Ovine forestomach matrix biomaterial is a broad spectrum inhibitor of matrix metalloproteinases and neutrophil elastase

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Key words
Enzyme inhibitor; Inhibition; Matrix metalloproteinase; Neutrophil elastase; Ovine forestomach matrix; Wound healing

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Abstract
Proteases play a critical role in the ordered remodelling of extracellular matrix (ECM) components during wound healing and tissue regeneration. However, the usually ordered proteolysis is compromised in chronic wounds due to over-expression and high concentrations of matrix metalloproteinase’s (MMPs) and neutrophil elastase (NE). Ovine forestomach matrix (OFM) is a decellularised extracellular matrix-based biomaterial developed for tissue regeneration applications, including the treatment of chronic wounds, and is a heterogeneous mixture of ECM proteins and proteoglycans that retains the native structural and functional characteristics of tissue ECM. Given the diverse molecular species present in OFM, we hypothesised that OFM may contain components or fragments that inhibit MMP and NE activity. An extract of OFM was shown to be a potent inhibitor of a range of tissue MMPs (IC50 = 23 ± 5 to 115 ± 14 μg/ml) and NE (IC50 = 157 ± 37 μg/ml), and was more potent than extracts prepared from a known protease modulating wound dressing. The broad spectrum activity of OFM against different classes of MMPs (i.e. collagenases, gelatinases and stromelysins) may provide a clinical advantage by more effectively addressing the protease imbalance seen in chronic wounds.

Key Messages
- wound proteases, including matrix metalloproteinases (MMPs) and neutrophil elastase (NE) are tightly controlled during the ordered sequence of wound healing
- in contrast, chronic wounds contain elevated wound protease concentrations that lead to rampant degradation of the extracellular matrix (ECM) molecules and a prolonged inflammatory phase
- agents that modulate protease activity in chronic wounds have clinical benefit in reducing the hyper-proteolytic state of chronic wounds
- ovine forestomach matrix (OFM) is a decellularised extracellular matrix biomaterial
- OFM mimics the native structure and function of tissue ECM, and provides a provisional scaffold and secondary molecules to kick start the healing process in chronic wounds
- extracts of OFM were shown to inhibit a range of MMPs, including the stromelysins, collagenases and gelatinases, while a dressing comprising oxidised

Introduction
Tissue proteases, for example, matrix metalloproteinases (MMPs) and neutrophil elastase (NE) play a critical role in normal tissue turn over and during ordered tissue regeneration and repair (1). Protease digestion of extracellular matrix (ECM) components facilitates cell migration and proliferation, and plays a role in the regulation of inflammatory processes (2,3). Conversely, over-expression of tissue proteases and increased protease concentrations has been associated with a number of pathologies, including chronic wounds (4,5), cancer (6) and vascular disease (7). MMPs are a large family of 23 closely related zinc-fingered proteases that digest ECM components including collagens I, III and IV, fibronectin, laminin and proteoglycans (5). The serine protease NE targets structural elements of the ECM, but has additionally been shown to degrade fibronectin (8) and peptide growth factors (9). In typically ordered tissue regeneration, the proteolytic activity of MMPs and NE are maintained by endogenous protease inhibitors, including tissue inhibitors of MMPs (TIMPs), α2-macroglobin and α1-proteinase inhibitor (4).
Materials and methods

