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Increased matrix metalloproteinase-9 (MMP-9) activity observed in chronic wound fluid is related to the clinical severity of the ulcer

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Short Title: Increased MMP-9 activity in chronic ulcers

Abbreviations: acute wound fluid, AWF; chronic wound fluid, CWF; N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide, GM6001; human serum, HS; matrix metalloproteinase, MMP; sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE; Tris-buffered saline solution with 0.1% Tween-20, TBST.

Abstract

Background: The pathology of chronic wounds is often characterised by <u>elevated levels of pro-inflammatory cytokines (e.g. tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)), proteases (e.g. matrix metalloproteinases (MMPs)) and neutrophil elastase. MMPs specifically have been implicated by a number of studies as the major protease family responsible for</u> the degradation of key factors critical to the ulcer's ability to heal.

Objectives: To assess individual MMPs in CWF to develop improved treatments for chronic ulcers.

Methods: Collagen Type I and IV zymography, immunoprecipitation followed by a substrate activity assay, and an indirect enzyme-linked immunosorbant assay (ELISA) were all used to analyse MMP levels in CWF.

Results: Our studies demonstrate that there is excessive protease activity in chronic wound fluid (CWF) compared with both human serum (HS) and acute wound fluid (AWF), which can be specifically attributed to MMPs as determined through a MMP-inhibitor study. Multiple MMPs were then immunoprecipitated from the CWF samples and MMP-9 was identified as the predominant protease in CWF, with significantly elevated activity levels in CWF compared with AWF. In addition, the clinical status of the ulcer is directly associated with the amounts of MMP-9 present in the wound fluid. <u>Therefore, this study suggests that higher levels of MMP-9 in chronic wound fluid correlate with a clinically worse wound</u>.

Conclusions: In view of these results, it is hypothesised that a specific inhibitor of MMP-9 could potentially be more therapeutically effective than general MMP inhibitors in modulating chronic ulcers towards a healing state.

1.   Introduction

Wound healing is a highly complex process that can lead to chronic ulcers if any stage in this intricate series of events is interrupted[1]. While there is no single reported cause of these ulcers, many factors are considered important when distinguishing chronic wounds from their acute counterparts. Predominantly, these wounds are characterised by excessive granulation tissue and increased fibrosis[2], along with significant increases in pro-inflammatory cytokines, proteases and neutrophil elastase[3-6]. Furthermore, excessive inflammation caused by an hyper-stimulated neutrophil response has also been suggested as a potential cause for a wound's chronicity[7]. This can be further exacerbated by susceptibility of open wounds to infection[8]. It is this protease activity, primarily caused by a specific group of proteases called matrix metalloproteinases (MMPs), that is believed to be responsible for the increased extracellular matrix destruction observed in chronic wounds[3]. However, there is considerable conflicting information in the literature concerning both the under- and over-expression of specific MMPs in chronic wounds.

Gelatinases, also referred to as MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B), are thought to have an extremely important function in normal wound healing during both the remodelling and re-epithelialisation phases, and it has been proposed that wound healing would be impaired if they were inhibited[9]. Conversely, other reports have suggested that both MMP-2 and MMP-9, when present at the significantly higher activity levels reported for chronic wounds, need to be inhibited to allow normal healing to occur[6,10]. The main reason why gelatinases are so important is that they are able to break down collagen more effectively than other MMPs[11], as well as cleave

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the major constituent of the basement membrane – collagen type IV[4]. Indeed, MMP-9 was shown to be able to cleave collagen types I-V into small peptides at the physiological temperature of 37 ºC[12]. In addition, MMP-2 has been shown to play an important role in the reorganisation of collagen lattices *in vitro*[13], as well as being only one of two MMPs found to play an active role in non-injured skin[11].

MMP-9 is produced by a number of different cell types, namely inflammatory cells, neutrophils, macrophages, monocytes and keratinocyte[10,14]. As chronic ulcers are classified as non-healing, the MMP-9 present is CWF is thought to be either neutrophil- or macrophage-derived, since these are the main cells present in an open non-healing ulcer[14]. In terms of MMP-9 expression in chronic ulcers, one study demonstrated that MMP-9 is temporally expressed during the healing of chronic wounds[10]. This also correlates with another report that showed MMP-9 activity levels in CWF were 25-fold elevated over a normal control[15]. In contrast, Cullen *et al.* (2002) reported a lack of MMP-2 in CWF, which as MMP-2 is fibroblast-derived, suggests that there must a lack of fibroblasts in these chronic wounds and hence perhaps underlying the lack of wound closure[14].

