identified proteins is shown in Supplementary Material. Those proteins that were identified in at least three out of four samples are listed above, along with Uniprot identifiers. The identified proteins have been grouped according to biological function. For each protein, the maximum Mowse score detected in any gel slice is given, as an approximate indication of relative abundance.

Figures

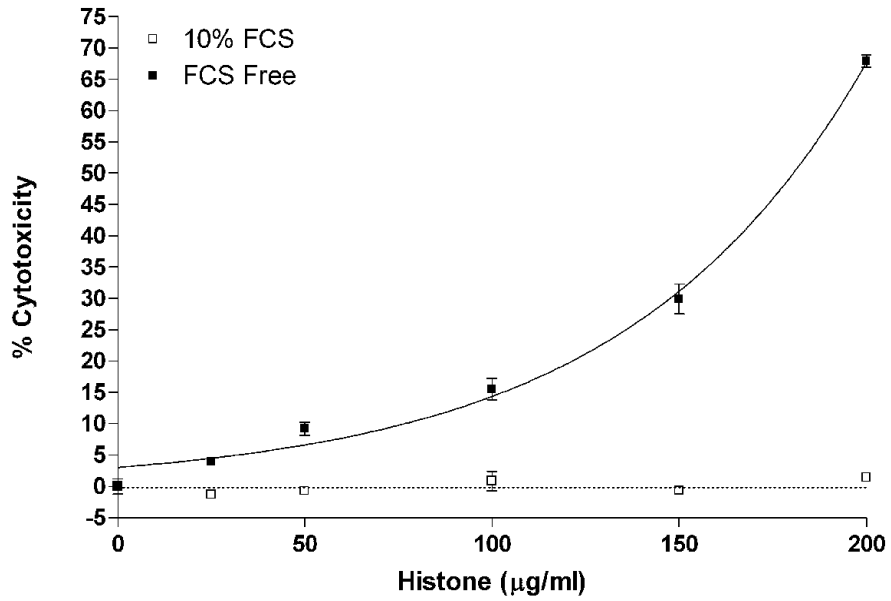

Figure 1: Foetal calf serum protects epithelial cells from histone induced cytotoxicity. Confluent monolayers of MODE-K cells were washed with sterile PBS and incubated with calf thymus histones (0 to 200 μg/ml final concentration) in DMEM with (open boxes) or without (filled boxes) 10% FCS. Cytotoxicity was determined after 24 hours using a Roche cytotoxicity detection kit (LDH). In the absence of serum, histones induced significant cytotoxicity ($P<0.01$) at concentrations of 25 μg/ml and above, but there was no evidence of cytotoxicity when serum was present in the culture medium. Curve fitted by non-linear regression ($R^2=0.98$) for the dose-dependent effects of histones under serum-free conditions.

