Matrix metalloproteinase-13 (MMP-13) is fully activated by neutrophil elastase and inactivates its serpin inhibitor, alpha-1 antitrypsin: Implications for osteoarthritis

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Matrix metalloproteinase-13 (MMP-13) is a uniquely important collagenase that promotes the irreversible destruction of cartilage collagen in osteoarthritis (OA). Collagenase activation is a key control point for cartilage breakdown to occur, yet our understanding of the proteinases involved in this process is limited. Neutrophil elastase (NE) is a well-described proteoglycan-degrading enzyme which is historically associated with inflammatory arthritis, but more recent evidence suggests a potential role in OA. In this study, we investigated the effect of neutrophil elastase on OA cartilage collagen destruction and collagenase activation. Neutrophil elastase induced significant collagen destruction from human OA cartilage ex vivo, in an MMP-dependent manner. In vitro, neutrophil elastase directly and robustly activated pro-MMP-13, and N-terminal sequencing identified cleavage close to the cysteine switch at 72MKKPR, ultimately resulting in the fully active form with the neo-N terminus of 85YNVFP. Mole-per-mole, activation was more potent than by MMP-3, a classical collagenase activator. Elastase was detectable in human OA synovial fluid and OA synovia which displayed histologically graded evidence of synovitis. Bioinformatic analyses demonstrated that, compared with other tissues, control cartilage exhibited remarkably high transcript levels of the major elastase inhibitor, (AAT) alpha-1 antitrypsin (gene name SERPINA1), but these were reduced in OA. AAT was located predominantly in superficial cartilage zones, and staining enhanced in regions of cartilage damage. Finally, active MMP-13 specifically inactivated AAT by removal of the serine proteinase cleavage/inhibition site. Taken together, this study identifies elastase as a novel activator of pro-MMP-13 that has relevance for cartilage collagen destruction in OA patients with synovitis.

Abbreviations
AAT, alpha-1 antitrypsin; ANOVA, analysis of variance; APMA, 4-aminophenylmercuric acetate; BSA, bovine serum albumin; GAG, glycosaminoglycan; HRP, horseradish peroxidase; MMP, matrix metalloproteinase; NOF, neck of femur; OA, osteoarthritis; OHP, hydroxyproline; SD, standard deviation; serpin, serine proteinase inhibitor; TPM, transcripts per million; Tris, tris (hydroxymethyl) aminomethane.
Introduction

Osteoarthritis is a highly prevalent, degenerative joint disease which causes significant pain and disability. The progressive and irreversible destruction of the articular cartilage is the central characteristic of the disease. Cartilage is composed of two major components, the highly charged proteoglycan aggregate aggregan and type II collagen, which is the major structural component of cartilage exhibiting a tightly interlocking triple helix, making it particularly resistant to proteolysis. Due to slow rates of synthesis, the loss of collagen is an essentially irreversible process [1], tant to proteolysis. Due to slow rates of synthesis, the loss of collagen is an essentially irreversible process [1], making it a critical therapeutic intervention point to prevent further cartilage breakdown. The crucial effector proteinases in this destruction are matrix metalloproteinases (MMPs) – specifically a subgroup known as the classical collagenases (MMP-1, MMP-8 and MMP-13). A wealth of evidence implicates MMP-13 (collagenase-3) with OA development and progression [2]. Indeed, this collagenase has the highest efficiency for cleavage of type II collagen [3], and MMP-13-deficient mice are protected from cartilage destruction in experimental OA [4]. However, MMP inhibition has failed clinically, with patients presenting with a range of off-target effects including arthralgia – referred to as ‘musculoskeletal syndrome’ [5]. This failure is attributed to a lack of inhibitor selectivity, which is a major hurdle, due to a common active-site architecture amongst different MMPs, many of which are considered ‘antitargets’ – proteins to be avoided for therapeutic inhibition [6]. Although new generations of MMP-13 inhibitors exhibit markedly improved selectivity [7], none have been clinically successful thus far.

An essential control point in the degradation of cartilage collagen is the activation of pro-MMPs [8–11] and may represent a novel method to reduce or block cartilage breakdown. Although some MMPs, such as MMP-3, are capable of collagenase activation [3], serine proteinases have long been postulated as putative in vivo activators [12,13] and are more amenable for drug development [14,15]. These enzymes are often assumed to be cartilage-derived, but the synovium can also be a source of proteinases [16]. Despite historically being referred to as a ‘noninflammatory’ arthritis, recent evidence suggests a significant inflammatory component to OA progression, at least within certain subgroups of patients [17]. Several studies have recently reported that activated macrophages and neutrophils within the synovium contribute to the severity of knee OA and that the serine proteinase neutrophil elastase may act as a functional biomarker for OA progression in this joint [18–22].

Neutrophil elastase is located primarily in azurophilic granules of polymorphonuclear leukocytes and released upon cell degranulation [23]. The proteinase cleaves preferentially after small hydrophobic residues and has a range of substrates and well-established roles such as pathogen digestion and cellular extravasation [24,25]. The major inhibitor of elastase is alpha-1 antitrypsin (AAT), a member of the serine proteinase inhibitor (serpin) superfamily, encoded by the SERPINA1 gene. This is perhaps most evident in patients with AAT deficiency, who often present with early-onset emphysema, thought to be driven by uncontrolled neutrophil elastase activity in the lung [26]. This inflammatory proteinase has been proposed as a drug target in numerous disorders, and a commercially available inhibitor, Sivelestat, has been approved for acute respiratory distress syndrome (ARDS), but only in specific jurisdictions [27,28].