In order to provide some clarity as to the role of MMPs in chronic ulcers, we elected to more fully examine these proteases in CWF. This choice of clinical sample was adopted as the longer term goal of this research was to develop a wound dressing to modulate this fluid. In addition, most investigators focus on total protease activity in CWF rather than individual proteases; therefore we were interested in examining individual MMP species using multiple detection techniques. It was hoped that this approach would help clarify the variable and conflicting data previously published on

MMPs in chronic wounds. Furthermore, our studies focussed on MMP-9 as it was identified through our studies to be the major MMP in CWF. In addition, it has the ability to cleave a number of substrates, as well as being produced mainly in inflammatory cells – with these cells hypothesised as being major contributors to an ulcer's chronicity. Finally, the clinical status of the ulcer was considered in relation to MMP-9 protease activity to establish if there was an association between the two.

Materials and Methods

Wound fluid sample collection and preparation

Chronic wound fluid (CWF) samples were obtained from consenting patients of the St. Luke's Nursing Services (Brisbane, QLD, Australia) suffering from chronic venous ulcers undergoing compression therapy *(Table 1)*. Ethical approval to collect these samples was obtained from both the Queensland University of Technology (QUT) and St. Luke's Nursing Services. A standard wound fluid collection technique has been established and was carried out at the clinical site. In addition, patients exhibiting infections in their ulcers were excluded from the study. Briefly, ulcers were washed with sterile water prior to collecting wound fluid, followed by the application of an occlusive dressing over the wound[16]. Exudate accumulated under the dressing after 30 min to 1 h was recovered by washing with 1 mL of saline. Three separate acute wound fluid (AWF) samples were collected from naturally occurring sub-epidermal blisters on the feet of volunteers. The fluid was removed with 26G x 0.5" needle and syringes (Terumo Medical, Somerset, NJ, USA) and collected in 1.5 mL Protein LoBind tubes (Eppendorf, Hamburg, Germany). The wound fluid samples were centrifuged at 14,000 g for 10 min, then the supernatant filtered using 0.45 μm cellulose acetate filters (Agilent Technologies, Wilmington, DE, USA). This wound fluid collection technique was chosen as being the most suitable approach, primarily because it does not rely on the absorption of wound fluid on to a porous, hydrophilic wound dressing. In particular, the use of absorption techniques has been reported to result in lower sample volume and protein amount[17]. In addition, other techniques that rely on this absorption approach do not take into account that proteins may

differentially adhere to these dressings, which will then influence the CWF sample in later analyses[18]. A pooled human serum (HS) sample was purchased commercially from Sigma-Aldrich (St Louis, MO, USA). Protein content for all samples was quantitated and standardised using the BCA Protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The samples were then sub-aliquoted and stored at -80 ºC until further analysis.

Identification of protease activity using collagen zymography

Collagen zymography was performed as previously described[19] using Collagen Type I and Collagen Type IV (Sigma-Aldrich) at a final concentration of 0.5 mg/mL in 10% total acrylamide gels under non-reducing conditions. Briefly, electrophoresis was performed at 4 ºC under Laemmli conditions[20]. The gels were then washed in 2.5% Triton X-100 for 30 minutes, then a further 60 minutes, prior to incubation in 50 mM Tris-HCl, 10 mM $CaCl_2$, 50 mM NaCl at pH 7.6 for 24 hours at 37 ºC. The gels were then stained using 0.25% Coomassie brilliant blue R-250 (Bio-rad Laboratories, Hercules, CA, USA) (40% methanol, 10% acetic acid) and destained appropriately (40% methanol, 10% acetic acid). Protease activity was visualised as clear (unstained) bands.

Analysis and quantitation of collagen zymography

Gels were scanned using GeneSnap version 6.07 (SynGene, Cambridge, UK) and analysed using GeneTools version 3.07 to quantitate the clear bands (SynGene). All quantitations were performed using the analysis of 3 separate gels per treatment and

values were expressed as a relative % of the MMP standard, similar to previously published reports[13,21]. The data was also analysed in terms of grouping the samples based on the "PUSH" score of the wounds from which the CWF was collected. Clinicians use the PUSH (Pressure Ulcer Scale of Healing) score to grade the wounds (1 = best, 17 = worst) with this value taking into account a number of factors to score the ulcer[22]. While this measurement technique was originally designed by the National Pressure Ulcer Advisory Panel to grade pressure ulcers, it has also been validated as an effective tool to monitor healing trends in venous ulcers[22]. Thus, Ratliff *et al.* (2005) noted that this system was a simple, valid and reliable tool for monitoring venous ulcer clinical status[22]. In view of this, the data in our study was organised in terms of PUSH score, with five samples (CWF-1, -2, -3, -4, -5) exhibiting a high PUSH score (≥12) and four samples (CWF-6, -7, -8, -9) showing a lower PUSH score (≤11). While we ideally would have preferred to assess a greater number of samples, CWF sample numbers were restricted due to patient availability, as well as the amount of protein required from individual samples to complete the full spectrum of experiments reported here.