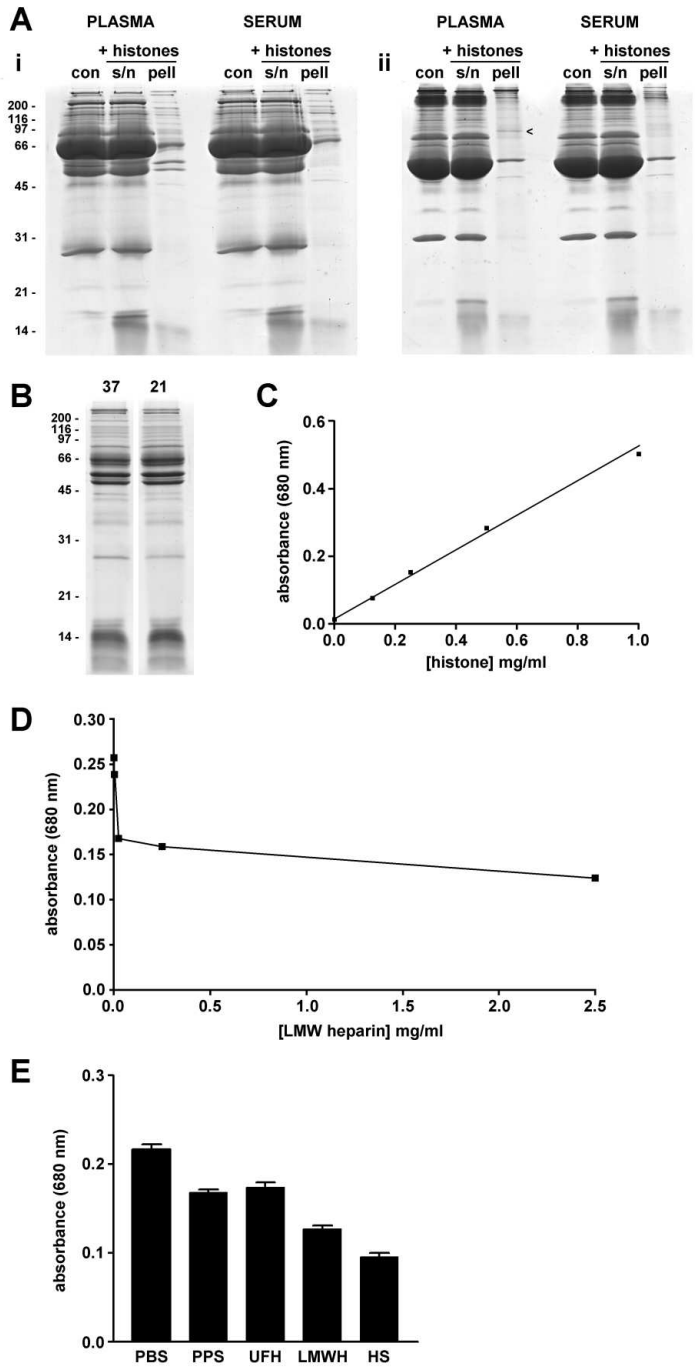

Figure 2: Precipitation of human plasma and serum proteins following addition of histones. A: SDS-PAGE (12%) comparison of EDTA plasma and serum, and the supernatants (s/n) and pellets (pell) following precipitation of proteins by addition of calf thymus histones. Incubations were carried out for 1 hr in PBS at 21°C, as described in Materials & Methods. Gels were run under reducing (i) and non-reducing (ii) conditions. The band marked (<) in (ii), selectively depleted by histone treatments, was identified as prothrombin by peptide mass fingerprinting. **B:** Histone

induced plasma protein pellets generated at 37°C and 21°C were compared by SDS-PAGE, revealing little evidence of temperature dependency. **C:** The titration of histones into diluted human EDTA plasma was observed by turbidity measurements at 680 nm, after incubation for 1 hr at 21°C. **D:** Incubations of histones and plasma proteins were carried out for 1 hr at 21°C in the presence of varying amounts of low molecular weight heparin (LMWH). Turbidity measurements indicated a strong inhibition of aggregation, particularly at lower concentrations of LMWH. **E:** Incubations of histones and plasma proteins were carried out for 1 hr at 21°C in the presence of 0.01 mg/ml of pentosan polysulfate (PPS), unfractionated heparin (UFH), LMWH and heparan sulfate (HS). All of the sulphated glycosaminoglycans tested significantly reduced protein aggregation compared to vehicle control (PBS); $p<0.01$.