The potent, direct cleavage of cartilage proteoglycan by neutrophil elastase has been demonstrated previously, both in vitro and in vivo [29,30]. Indeed, a role for neutrophil elastase in cartilage destruction in rheumatoid arthritis (RA) has long been postulated [31–33]. More recently, it has been demonstrated that neutrophil elastase inhibition reduces chronic pain in mice that have undergone monoiodoacetate injection to induce OA [34], and reduces structural changes in rats following induction of post-traumatic knee OA [35]. Considering these observations, we investigated the effect of neutrophil elastase in OA cartilage collagenolysis, and in particular explored the biochemical interplay with the major collagenase in OA, MMP-13.

Results

Neutrophil elastase induces collagen release from human OA cartilage in explant culture in an MMP-dependent manner

To investigate the effect of neutrophil elastase on human OA knee cartilage, explant culture experiments were performed. Neutrophil elastase potently and reproducibly induced the destruction of cartilage proteoglycan and collagen. To investigate whether this destruction was direct or indirect, a broad-spectrum MMP inhibitor, GM6001 was utilised. Elastase-induced collagen release was markedly reduced in the presence of this inhibitor (Fig. 1A) which correlated with reduced MMP activity in the conditioned medium (Fig. 1B), whereas proteoglycan release was unaffected (Fig. 1C). GM6001 does not affect the activity of neutrophil elastase directly (Fig. 1D), and its vehicle
control (DMSO) had no effect on cartilage matrix destruction. When the serpin neutrophil elastase inhibitor AAT was also included, ECM degradation was abrogated, demonstrating the requirement for elastolytic activity to induce cartilage degradation, both directly (proteoglycan release) and indirectly (collagen release).

**Neutrophil elastase activates pro-MMP-13**

As collagen release was MMP-dependent, we hypothesised that neutrophil elastase could activate pro-MMPs that promote collagenolysis. To determine whether elastase can directly activate procollagenases, *in vitro* incubation experiments were performed using recombinant enzymes followed by an MMP-specific fluorogenic peptide substrate. In this system, neutrophil elastase induced very modest activation of pro-MMP-1 (Fig. 2A) and no activation of pro-MMP-8 (Fig. 2B). However, when incubated with pro-MMP-13 it induced robust activation of the collagenase, to a similar level as the chemical activator 4-aminophenylmercuric acetate (APMA; Fig. 2C). Remarkably, neutrophil elastase induced approximately threefold higher MMP-13 activity than an equimolar amount of active MMP-3, while matriptase and hepsin failed to significantly activate pro-MMP-13 (Fig. 2D), in line with previous observations [36,37].

**Neutrophil elastase fully activates MMP-13, generating the 85YNVFP neo-N terminus**

To demonstrate that pro-MMP-13 was processed into its active form, an activation time course was performed. Neutrophil elastase rapidly converted pro-MMP-13 into a 45 kDa species which corresponds to the fully active form as demonstrated by treatment with APMA (Fig. 3A,B). APMA also generated an additional band of a lower molecular weight, likely due to continued autoproteolysis of MMP-13. A dose–response experiment was conducted to assess the kinetics of processing in more detail. We observed optimum activation at a 1:5 enzyme to substrate ratio, but processing and increased MMP activity were observed at as low as 1:100. A 1:1 ratio resulted in slightly lower MMP activity, consistent with a reduced level of active MMP-13, suggesting a degree of nonspecific proteolysis at this higher concentration (Fig. 3C,D). The inclusion of the metalloproteinase inhibitor GM6001 led to the formation of an intermediate species (Fig. 3E). N-terminal sequencing was performed to identify cleavage sites. Neutrophil elastase alone generated the 85YNVFP neo-N terminus, while the predominant form in the presence of GM6001 was 725M KKPR (Fig. 3F). To demonstrate the activation of pro-MMP-13 following neutrophil degranulation, blood-purified neutrophils were primed with GM-CSF then subsequently stimulated with a degranulation cocktail of...
cytochalasin B and the bacterial peptide-stimulant fMLP. Neutrophil elastase protein (Fig. 4A) and proteolytic activity against an elastase-specific quenched-fluorescent peptide MeOSuc-AAPV-AMC (Fig. 4B) were detectable in supernatants from degranulated neutrophils. When degranulation supernatants were incubated with pro-MMP-13, they induced rapid activation, as determined by an MMP-specific fluorogenic assay and a shift to the active 45 kDa form, an effect that was abrogated by the inclusion of AAT (Fig. 4C, D).