Confirmation of MMP-specific collagen degradation using GM6001 inhibitor

To confirm that the collagen degrading activity visualised through zymography was due to MMPs, a specific MMP inhibitor, GM6001 (Ilomastat or N-[(2R)-2-(hydrox-amidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide) (Chemicon, Temecula, CA, USA) was used. Increasing concentrations of GM6001 (1, 3, 10, 30, 100, 300 and 1000 µM) were incubated with CWF-1 samples at 37 ºC for 24 hours.

These samples were then run on Collagen Type I zymograms and analysed as described above.

Specific identification of individual MMPs through immunoprecipitation

Further confirmation of MMP activity was established through the immunoprecipitation of specific MMPs followed by collagen zymography. UltraLink Immobilized Protein A/G resin (Pierce Biotechnology) was incubated with individual MMP antibodies in a Tris-buffered saline solution with 0.1% Tween-20 (TBST) and rotated for 5 hours at 4 ºC in 0.5 mL Protein LoBind tubes (Eppendorf). The antibodies were: a mouse mAb for human MMP-9 (Ab-7) (Calbiochem, San Diego, CA, USA); a rabbit pAb for human MMP-8 (Chemicon); and a rabbit pAb for human MMP-13 N-terminal (Chemicon). Following incubation, the solution was centrifuged at 2,000 rpm for 2 minutes, the supernatant removed, and the resin then washed twice with TBST. This "charged" resin was then incubated with the human serum, AWF and CWF samples for 18 hours at 4 ºC, constantly rotating. As before, following incubation the solution was centrifuged at 2,000 rpm for 2 minutes, the supernatant removed, and the resin was washed twice with TBST. The resin was then mixed with a 2X SDS sample buffer and left at room temperature for 1 hour. To examine the bound proteins, these resin samples were run on Collagen Type I zymograms and analysed as described above.

Quantitative confirmation of MMP-9 levels through a direct enzyme‐ linked immunosorbant assay (ELISA)

Quantitative MMP-9 levels in HS, AWF and CWF samples were obtained using a direct ELISA. Briefly, 96-well plates (Nunc) were coated with 40 ng of the sample/well and left overnight at 4 ºC. Next, the wells were washed three times with TBST and blocked with 5% skim milk powder (SMP) in TBST for 1 hour at room temperature. Wells were again washed with TBST and then probed with a rabbit polyclonal antibody (1:1000) raised against the MMP-9 whole molecule (Abcam, Cambridge, UK) for 1 hour at room temperature. Following washing, the wells were probed with polyclonal goat anti-rabbit immunoglobulins/HRP (1:1000) (Dako Denmark A/S, Glostrup, Denmark) for 1 hour at room temperature. Then, the wells were again washed and rinsed a final time with TBS before adding the substrate solution, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich) for 30 minutes at room temperature. The optical density of the plate was then read at 405 nm on a standard plate reader (Bio-rad Laboratories). The amount of MMP-9 in the samples was calculated from a standard curve and then converted to MMP-9 (ng)/total sample protein (μg). Data was organised in terms of the sample's PUSH score.

Results

Elevated proteolytic activity in chronic wound fluid

In order to analyse the proteolytic activity in wound fluid samples, CWFs from nine separate patients *(Table 1)* were analysed using Collagen Type I and Collagen Type IV zymography to identify protease activity. In addition, human serum (HS) and acute wound fluid (AWF, n=3) samples were analysed. Collagens were used for the zymography studies as they are the major extracellular matrix constituents of the skin, contributing to approximately 70% of its dry weight. Furthermore, Collagen Type I accounts for approximately 80% of the skin collagens, while Collagen Type IV forms the basement membrane[23], an important structure that is commonly disrupted in large non-healing ulcers. It is noted that there are other extracellular matrix proteins important in epithelial wound healing, e.g. Collagen Type III[24] and fibronectin[25], but for the purposes of this study only Collagens Type I and IV were investigated. Collagen degrading activity was identified as clear bands on a dark background in the zymograms, with some smearing evident in the standards, as seen previously by others[26]. Ten times more total protein of both the HS and AWF samples had to be loaded on the gels in order to adequately visualise the collagenase-like activity on both substrates. As shown in *Figures 1-2*, the degradation profiles vary between the patient samples in terms of both intensity and molecular weight of the visualised bands. However, the bands appear to be consistent between the two different substrates.

More specifically, Collagen Type I zymography revealed a high level of protease activity in the CWF samples (lanes 6-14), especially considering the lower amount of the sample loaded (*Figure 1*). In addition to the main band at approximately 102 kDa, there are also smaller and potentially more active forms visible at 75 kDa, 66 kDa and 54 kDa across the samples. Compared to CWF, less protease activity was evident in both the HS (lane 2) and AWF samples (lanes 3-5), especially considering the increased amount of protein loaded in these lanes for these samples. The main band in the AWF samples correlates with a mass of 66 kDa, similar to the main band seen in the HS. CWF-6 (lane 11) appears to have less proteolytic activity than the other CWF samples, however, the activity present was greater than that found with the HS and AWF samples.