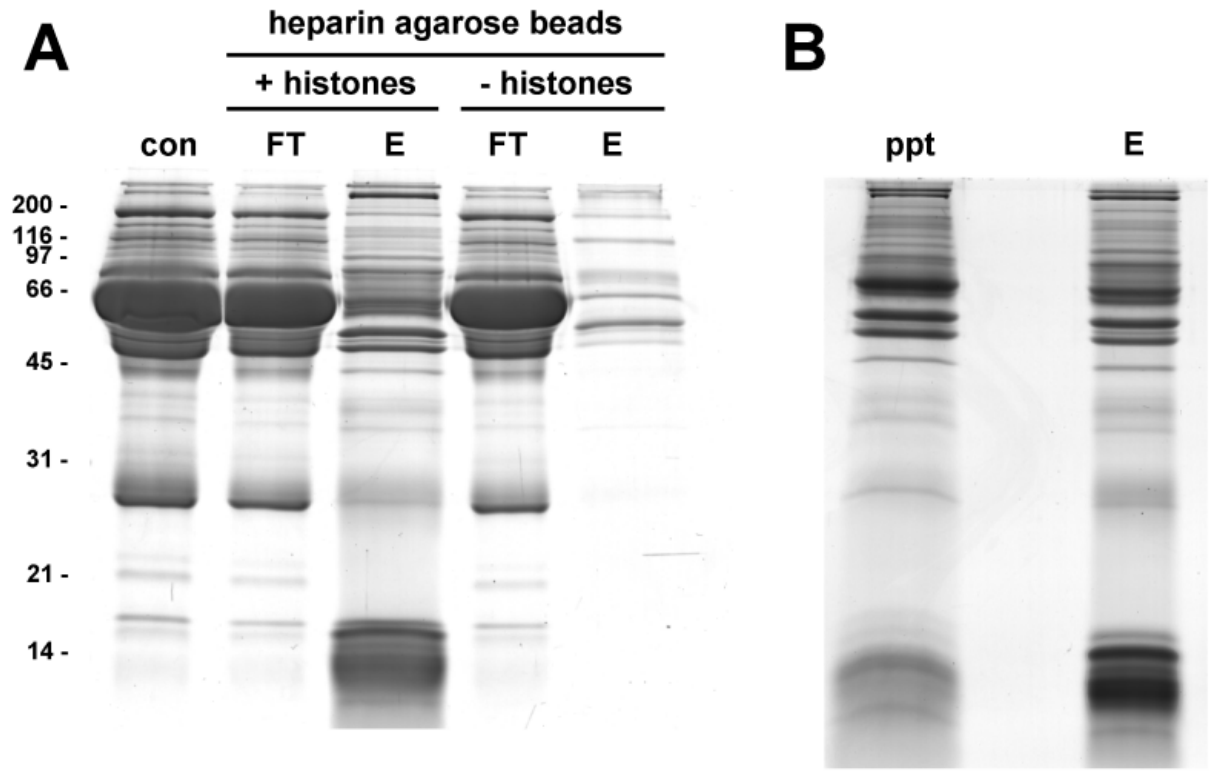

Figure 3: Histones bound to heparin agarose beads pull down plasma proteins from solution.

A: Heparin agarose beads were first incubated with or without histones, then after washing, the beads were exposed to diluted human EDTA plasma. Unbound proteins were collected from the flow through (FT) and after further washing, proteins that remained bound were eluted (E) with SDS-PAGE reducing buffer. Control plasma (con) was compared with the FT and E fractions by SDS-PAGE (12%). **B:** SDS-PAGE analysis shows similar banding patterns in the precipitate formed by adding histones to plasma proteins in solution (ppt) and the eluate from histone-charged heparin agarose treated with plasma proteins (E).

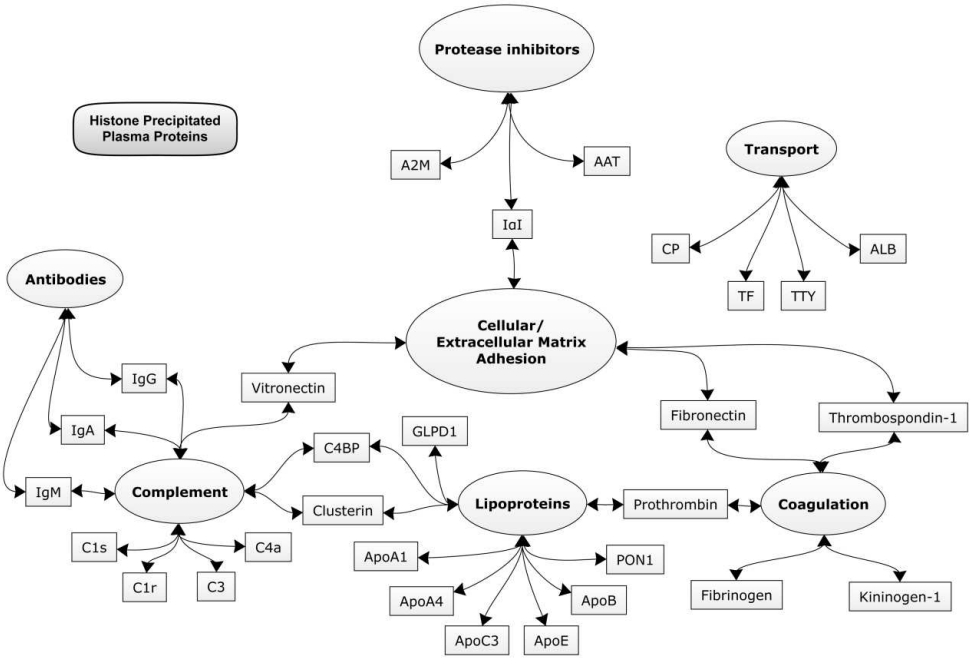

Figure 4: Functional classification of histone-precipitated plasma proteins. Histone-precipitated plasma proteins identified by shotgun proteomic analysis (see Table 1) were grouped according to biological function, as determined from UniProt annotations. A strong representation of complement, lipoprotein and coagulation-associated proteins was observed.