**Neutrophil elastase is detectable in inflamed OA synovium and OA synovial fluid**

Neutrophil elastase is poorly expressed in cartilage [38,39], so to determine whether neutrophil elastase could be detected in the OA joint, an immunohistochemical examination of human OA synovial tissues was performed. In total, 17 human OA synovia were scored histologically using a previously described synovitis scoring system [37,40]. Neutrophil elastase was virtually undetectable in tissues with no synovitis but was detectable in invading leukocytes and in blood vessels of samples categorised as having ‘slight’ or ‘moderate’ synovitis. MMP-13, however, was detectable at all levels of synovitis, although staining was observed predominantly in fibroblasts in no/low synovitis samples and often in invading leukocytes in higher grades (Fig. 5A). To determine the presence of neutrophil elastase and MMP-13 in OA synovial fluid, ELISAs were performed on fluid which was taken at the time of joint arthroplasty. Neutrophil elastase was detectable in all OA synovial fluids although this varied significantly between patients (mean ± standard deviation; SD): 6374 (± 5745) pg·mL⁻¹, n = 8; Fig. 5B). MMP-13 was detectable in all except three OA synovial fluid
Fig. 3. Neutrophil elastase (NE) cleavage results in an N terminus indicative of a fully active MMP-13. (A) For time course experiments, neutrophil elastase (NE) was incubated with pro-MMP-13 in a 1 : 5 enzyme substrate ratio in a final volume of 20 µL for the time points indicated. Reactions were stopped by the addition of 5x sample buffer and the products separated by SDS/PAGE and stained with silver. (B) Densitometry of the activation time course calculated as percentage of active MMP-13 compared to pro-MMP-13 at each timepoint. Data presented as mean ± SD from three independent experiments. (C) Dose–response experiments were conducted over 4 h with 1 : 1000, 1 : 100, 1 : 50, 1 : 20, 1 : 10, 1 : 5 and 1 : 1 neutrophil elatase:MMP-13 ratios, followed by silver stain (C) and MMP activity assay (D). (E) The inclusion of the broad-spectrum metalloproteinase inhibitor GM6001 was used to determine direct and indirect cleavage. (F) To identify cleavage sites, similar experiments were performed, with products separated by SDS/PAGE and blotted onto PVDF. Blots were briefly stained with Coomassie, and both the full cleavage product (full) and the intermediate species (int) generated in the presence of GM6001 were subject to analysis by N-terminal sequencing. Sequencing gave five unambiguous amino acid reads at each site which correspond to the cleavage sites as depicted. Solid arrow corresponds to the final N terminus in the absence of metalloproteinase inhibition, whereas the dashed arrow corresponds to cleavage by neutrophil elastase alone in the absence of metalloproteinase activity (i.e. in the presence of metalloproteinase inhibitor, GM6001).
samples [mean (± SD): 584 (± 760) pg mL⁻¹, n = 8; Fig. 5C]. In this small sample size, neutrophil elastase did not correlate with MMP-13 (Fig. 5D) and neither proteinases significantly correlated with erythrocyte sedimentation rate (ESR), a marker of systemic inflammation (Fig. 5E,F).

**The neutrophil elastase inhibitor, alpha-1 antitrypsin, is abundant in cartilage and is observed predominantly in the superficial layer**

Serine proteinase activity is regulated by endogenous inhibitors, the largest family of which are the serpins.

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**Fig. 4.** MMP-13 is activated by neutrophil degranulation products. Purified neutrophils were primed with GM-CSF for 30 min, stimulated with cytochalasin B + fMLP for a further 30 min, and supernatants harvested. (A) Neutrophil elastase was detected in supernatants from degranulated neutrophils and elastase activity could be detected in these samples using an elastase-specific quenched-fluorescent peptide, MeOSuc-AAPV-AMC (B; timepoints shown as mean ± SD). Degranulation products were incubated with pro-MMP-13 for 2 h for donors one and three (blue and red, respectively) and 30 min for donor three (green), due to high proteolytic activity in degranulated neutrophils of this donor. MMP activity was measured with an MMP-specific quenched-fluorescent substrate (FS-6; C, upper panel) and neutrophil elastase activity was also determined using an MeOSuc-AAPV-AMC (C, lower panel). MMP-13 activation was further demonstrated using western blotting (D, upper panel), and a neutrophil elastase western blot was performed to act as a loading control (D, lower panel). Treatment with stimulation cocktail alone did not cause MMP-13 to be activated, and inclusion of AAT blocked processing of pro-MMP-13. Activity shown from three independent donors (bars represent mean ± SD) while the western blots are representative of all three donors. Statistical analyses of MMP activity were performed using a one-way ANOVA with Tukey’s post hoc test where ***P < 0.001 compared to pro-MMP-13 alone, ###P < 0.001 compared to pro-MMP-13 with degranulation products (n = 3).
To determine relative serpin expression in human cartilage, we interrogated our previously published RNAseq data (GSE111358; [39]). In non-OA control human hip cartilage, several serpins were highly abundant, with 10 serpins having transcripts per million (TPM) counts of 10 or more. Of these, the SERPINA1 gene, encoding AAT, the major neutrophil elastase inhibitor, was particularly abundant with a TPM count exceeding 7000. Indeed, SERPINA1 was the 9th most abundant transcript within this dataset (Fig. 6A). Using the human protein atlas, cross-tissue expression levels were determined. Interestingly, SERPINA1 expression was markedly enriched in cartilage, second only to expression within the liver (Fig. 6B). When comparing macroscopically intact hip OA cartilage to control non-OA (NOF) hip cartilage, levels of SERPINA1 transcript were markedly downregulated (adj \( \text{F} = 0.004; \) Fig. 6C).

To determine specific localisation of the AAT protein within human cartilage, immunohistochemistry revealed that the staining was particularly strong in the upper regions of the cartilage matrix, consistent with expression predominantly in the superficial cartilage layer. In undamaged cartilage, the staining was principally localised to the chondrocyte, but interestingly, staining intensity was markedly increased in areas of damaged ECM, with intense AAT staining in the most damaged areas of OA cartilage (Fig. 6D).