Similar to the results found with the Collagen Type I zymography, Collagen Type IV zymography also revealed increased levels of protease activity in CWF (lanes 6-14) as compared with HS (lane 2) and AWF samples (lanes 3-5) (*Figure 2*). The main band in CWF is visualised at 102 kDa, suggesting that this is the same protease as identified with Collagen Type I zymography. The main differences observed with Collagen Type IV zymography, as compared to Collagen Type I zymography, are that firstly, there seem to be less bands of lower molecular weight activity with this substrate, and also, the amount of proteolytic activity is reduced when taking into account the fact that twice the amount of sample was loaded.

MMPs are responsible for collagen degradation as shown through a MMP specific inhibitor study

The MMP-specific inhibitor, GM6001, was used to confirm that the collagen degradation revealed by zymography was indeed due to MMPs and not another group of proteases present in the wound fluid samples. Through incubation with increasing concentrations of GM6001 at 37 ºC over 24 hours, the proteolytic activity in CWF-1 gradually decreased as revealed by Collagen Type I zymography. Indeed, no visible degradation of collagen was apparent at 1 mM GM6001 (lane 9) (*Figure 3a*). Densitometry was used to quantify the reduction in proteolytic activity revealed by zymography, and the levels are represented graphically in *Figure 3b*. Both the total amount of protease activity, plus the protease activity in individual molecular weight species, was followed across the increasing concentrations of 1, 10, 100 and 1,000 µM concentrations of GM6001 and the data is expressed as relative % of the untreated CWF-1 ± SEM. In all cases, the collagen degrading activity decreases when increasing concentrations of the MMP-specific inhibitor are present, confirming that the majority of the proteases responsible for collagenase-like activity in these CWF samples are from the MMP family.

Initial immunoprecipitation of MMPs from CWF samples

Following confirmation that the majority of collagen-degrading activity seen in CWF samples was due to MMP-specific activity, individual MMPs were immunoprecipitated from the CWF samples. Thus, individual MMP antibodies were linked to a protein A/G resin and then incubated with the CWF samples. Antibodies for MMP-1, -8 and -13 were used to identify particular species in terms of relative abundance when compared to the complete samples *(Figure 1)*. In view of this, six CWF samples were analysed separately using specific immunoprecipitation of MMP-

8, followed by analysis through Collagen Type I zymography as described previously. There appeared to be minimal MMP-8 activity across all 6 CWF samples containing the MMP-8 enriched fractions *(Figure 4a)*. MMP-13 was also analysed across the same 6 CWF samples and revealed similar results *(Figure 4b)*. Similarly, MMP-1 was examined in select samples *(data not shown)* and again, minimal activity was present. Positive controls of individual purified MMP proteins were also run in parallel to ensure that these reduced results were not simply due to laboratory analysis, and therefore were indeed indicative of the MMP levels in the CWF samples. Thus, the levels of MMP-1, -8 and -13 present in the samples were insufficient to cause the majority of Collagen Type I degrading activity as revealed in the complete CWF samples through zymography *(Figure 1)*.

MMP-9 is the predominant protease responsible for matrix degradation in chronic wound fluid

The CWF samples were then investigated for MMP-9 activity levels since MMP-9 can cleave collagen types I-V [12], and because previous reports suggested excessive levels of MMP-9 were present in CWF [15]. Through incubation with a MMP-9 monoclonal antibody linked to a A/G protein resin, MMP-9 was analysed in terms of relative abundance within HS, AWF and CWF samples. Both the bound and unbound fractions from the MMP-9 immunoprecipitation step were analysed through Collagen Type I zymography and then quantitated through densitometric analysis. The degradation profiles obtained via zymography varied in terms of both abundance and molecular weight of the proteolytic activity. Samples were also analysed in terms of grouping the samples based on the "PUSH" score of the wounds from which the CWF

was collected. Clinicians use the PUSH (Pressure Ulcer Scale of Healing) score to grade the wounds (1 = best, 17 = worst) with this value taking into account a number of factors, i.e. ulcer size, exudate amount and tissue type, to score the ulcer[22]. This therefore allows a more complete clinical picture to be shown, instead of simply relying on single parameters, e.g. ulcer duration. However, previously reported indicators, e.g. ulcer size[27], also score quite highly on the PUSH scale, which is shown through further analysis of the patient data in *Table 1* (Patient 3, ulcer size = 84 cm^2, PUSH = 15).

The MMP-9-enriched fractions of CWF, that is the fraction that bound to the MMP-9 antibody-linked resin, showed high levels of Collagen Type I degrading activity *(Figure 5a)*. However, there was minimal activity in the MMP-9-enriched fractions of human serum and AWF samples, except for AWF-2. The molecular weight profile in these bound fractions appeared quite similar to the Collagen Type I zymograms of the complete samples *(Figure 1)*, possibly indicating that MMP-9 was indeed the major constituent of the collagen degrading activity in the wound fluids. Quantitatively, there were significant differences between HS and AWF-2 (p<0.05), and with all CWF samples (p<0.01), except for CWF-6 *(Figure 5b)*. In terms of grouped samples, the CWF samples showed statistically significant increased collagenase-like activity (p<0.01) when compared to both HS and AWF samples *(Figure 5c)*. Further detailed analysis of the samples in terms of the PUSH scores revealed that both the lower PUSH score (PUSH ≤11) grouped samples and the higher PUSH score (PUSH ≥12) grouped samples both had significantly increased MMP-9 activity compared to both HS and AWF samples (p<0.01) *(Figure 5d)*.

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MMP-9-depleted fractions, that is the fraction that did not bind to the MMP-9 antibody-linked resin, showed lower levels of Collagen Type I degrading activity *(Figure 6a)* than both the unfractionated samples *(Figure 1)* and MMP-9-enriched fractions *(Figure 5a)*. Furthermore, there were increased levels of collagenase-like activity in the depleted fractions of CWF samples 1-5 and 7-9, compared with HS, AWF samples and CWF-6. Quantitatively, the levels of MMP-9 activity were significantly increased in CWF samples 1-3 and 8-9 ($p<0.01$) and CWF-7 ($p<0.05$), with similar trends evident in CWF samples 4 and 5, although they were not statistically significant *(Figure 6b)*. When the samples were grouped into their relative types, that is CWF versus AWF versus HS, the CWF samples displayed highly significant Collagen Type I degrading activity when compared with HS and AWF samples ($p<0.01$) *(Figure 6c)*. In addition, analysis of the samples in terms of PUSH scores revealed that the higher PUSH score (PUSH ≥ 12) group had significantly increased MMP-9 activity compared to HS and AWF samples ($p<0.01$), as well as the lower PUSH score (PUSH ≤ 11) group ($p<0.05$). In addition, the lower PUSH score (PUSH ≤ 11) group was not significantly different from either HS or AWF samples. The main differences in the zymography profiles of the bound and unbound fractions were at an approximate molecular weight of 65 and 55 kDa respectively. Therefore, from these results it appears that increased levels of MMP-9 activity are significantly different in CWF, as compared with both HS and its acute counterpart, in terms of both activity and molecular weight species. In addition, the fractions that had been depleted of MMP-9 appear to display a trend towards a higher level of Collagen Type I degrading activity correlating with a higher PUSH score, i.e. a clinically worse ulcer.

MMP-9 levels present in chronic wound fluid correlate with the clinical severity of the ulcer

To confirm the findings found by immunoprecipitation, a direct ELISA was used with a different MMP-9 antibody to ensure accuracy in antibody specificity. MMP-9 levels for all samples ranged from approximately 70 ng/µg total protein to 370 ng/µg total protein. In all AWF samples, there were no statistically significant differences in MMP-9 levels compared to HS. Concerning the CWF samples, CWF-8 displayed significantly increased levels of MMP-9 compared with HS ($p<0.05$), with CWF samples 1-3, 5 and 9 also showing statistically increased MMP-9 levels ($p<0.01$) *(Figure 7a)*. When grouped samples were analysed, CWF showed a statistically significant increase in MMP-9 levels compared with both AWF and HS ($p<0.01$) *(Figure 7b)*. In addition, when the samples were grouped according to their clinical PUSH score, the lower PUSH score (PUSH ≤11) group was not significantly different from HS, but did have statistically significant differences from both AWF and the higher PUSH score (PUSH ≥12) group ($p<0.01$) *(Figure 7c)*. Furthermore, the higher PUSH score (PUSH ≥12) group showed a significant increase in MMP-9 levels compared with both HS and AWF ($p<0.01$) *(Figure 7c)*. From these results it appears that there is a trend towards increased levels of MMP-9 correlating with an increased PUSH score, and hence a clinically worse ulcer.

Discussion

A major factor responsible for the chronic nature of ulcers is believed to be the tissue-destructive events mediated by certain endopeptidases[28,29]. These neutral endopeptidases are able to degrade the extracellular matrix at the ulcer's physiological pH and can be classified into two groups: serine proteases and matrix metalloproteinases[29]. A previous study by Weckroth *et al.* (1996) showed that serine proteases of polymorphonuclear neutrophil origin, e.g. cathepsin G and elastase, were both low in activity in CWF[30]. For this reason, the studies reported herein focussed on MMPs.