**MMP-13 inactivates AAT at two sites, removing the reactive-centre loop**

Matrix metalloproteinases have previously been demonstrated to inactivate AAT by removing the reactive-centre loop required for irreversible inhibition of target proteinases [41]. Here, we demonstrate that \( \text{in vitro} \) incubation of AAT with active MMP-13 results in the generation of a single protein band – even after a 16-h incubation – with a lower molecular size than that of native AAT (Fig. 7A), and AAT is no longer able to inhibit neutrophil elastase (Fig. 7B). A time course experiment was performed, and products separated using tris-glycine (protein) or tris-tricine (peptide) SDS/PAGE (Fig. 7C). N-terminal sequencing revealed that MMP-13 cleaved AAT at two sites, \( 377\text{LEAIP} \) and \( 382\text{MSIPP} \) (Fig. 7D), upstream of the canonical neutrophil elastase cleavage site. Thus, active MMP-13 induces a specific inactivation of AAT, removing the inhibitory bait region of the serpin.

**Discussion**

In this study, we demonstrate that neutrophil elastase is a potent activator of the major OA collagenase, MMP-13. Historically described as noninflammatory arthritis, it is becoming clear that OA is a disease of the whole joint and that inflammation contributes to the disease processes, at least in a significant subgroup of patients [16]. The importance of the synovium in OA is becoming increasingly apparent, making factors often thought of having a role only in inflammatory arthritis progression – including proteinases – of renewed interest for study in OA.

Serine proteinases have been shown to be important in the proteolytic cascades leading to cartilage destruction previously [8,9,12,36,37,42], although identifying the specific proteinases involved is challenging. Indeed, the conversion of pro-MMP-13 into its active form is an essential, yet often overlooked, control point for the breakdown of cartilage collagen in OA. To our knowledge, the only described direct serine proteinase activators of MMP-13 are trypsin [3] and plasmin [43], with the former lacking physiological relevance to the joint, while the latter has a complex and debated role in arthritis [44]. This is the first study to describe the direct and full activation of pro-MMP-13 by neutrophil elastase. This serine proteinase has often been linked to the pathophysiology of RA and has been previously shown to be present in patient synovial fluid [45–47]. Indeed, systemic administration of human plasma AAT, reduced arthritis scores and joint destruction in murine collagen-induced arthritis [48], and mice deficient for neutrophil elastase and the related proteinase cathepsin-G, are also protected in this model [32]. As a relatively broad-spectrum serine proteinase, the role of neutrophil elastase has been considered one of matrix destruction, and it has been shown to directly degrade proteoglycans [30,49]. The data in our study are consistent with this, as neutrophil elastase-induced proteoglycan release in human OA cartilage was insensitive to metalloproteinase inhibition, and therefore direct. Elastase has previously been shown to directly cleave type III collagen \( \text{in vitro} \), but was unable to cleave type I collagen [50]. The hydroxyproline (OHP) assay is a sensitive and specific way to measure collagen breakdown from cartilage in explant culture [37,42,51]. Cartilage is composed overwhelmingly of type II collagen (> 90%; [52]), but does include other minor collagen types such as type IX and type XI, which elastase has been shown to degrade directly, at least \( \text{in vitro} \) [53]. In our study, we observed up to 15% collagen release from human OA cartilage, which was mediated by metalloproteinase activity, not the direct activity of elastase. The unique structure of triple-helical collagen makes it susceptible to significant proteolysis from only a very restricted number of collagenases, likely due to important.
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interactions of exosites required for unfolding of the triple-helix [54,55]. Although proteoglycan breakdown by neutrophil elastase is well established, the finding that this proteinase can induce MMP-dependent collagen degradation is important, as this is an irreversible step in the process of joint destruction [1]. Indeed, explant data in this study indicate that a pool of latent collagenases exists within diseased OA cartilage, something which has been observed in other studies through the addition of MMP activators to the diseased tissue [37,56]. Correlation between elastase activity and collagen neo-epitopes generated by a collagenase has been observed in the synovial fluids of patients with noninfectious knee joint synovitis previously [57]. Although neutrophil elastase has been shown to be an activator of other MMPs [13,58], here we demonstrate that it is a direct activator of the collagenase most commonly associated with OA, MMP-13. [12]. Under the described experimental conditions, neutrophil elastase was unable to significantly activate pro-MMP-8 while providing only modest activation of pro-MMP-1, further supporting a role for MMP-13 in neutrophil elastase-induced collagen destruction. Degranulation experiments further demonstrated a rapid activation of pro-MMP-13, which could be blocked in the presence of AAT. Neutrophil degranulation products have previously been shown to induce cartilage breakdown in porcine cartilage explants, with neutrophil elastase implicated in the process [59]. Neutrophil elastase was detectable in all synovial fluid samples tested. While these levels are likely to be significantly lower than concentrations observed in RA [45], it is important to recognise that OA is disease of slow progression. Furthermore, the timing of sample collection will likely be of critical importance. For example, levels of neutrophil elastase have been shown to be high immediately after sports injury, but then gradually reduce over a 90-day period [60]. Periodic, localised inflammation could contribute to temporary release of neutrophil elastase into the OA joint, which could contribute to collagen degradation through MMP-13 activation.