In terms of MMPs, there have been a number of studies measuring MMP activity in CWF, but many of these studies have only used general techniques to identify a broad range of MMPs e.g. the Azocoll assay[1,4], gelatin zymography[1,10,15,31] and antibody detection[32]. As most members of the MMP family are structured into three essential, characteristic and highly conserved domains[33], it is often quite difficult to differentiate MMP species based on molecular weight alone. In addition, antibody assays that are based on specific MMPs often find it difficult to differentiate between closely related species, as there is a very close evolutionary relationship within the family, as is reflected by their highly conserved domains[34]. Indeed, this high degree of structural similarity is likely to be the main reason underlying the controversy surrounding levels of specific MMPs in chronic wounds. In view of this, the studies reported herein used multiple techniques to identify specific MMPs species. Together, the combination of an antibody-based identification assay with an activity assay has allowed more conclusive and robust results. Indeed, the findings reported herein

indicate that there are minimal levels of MMP-1, -8 and -13 activity in CWF, with MMP-9 being the most likely candidate responsible for Collagen Type I degradation in CWF. This was further confirmed by using a different MMP-9 antibody for the ELISA as compared with that employed for the immunoprecipitation studies.

All nine CWF samples examined exhibit higher levels of MMP activity than the AWF and HS samples. While CWF-6 appears to have less MMP activity than the other CWF samples, it still contains more MMP activity than the HS and AWF samples. These differences within the sample groups, however, highlight the difficulties of dealing with CWF as a diagnostic sample; due to the heterogeneity of these samples, and the wounds from which they are obtained, it is often quite difficult to achieve statistically conclusive results. While there have been many studies using CWF from venous ulcers as a clinical indicator of the ulcer's status, there is still a large amount of ambiguity in this area concerning sample collection, sample standardisation, and long-term storage[35]. Furthermore, variations in patient wound care, clinical measures and sample collection all contribute to the complications shown in this area of research[36].

While these sample limitations have to be considered, in the study we report here, it is clear that Collagen Type I and IV degradation by CWF samples is higher than that observed with HS and AWF samples. In addition, our data demonstrate that the collagen degradation can be specifically attributed to MMPs as shown through the targeted inhibitor study using GM6001, a specific MMP inhibitor[37]. Thus, GM6001 was shown to inhibit the CWF-induced collagen degradation in a dose-dependent manner. Together, these data confirm the hypothesis that elevated MMP levels in

CWF are indicative of a clinically worse chronic ulcer and furthermore, suggest that inhibition of these proteases holds promise as a therapeutic treatment for these debilitating chronic wounds.

More specifically, these experiments suggest that MMP-9 appears to be the major protease responsible for matrix degradation in CWF. This is similar to previous reports by Ladwig *et al.* (2002), which suggested that there was a statistically significant correlation between poor healing and elevated levels of MMP-9 in patients, regardless of the treatment regime[6]. Moreover, Wysocki *et al.* (1999) state that as healing proceeds, the levels of MMP-9 decrease in CWF and reach those found in acute wounds[10]. However, both of these studies relied on semi-quantitative data from quantitation of either gelatin zymography[10] or both zymography and western blotting[6]. Therefore, while their results are important, they do not provide definitive evidence of statistically significant differences between ulcers with different clinical scores, which underpins the novelty of what we report here. Further evidence suggests that the MMP-9 present in CWF is derived from macrophages infiltrating the wound bed[38], which considering the excessive levels of MMP-9 in CWF compared with AWF, could further confirm the hypothesis that chronic wounds are partly caused by disproportionate inflammation and the inability to proceed into the normal remodelling stage of wound healing[3]. Therefore, the clinically worse ulcers are those that show prolonged inflammation, presenting as increased levels of macrophage-derived MMP-9 in the resultant CWF.

In this study, CWF samples were organised according to their PUSH score, which is a clinical score used to grade the ulcer taking into account a number of factors[22]. While

this system was originally created for use with pressure ulcers, it has since been validated as a straightforward, reliable tool for observing venous ulcer clinical status[22]. In the results reported herein, the lower and higher PUSH score groups were indistinguishable through immunoprecipitation, as both groups showed a significant increase in MMP-9 activity compared with HS and AWF samples ($p<0.01$). Similar results have been obtained in previous studies, with MMP-9 in CWF showing a minimum of a 2-fold increase when compared with AWF and HS[6,10]. When considering the MMP-9-depleted fractions, there were significant MMP-9 activity differences between the lower PUSH score (PUSH ≤ 11) group and the higher PUSH score (PUSH ≥ 12) group ($p<0.05$), with the higher PUSH score (PUSH ≥ 12) group displaying a significant increase in Collagen Type I degrading activity as compared with both HS and AWF ($p<0.01$). This could be due to the large levels of MMP-9 present in these samples, which in turn suggests that the zymograms may well have been overloaded. If indeed this did occur, this may explain why statistically significant differences between the two clinical groupings were not obtained.

Nevertheless, Tarlton *et al.* (1999) were able to use gelatin zymography to show a statistical correlation between pro-MMP-9 and the increasing severity of the ulcer ($p=0.006$)[39]. However, the Tarlton *et al.* (1999) study used wound site specific collection techniques, i.e. they collected CWF from the advancing wound margin in a deteriorating ulcer and not the ulcer it its entirety; so the results are not directly comparable[39]. In this study, we specifically chose to use a standardised collection technique that recovered the wound fluid in its entirety without any added protein selection processes, i.e. differential protein absorption and retrieval from wound dressings. Furthermore, while the samples were not collected from clinically infected

wounds, it is important to recognise that there would still be a bacterial presence in the wound, which may then contribute to the wound fluid's proteolytic activity. The extent to which bacterial proteases contribute to the overall amount of MMP activity in CWF will be a focus of future studies. Another interesting point to note is that while some MMPs have been known to degrade IgG proteins, namely MMP-3 and MMP-7[40], this was not observed to contribute to cleavage of the antibody from the resin in these experiments. This could be due to lower levels of these specific MMPs, i.e. MMP-3 and -7, in the CWF samples, or indeed, competitive binding of MMP-9 to the antibody, which could then potentially block any further interactions.

To further investigate any potential differences in CWF collected from wounds with different clinical status, a direct ELISA was used with a different MMP-9 antibody to show quantitative levels of MMP-9 in these samples. This technique showed clear differences between the levels of MMP-9 present in the two separate groupings. The lower PUSH score (PUSH ≤ 11) group displayed significantly higher levels of MMP-9 than AWF ($p < 0.01$), as well as significantly lower levels of MMP-9 than the higher PUSH score (PUSH ≥ 12) group ($p < 0.01$). As a result, it appears that there is a strong correlation between increased MMP-9 levels in CWF from wounds in the higher PUSH score (PUSH ≥ 12) group compared to AWF samples ($p < 0.01$). This therefore suggests that MMP-9 is the predominant protease involved in degrading the extracellular matrix in the chronic wound environment and healing could potentially be promoted by its attenuation. In contrast, Fray *et al.* (2003) propose that MMP-2 and -9 are both essential for wound healing, a process that would therefore be impaired if these two proteases were inhibited in chronic ulcer treatments[9]. However, their hypothesis is based on the fact that gelatinases are required for tissue

remodelling and for the onset on re-epithelialisation – stages of wound healing that normally occur when inflammation has subsided[1]. Therefore, if significantly increased levels of MMP-2 and -9 are present before inflammation has abated, an excessively proteolytic environment will continually degrade key growth promoting agents and thus will not allow normal wound healing to occur.

In summary, the study reported here demonstrates that the levels of MMP-9 present in the CWF samples is statistically significantly higher compared with both HS and AWF. When these CWF samples were separated by their clinical score, the fluids from the clinically worse ulcers, i.e. those with the higher PUSH scores (PUSH ≥12), displayed significantly higher levels of MMP-9 than those from the lower PUSH score (PUSH ≤11) group ($p<0.01$). In addition, it appears that high levels of MMP-9 activity in CWF are a better indicator of a wound's chronicity than the total protease activity levels alone. Further, these data suggest it is not surprising that current topical treatments of chronic ulcers with bioactives, e.g. growth factors, have proven to be only slightly effective in treating chronic wounds since high levels of proteases can rapidly degrade these growth promoting agents[41].

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Tables

Table 1.   Patient clinical data from chronic wound fluid samples.

Patient	Age	Sex	Sample Code	Ulcer Duration (Weeks)	Ulcer Size (cm²)	% Reduction in wound area (from baseline)	Compression (mmHg)	PUSH Score
1	54	F	33	102	44	0	<10	15
2	79	F	34	102	15	-25	30 - 35	12
3	76	F	57	162	84	64	30 - 35	15
4	79	F	89	261	16	0	30 - 40	14
5	88	M	95	320	10.7	83.7	30 - 35	13
6	67	F	96	37	16	0	30 - 40	10
7	77	M	131	550	8	0	20 - 25	10
8	86	F	142	100	7.1	0	30 - 35	9
9	75	M	173	20	7	0	30 - 40	10

Figure Legends

Figure 1. **Collagen Type I zymography demonstrating protease activity present in wound fluid samples.** Lane 1 is the positive control of MMP-8 (100 ng). Lane 2 is HS and lanes 3-5 are AWF samples 1-3 with 5 µg of total protein loaded per sample. Lanes 6-14 are CWF samples 1-9 with 500 ng of total protein loaded per sample.

Figure 2. **Collagen Type IV zymography demonstrating protease activity present in wound fluid samples.** Lane 1 is the positive control of MMP-9 (10 ng). Lane 2 is human serum and lanes 3-5 are AWF samples 1-3 with 10 μg of total protein loaded per sample. Lanes 6-14 are CWF samples 1-9 with 1 μg of total protein loaded per sample.