The serpins are a superfamily of structurally similar proteins, the majority of which inhibit serine proteinases, and we have recently described their emerging role in cartilage [61]. In this study, we have identified remarkably high transcript expression levels of SERPINA1 gene in human cartilage. Indeed, others have demonstrated high levels of the AAT protein within synovial fluid previously [62,63]. This serpin is a serum protein with levels in the blood of approximately 0.9–2 mg mL⁻¹ [64], the vast majority of which is produced in the liver [65]. Here, we show that levels of SERPINA1 transcript in human cartilage are second only to those in the liver. An interesting study which used microdissection and iTRAQ proteomics to investigate the protein composition of human cartilage layers found AAT to be mainly located in the upper levels of cartilage [66]. Indeed, this is something we also observed in our current study. It is important to recognise that AAT has multiple proteinase targets, although neutrophil elastase is by far the most kinetically favourable (Kₐ = 6.5 × 10⁷ M⁻¹ s⁻¹; [24,67]). It is plausible that AAT may act as a ‘first line of defence’ against damaging immune cell-derived serine proteinases following degranulation. We also observed that AAT localises to damaged cartilage regions which requires further investigation. Possible reasons could include a localised damage response or that greater levels of AAT from the synovial fluid are able to penetrate the cartilage following significant degradation of the proteoglycan matrix (as evidenced by loss of safranin-O staining in these areas).
Fig. 6. *SERPINA1* gene expression is abundant in human cartilage and the alpha-1 antitrypsin (AAT) protein localised predominantly in the superficial layer. (A) The top 50 most abundant genes within a control non-OA cartilage data set (GSE111358). When ordered by TPM, *SERPINA1* is the 9th most abundant transcript in hip cartilage. (B) Comparison of *SERPINA1* expression in human tissues using TPM values in control non-OA (neck of femur fracture; NOF) hip cartilage samples was generated using Salmon as described [39], and Human Protein Atlas TPMs were retrieved from the HPA website [78]. (C) *SERPINA1* transcript expression is significantly reduced in macroscopically intact OA cartilage compared to intact control NOF fracture cartilage. Data shown as individual TPM from each donor with bars representing mean ± SD. Adjusted P-value shown is calculated by DESeq2 analysis using a Benjamini post hoc test. (D) Immunohistochemistry of damaged regions from cartilage samples (both OA and NOF controls) shows that AAT staining localises to areas of damage and staining intensity increases with level of cartilage destruction. Sections where no primary antibody was included, as well as an isotype control antibody at the same concentration as primary antibody, served as negative controls. Cartilage proteoglycan loss was determined by safranin-O (Saf-O) staining. AAT is localised predominantly to chondrocytes in the superficial and upper middle zone of the tissue. Images taken at 20x magnification and scale bars represent 100 µm.
Fig. 7. MMP-13 inactivates alpha-1 antitrypsin (AAT). (A) Human purified AAT was incubated with pro-MMP-13 in a 1 : 5 ratio for 16 h at 37 °C in a final volume of 20 µL. Pro-MMP-13 was activated by APMA (0.67 mM). Products were separated by SDS/PAGE (10 µL) and stained with silver. (B) In similar experiments, 5 µL of incubation products was added to activity assays, in which neutrophil elastase (NE; 5 nM) was incubated with an elastase-specific fluorogenic substrate (20 µM). Reaction velocities were calculated from linear progress curves of three independent experiments, and data plotted as mean ± SD. Statistical analysis calculated by one-way ANOVA with a Tukey’s post hoc test where ***$P < 0.001$ (n = 3). (C) A time course experiment was performed, and incubation products were separated by both (i) 10% tris-glycine (protein) and (ii) 20% tris-tricine (peptide) SDS/PAGE. All gels and activity assays are representative of three independent experiments. (D) Cleavage sites were identified by N-terminal sequencing of the peptide products. MMP-13 cleaves upstream of the neutrophil elastase (NE) cleavage site in two positions, disarming the inhibitor, as depicted in both protein sequence (upper panel) and structural view (lower panel). SERPINA1 was visualised using PYMOL software (PDB ID: 1QLP). The flexible reactive-centre loop (bait region) is shown in green, and P1 residues at cleavage sites are shown for both MMP-13 (blue – Phe$^{376}$ and Pro$^{381}$) and neutrophil elastase (NE; red – Met$^{382}$). Images taken using Ray Trace mode. (E) Schematic showing working hypothesis for the interplay between neutrophil elastase, MMP-13 and AAT in cartilage. NE fully activates MMP-13, which can result in collagen breakdown. Rapid and specific inactivation of AAT by MMP-13 (along with other MMPs) may add to the proteolytic burden and further enhance activation of other latent MMP-13 within the cartilage.
High SERPINA1 expression in cartilage raises important questions about the physiological relevance of neutrophil elastase deposition from an inflamed synovium. Indeed, using the total protein ELISAs undertaken in this study, it is not possible to determine the level of active neutrophil elastase or the level of functional AAT protein. However, it has been previously demonstrated that cartilage-bound neutrophil elastase is protected from the activity of proteinase inhibitors [68]. Moreover, serpins can be inactivated by other proteinases, as well as by oxidation, both of which have been demonstrated in the RA joint previously [69–71]. These mechanisms suggest total serpin levels may not be representative of the effective inhibitor concentrations within the joint. In this study, we describe the inactivation of AAT by MMP-13. While this finding is novel, it is likely that in vivo, several MMPs may contribute to the inactivation of this inhibitor. Indeed, this serpin has been shown to be inactivated by MMP-1, MMP-2 and MMP-3 previously and the inactivated form has been observed in the synovial fluid of arthritis patients [70,71]. Here, we have identified cleavage sites generated by MMP-13 which correspond to regions N-terminal of the canonical cleavage site of neutrophil elastase (M382 is the neo-N terminus generated following this cleavage/inhibition). Both sites have been identified as cleavage sites for other MMPs [71]. Future studies could involve the generation of neo-epitope antibodies which specifically recognise MMP-cleaved AAT. Such antibodies could be used to determine the level of AAT inactivation within the synovial joint of OA patients and the likely proteolytic source. Alternatively, the relative levels of different AAT (cleaved/uncleaved or complexed) could be determined by mass spectrometry approaches. The disarming of cognate inhibitor(s) will make joints more susceptible to the damaging effects of serine proteinases which present during periods of localised inflammation. A working hypothesis regarding the interplay between MMP-mediated AAT inactivation and pro-MMP-13 activation by neutrophil elastase is outlined in Fig. 7E. A corollary of this hypothesis is the crucial role of functional AAT in mediating joint protection. To our knowledge, studies investigating the susceptibility of cartilage for degradation in OA (or indeed other arthritic diseases) in patients with AAT deficiency have not been thoroughly investigated previously. This too should be the focus of future endeavours. The high level of SERPINA1 expression within cartilage suggests mutations within the SERPINA1 gene (rendering a weaker/nonfunctional AAT) may have significant consequences for the integrity of the tissue during periods of inflammation.