Figure 3.   **GM6001 inhibition of protease activity in CWF-1 confirms the MMP specific degradation of Collagen Type I as revealed through zymography.**

(a)   Lane 1 is the positive control of MMP-8 (100 ng). Lane 2 is the untreated sample of CWF-1 with 500 ng of total protein loaded. Lanes 3-9 are CWF-1 samples (500 ng) treated with increasing concentrations of the specific MMP-inhibitor, GM6001, at 1, 3, 10, 30, 100, 300, and 1000 μM at 37 ºC for 24 hours.

(b)   Relative levels of protease activity in CWF-1 samples treated with increasing concentrations of GM6001. The MMP-specific inhibition of collagen degrading activity was represented quantitatively through densitometric analysis. Levels are shown as the % collagen degrading activity, of both the total and also individual molecular weight bands, as compared to the untreated sample, ± SEM (n=3).

Figure 4. Initial immunoprecipitation of MMP-8 and -13 from CWF samples and analysis of enriched fractions through Collagen Type I zymography.

(a) Lane 1 contains the positive control of MMP-8 (100 ng). Lanes 2 and 3 are the negative controls of Protein A/G and Protein A/G + MMP-8 pAb. Lanes 4 -9 are the bound fractions of CWF samples 1-6 with 500 ng of total protein loaded per sample.

(b) Lane 1 contains the positive control of a 27 kDa truncated version of MMP-13 (100 ng). Lanes 2 and 3 are the negative controls of Protein A/G and Protein A/G + MMP-13 pAb. Lanes 4 -9 are the bound fractions of CWF samples 1-6 with 500 ng of total protein loaded per sample

Figure 5. **Immunoprecipitation of MMP-9 from wound fluid samples and analysis of MMP-9 enriched fractions through Collagen Type I zymography.**

(a) Lane 1 contains the positive control of MMP-9 (500 pg). Lanes 2 and 3 are the negative controls of Protein A/G and Protein A/G + MMP-9 mAb. Lanes 4 -16 are the bound fractions of HS, AWF samples 1-3 and CWF samples 1-9 with 500 ng of total protein loaded per sample

(b) Relative levels of MMP-9-bound fractions in individual samples represented quantitatively through densitometric analysis. Levels are shown as the mean activity $\pm$ SEM (n=3). Statistical significance is relative to HS and shown as either * ($p<0.05$) or # ($p<0.01$) as determined by Tukey's test.

(c) Data from **(b)** is separated into HS, AWF and CWF pooled samples. Statistical significance is relative to both HS and AWF and shown as # ($p<0.01$) as determined by Tukey's test.

(d) Data from **(b)** is separated into samples with a higher PUSH score (CWF samples 1-5, PUSH ≥ 12) and lower PUSH scores (CWF samples 6-9, PUSH ≤ 11). A higher PUSH score indicates a clinically worse ulcer. Statistical significance is relative to both HS and AWF and shown as # ($p<0.01$) as determined by Tukey's test.

Figure 6. **Immunoprecipitation of MMP-9 from wound fluid samples and analysis of MMP-9 depleted fractions through Collagen Type I zymography.**

(a) Lane 1 contains the positive control of MMP-9 (500 pg). Lanes 2 -14 are the unbound fractions of HS, AWF samples 1-3 and CWF samples 1-9 with 500 ng of total protein loaded per sample

(b) Relative levels of MMP-9-unbound fractions in individual samples represented quantitatively through densitometric analysis. Levels are shown as the mean activity ± SEM (n=3). Statistical significance is relative to HS and shown as either * ($p<0.05$) or # ($p<0.01$) as determined by Tukey's test.

(c) Data from **(b)** is separated into HS, AWF and CWF pooled samples. Statistical significance is relative to both HS and AWF and shown as # ($p<0.01$) as determined by Tukey's test.

(d) Data from **(b)** is separated into samples with a higher PUSH score (CWF samples 1-5, PUSH ≥12) and lower PUSH scores (CWF samples 6-9, PUSH ≤11). A higher PUSH score indicates a clinically worse ulcer. Statistical significance is relative to both HS and AWF and shown as # ($p<0.01$) as determined by Tukey's test.

Figure 7.   MMP-9 levels in wound fluid samples analysed through an ELISA.

Levels are shown as MMP-9 (ng)/total protein (μg) ± SEM (measured in triplicate and repeated).

(a) Data is represented as individual samples. Statistical significance is relative to HS and shown as either * ($p < 0.05$) or # ($p < 0.01$) as determined by Tukey's test.

(b) Data from **(a)** is separated into HS, AWF and CWF pooled samples. Statistical significance is relative to both HS and AWF and shown as # ($p < 0.01$) as determined by Tukey's test.

(c) Data from **(a)** is separated into samples with a higher PUSH score (CWF samples 1-5, PUSH ≥ 12) and lower PUSH scores (CWF samples 6-9, PUSH ≤ 11). A higher PUSH score indicates a clinically worse ulcer. Statistical significance is relative to both HS and AWF and shown as # ($p < 0.01$) as determined by Tukey's test.