There are limitations to this study. Although our observations regarding cleavages (and cleavage sites) are novel and important, we must be candid about extrapolating biochemical observations to the OA joint. To determine pathophysiological importance of neutrophil elastase on MMP-13 activation in OA, future studies would need to: (a) determine the level of active neutrophil elastase in the OA joint; (b) assess how this compares to the level of pro-MMP-13 in the cartilage; (c) establish the degree of AAT inactivation and therefore the viable inhibitor pool in the OA joint; and (d) how these change with stage and severity of OA (and the presence or absence of synovitis). Larger cohorts of human clinical samples and the use of different preclinical models of OA will prove important in this regard. Recently, Kaneva et al.[72] demonstrated that AAT protected cartilage in two animal models of inflammatory arthritis, including one induced by neutrophil elastase. Our study both supports and compliments these observations and provides novel insights into the proteolytic interplay between AAT, neutrophil elastase and MMP-13, with potential consequences for the destruction of OA cartilage. AAT can have a role beyond proteinase inhibition [61] and the authors conclude that AAT was not only chondroprotective but also anti-inflammatory and has a significant effect on pain [72]. This study and our own highlight the need to also investigate the effects of AAT administration using in vivo models of OA, with direct quantification of cartilage degradation, pain and inflammation.

The importance of synovial inflammation in OA progression is contested, and the central role of dysregulated chondrocyte biology in OA cartilage destruction is clear. However, we and others have demonstrated a variable degree of synovitis in OA patients (reviewed in [73]), and in these instances, it is credible that neutrophil elastase – given its activation potency and ability to generate a fully active MMP-13 – will contribute to OA cartilage collagen destruction through direct activation of latent MMP-13. The search for in vivo activators of MMP-13 is important, particularly as this represents a key control point for cartilage destruction and may represent a novel approach for therapy. The rapid, full activation of MMP-13 by neutrophil elastase may have significance in a subset of OA patients where an inflamed synovium contributes to the progression and exacerbation of the disease process.

Materials and methods

Reagents

Unless stated otherwise all reagents were of the highest purity and purchased from Sigma-Aldrich (Gillingham,
Human neutrophil elastase (EC3.4.21.37) purified from human sputum was purchased from Elastin Products (SE663; Owensville, MO, USA). AAT protein was purified as described previously [74]. Human recombinant pro-MMP-1 (E.C.3.4.24.7) and pro-MMP-13 (E.C.3.4.24.24) were expressed using an insect cell expression system (and purified) as previously described [11,37]. Recombinant human pro-MMP-8 (E.C.3.4.24.34) was purchased from R&D Systems (908-500-010; Abingdon, UK). Recombinant human active MMP-3 (EC3.4.24.17) was expressed and purified as previously described [75]. Recombinant human full-length tissue inhibitor of metalloproteinase (TIMP)-1 was a kind gift from Celltech Pharmaceuticals, Slough, UK. Elastase fluorescent substrate (CAS 72252-90-5), MMP fluorogenic substrate (FS-6; SCP0193) and GM6001 were purchased from Merck Millipore (Watford, UK). Neutrophil elastase (ab68672) and MMP-13 (Ab75606) antibodies were purchased from Abcam (Cambridge, UK). MMP-13 and neutrophil elastase DUOset ELISA kits were purchased from R&D systems. Ficoll-Paque was purchased from GE healthcare (GE Healthcare, Chalfont St Giles, UK). Cytochalasin B and f-Met-Leu-Phe (IMLP) were purchased from Sigma-Aldrich. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from Roche Applied Biosciences (Burgess Hill, UK).

**Human tissue and cartilage explant culture**

All tissue was taken with informed consent from patients undergoing total joint replacement at the Freeman Hospital in Newcastle upon Tyne. All OA tissues (cartilage, synovium and synovial fluid) were handled according to Newcastle University guidelines with ethical approval from NHS Health Authority NRES Committee North East – Newcastle and North Tyneside (REC14/NE/1212). Neutrophils were isolated from consenting healthy donors at the University of Liverpool, approved by the University of Liverpool Research Ethics Subcommittee for physical interventions (Ref: 1672). For cartilage explant cultures, macroscopically intact human OA cartilage was cut from the tibial plateau (approximately 2-mm³ pieces) and cultured overnight in 24-well plates (three chips per well) in serum-free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with glutamine (2 mM), penicillin/streptomycin (200 IU·mL⁻¹ and 200 μg·mL⁻¹, respectively) and nystatin (40 IU·mL⁻¹). The following day, explants were stimulated with neutrophil elastase for the time period and concentrations indicated. At the end of the experiment, medium was harvested and remaining cartilage digested with papain as previously described [51]. For both medium and digested cartilage, OHP assays were performed to assess percentage collagen destruction, while proteoglycan destruction was assessed by measuring glycosaminoglycan (GAG) levels using dimethyl methylene blue (DMMB) assay (see [51] and references therein).

**In vitro incubations, SDS/PAGE and N-terminal sequencing**

For *in vitro* incubation experiments, neutrophil elastase (20 nm) was incubated with pro-MMP-13 (100 nm) in ‘reaction buffer’ (100 mM Tris pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 0.01% (w/v) Brij35) for the indicated time points at 37°C, in a final volume of 20 μL. APMA was used as a positive control (0.67 mM). For inactivation experiments, AAT (50 nm) was incubated with MMP-13 (10 nm), APMA or both for 16 h at 37°C. The incubation products (10 μL) were subsequently incubated with neutrophil elastase to determine inhibitory capacity (see below for assay details). For protein gels, digestion products (10 μL for AAT inactivation and 20 μL for pro-MMP-13 activation experiments) were mixed with 5× sample buffer (625 mM Tris/HCl pH 6.8, 10% (w/v) SDS, 40% (v/v) glycerol, 0.5% (w/v) bromophenol blue, 3% (v/v) β-mercaptoethanol) and separated by 10% SDS/PAGE as described previously [37]. Gels were subsequently stained using a PlusONE silver staining kit according to the manufacturer’s instructions (GE healthcare). For low molecular mass products of AAT digests, peptides were detected by separating reaction products by Tris-Tricine SDS/PAGE as described previously [76]. For N-terminal sequencing (both activation and inactivation experiments), gels were blotted onto PVDF and stained with Coomassie Brilliant Blue for 2 min. Bands were excised with a clean scalpel blade and sent for N-terminal sequencing (Alphalyse, Odense, Denmark).

**Enzyme activity assays**

To test MMP activation, pro-MMP-1, pro-MMP-8 and pro-MMP-13 were incubated with elastase as described above, for 4 h. As a negative control, an excess of AAT (1.14 mg·mL⁻¹) was included, which demonstrated the requirement for elastase activity. Incubation products (5 μL) were added to white-walled plates and diluted to 80 μL in MMP assay buffer (reaction buffer with 0.1% (v/v) polyethylene glycol 6000). MMP fluorogenic substrate (FS-6) was added to start the reaction at a final concentration of 10 μM, to a total well volume of 100 μL. Reaction velocity was calculated by linear regression of initial rates of progress curves. For mole-per-mole comparison experiments, neutrophil elastase was titrated with freshly resuspended AAT protein (Merck Millipore; 178251). MMP-3 was titrated with full-length TIMP-1, while matriptase and hepsin were titrated with 4-methylumbelliferyl 4-guanidinobenzoate (MUGB). Pro-MMP-13 (100 nm) was incubated with 20 nm of each enzyme for 4 h in the reaction buffer described above prior to conducting the MMP

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activity assay. To measure MMP activity in human OA cartilage conditioned media, 50 µL of medium was used with FS-6 substrate as described above. To test AAT inhibitory capacity following MMP-13 incubation, elastase activity assays were performed in a similar manner using elastase activity buffer (100 mM Tris pH 7.6, 150 mM NaCl, 0.01% (w/v) Brij 35) and an elastase fluorogenic substrate (MeO Suc-AAPV-AMC) at a final concentration of 20 µM. Neutrophil elastase was used at a final concentration of 5 nM.

Neutrophil isolation, degranulation and MMP-13 activation

Neutrophils (purity typically > 97%, assessed by cytoospin) were isolated from heparinised whole blood using HetaSep and Ficoll-Paque as previously described [77] and resuspended at 5 x 10^6 cells·mL^-1 in 1 mL of RPMI 1640 media supplemented with HEPES (25 mM) and l-glutamine (2 mM). Neutrophils were incubated with GM-CSF (5 ng·mL^-1) for 30 min followed by cytochalasin B (6 µg·mL^-1) and f-Met-Leu-Phe (fMLP, 1 µM) for a further 30 min. Following incubation, cells were pelleted at 1000 g for 3 min and supernatant decanted into clean tubes. Degranulation products (supernatant; 5 µL) from either treatment were incubated with pro-MMP-13 (100 nm) for 2 h at 37 °C. Controls of the degranulation stimulants (cytochalasin/GM-CSF/fMLP mix in the absence of neutrophils) were also incubated with pro-MMP-13. AAT (50 nm) was included as a negative control. After the incubation, 5 µL was added to a white-walled 96-well plate and an MMP and elastase activity assays were undertaken as described above. Data are presented as mean ± SD using three independent donors of neutrophils. For SDS/PAGE, 10 µL of this incubation was mixed with 5x sample buffer (as above), boiled and kept at -20 °C until the products were separated by SDS/PAGE and western blotting performed.

Western Blotting

For western blotting, samples were separated by SDS/PAGE using 10% (w/v) precast acrylamide gels and transferred onto polyvinylidene fluoride (PVDF) using the Trans-Blot Turbo apparatus (Bio-Rad, Watford, UK). Blots were blocked in 5% (w/v) nonfat dry milk for 1 h, washed in tris-buffered saline-Tween 20 (TBS-T), prior to overnight incubation at 4 °C with an MMP-13 antibody (sc-30073, Santa Cruz, CA, USA), and diluted 1 : 2000 in 5% (w/v) bovine serum album (BSA). Blots were then further washed in TBS-T prior to incubation with an anti-rabbit secondary (GE Healthcare, Bucks, UK) at 1 : 2000 dilution in milk for 1 h. Blots were washed again in TBS-T and detected using enhanced chemiluminescent substrate (ECL; Thermo Fisher, Loughborough, UK).

ELISA

Levels of MMP-13 and neutrophil elastase in human OA knee synovial fluids (eight samples; six females/two males; age 69.5 ± 10.4) were determined by ELISA according to the manufacturer’s instructions (R&D Systems). Briefly, MaxiSorp plates (Thermo Fisher) were incubated with coating antibody overnight at 4 °C. Plates were washed in PBS with Tween (0.1% v/v) and blocked in 300 µL of 1% (w/v) BSA in PBS for 1 h. Plates were washed and standards, and samples (100 µL; neat or diluted in PBS) were loaded onto the plates and incubated overnight. Plates were washed and detection antibody diluted in PBS with 0.1% (w/v) BSA added to the wells. Plates were washed and streptavidin-horseradish peroxidase (HRP) diluted in PBS with 0.1% (w/v) BSA. Colour development was initiated by the addition of o-phenylenediamine dihydrochloride substrate solution, the reaction stopped with 3 mM HCl and the absorbance read at 490 nm.

Histology and Immunohistochemistry

Human synovial tissues (17 patients, 14 Female/three male, mean age = 65 years ± 9.2) were fixed in 10% formalin and embedded in paraffin, and sections were taken at 4-µm intervals. Haematoxylin and eosin (H&E) staining was performed as previously described [11] and synovitis scored according to a previously described system [37,40]. Immunohistochemistry was performed essentially as previously described [37], except antigen retrieval was performed using a mini autoclave (Aptum 2100 retriever; Aptum, Southampton, UK) for 30 min. Primary antibodies for neutrophil elastase and MMP-13 were used at 1 : 100 dilution (both 10 µg·mL^-1) for 1 h, and staining conducted using VECTASTAIN Elite ABC HRP Kit according to the manufacturer’s instructions (Vector Laboratories; Burlingame, CA, USA). To visualise AAT human OA cartilage, samples from patients undergoing total hip replacement (mean age 61.3 ± 7.5, 3 Female) or from hip replacement following fracture (NOF; mean age 74 ± 8.4, 1 Male, 1 Female) were fixed, embedded and sectioned as described above. Serial sections were stained with safranin-O or underwent IHC staining using an antibody against AAT protein (16382-1-AP; ProteinTech, Manchester, UK) at 1 : 150 dilution (3.2 µg mL^-1) for 2 h, followed by signal detection using VECTASTAIN Elite ABC HRP Kit. In all cases, sections treated in the same manner in the absence of a primary antibody, or with an isotype control antibody (R&D systems; AB-105-C) at the same concentration as the corresponding primary antibody, served as negative controls.
Images were taken using a Leica Biosystems DM4000B light microscope.

**Bioinformatic analyses and protein visualisation**

Transcripts per million values in control cartilage (NOF) samples were generated using Salmon as described [39] while Human Protein Atlas TPMs were retrieved from the Human Protein Atlas ([78]; www.proteinatlas.org). R software was used to generate figures from RNA sequencing data in Fig. 6A,B. For structural images, AAT was visualised using PYMOL software (version PyMOL v1.7.6.6 Enhanced for Mac OS X) using the structure of the intact protein [Protein Data Bank (PDB) ID 1QLP; [79]].

**Statistics**

Statistical analyses where multiple comparisons are required were performed using a one-way ANOVA with a Tukey’s post hoc test. In all cases, \( *P < 0.05, **P < 0.01, ***P < 0.001 \) and data are presented as mean ± SD. RNA-seq statistical analysis was performed using DESeq2 where \( P \)-values were adjusted using the Benjamini–Hochberg method which controls for the false discovery rate (\( < 0.05 \)).

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**Conflict of interest**

The authors declare no conflicts of interest.

**Author contributions**

DJW and ADR conceptualised the study; DJW, AMDF, HLW, HL, KY, KC, SHC, MdCA, SJ, RR, KSR, DAY and ADR investigated the study; DJW, AMDF, HL, KC and ADR involved in formal analysis; DJW, AMDF and ADR wrote – original draft; DJW, AMDF, HLW, HL, KY, SHC, MdCA, SJ, RR, KSR, DAY and ADR wrote – review and editing; DJW, KSR, DAY and ADR supervised the study; and DJW, DAY and ADR involved in funding acquisition.

**Ethics approval and consent to participate**

All tissues were taken with informed consent from patients undergoing total joint replacement at the Freeman Hospital in Newcastle upon Tyne. All OA tissue (cartilage, synovium and synovial fluid) was handled according to Newcastle University guidelines with ethical approval from NHS Health Authority NRES Committee North East – Newcastle and North Tyneside (REC14/NE/1212). Neutrophils were isolated from healthy donors at the University of Liverpool. This study was approved by the University of Liverpool Research Ethics Subcommittee for physical interventions (reference 1672). All participants gave written, informed consent in accordance with the declaration of Helsinki.

**Peer Review**

The peer review history for this article is available at https://publons.com/publon/10.1111/febs.16127.

**Data accessibility**

The data supporting the conclusions of this article are included within the article (and its additional files). The RNAseq dataset used has been published previously [39] and is publicly available (GSE111358). Data are available from the corresponding author upon reasonable request.

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