MMP solid state assay

Inhibition of MMPs by intact OFM was quantified according to Cullen et al. (14) with modifications. Samples of OFM (Mesynthes Ltd, Lower Hutt, New Zealand) and oxidised regenerated cellulose/collagen (ORC/C; PromogranTM; Systagenix, Gatwick, UK) were cut into 5-mm-diameter discs and rehydrated in phosphate-buffered saline (500 μl, PBS) for approximately 1 minute. Test materials were blotted to remove excess PBS and then added to 70 μl of either recombinant human MMP9 (0·4 μg/ml; Enzo Life Sciences, Farmingdale, NY) or recombinant human MMP8 (0·83 μg/ml; Enzo Life Sciences), both prepared in 50 mM Heps, 10 mM CaCl₂, 0·05% Tween 20, pH 7·5. Samples were incubated for 15, 30, 60, 120, 240 and 360 minutes at 37 ◦C with shaking. Samples were removed from solution, discarded and the residual enzymatic activity of the solution quantified using a fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpn-Ala-Arg-NH₂ (27) according to the manufacturer’s instructions. In a 96-well plate, 20 μl of the protease solution was mixed with 60 μl of MMP assay buffer (50 mM Heps, 10 mM CaCl₂, 0·05% Tween 20, pH 7·5) and fluorogenic substrate (20 μl, 20 μM). Relative florescence units (RFU) were quantified on a plate reader (SpectraMax® M4; Molecular Devices, Sunnyvale, CA) every 1 minute over a 10-minute period at 37 ◦C, using excitation and emission wavelengths of 328 and 420 nm, respectively. RFU was plotted versus time to derive the rate of formation of the fluorophore. Residual MMP activity was determined by comparing the rate of the test sample, relative to an untreated control, and expressed as percentage activity. P-values were determined by an unpaired t-test (Excel 2007; Microsoft Corporation, Redmond, WA) with a P-value of <0·05 considered statistically significant.

MMP inhibition assay

Aqueous extracts of OFM and ORC/C were prepared by incubating approximately 30–100 mg of sample in PBS (1 ml) for 1 hour at 37 ◦C with shaking. The samples were centrifuged at 9500 g for 10 minutes and the supernatant was transferred. Protein concentrations of the extracts were determined using bicinchoninic acid (BCA) protein assay, according to the manufacturer’s instructions (Pierce, Thermo Fisher Scientific, Waltham, MA). Extracts were serially diluted over a final concentration range of 3–600 μg/ml protein using MMP assay buffer (50 mM Heps, 10 mM CaCl₂, 0·05% Tween 20, pH 7·5). Test solution (20 μl) was added to recombinant human MMPs (20 μl) at the final concentration specified in Table 1. MMP assay buffer (40 μl) was added and samples were incubated for 60 minutes at 37 ◦C, prior to the initiation of enzymatic activity by addition of fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpn-Ala-Arg-NH₂ (20 μl, 20 μM). Production of the fluorophore was quantified every 1 minute over a 10-minute period at 37 ◦C, using excitation and emission wavelengths of 328 and 420 nm, respectively. RFU was plotted versus time to derive the rate of formation of the fluorophore (RFU/minute) for each concentration of the extract (Figure 2A). Percent activity was determined from the observed rate relative to the rate of an untreated control. Sigmoidal dose–response curves were plotted (SigmaPlot v11.0; Systat Software, San Jose, CA) of percent activity versus extract concentration and the half-maximal inhibitory concentration (IC₅₀) derived for test extracts against...
Table 1 Inhibitory activity of OFM and ORC/C extracts against tissue proteases*  

<table>
<thead>
<tr>
<th>Protease</th>
<th>IC50 OFM (μg/ml)</th>
<th>IC50 ORC/C (μg/ml)</th>
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<tbody>
<tr>
<td>MMP1 (Collagenase 1)</td>
<td>115 ± 14</td>
<td>~600</td>
</tr>
<tr>
<td>MMP8 (Collagenase 2)</td>
<td>86 ± 2</td>
<td>~600</td>
</tr>
<tr>
<td>MMP13 (Collagenase 3)</td>
<td>38 ± 2</td>
<td>~600</td>
</tr>
<tr>
<td>MMP3 (Stromelysin-1)</td>
<td>96 ± 8</td>
<td>&gt;600</td>
</tr>
<tr>
<td>MMP10 (Stromelysin-2)</td>
<td>45 ± 1</td>
<td>355 ± 51</td>
</tr>
<tr>
<td>MMP2 (Gelatinase-A1)</td>
<td>23 ± 5</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>MMP9 (Gelatinase-B)</td>
<td>50 ± 14</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>MMP12 (Macrophage metalloelastase)</td>
<td>24 ± 1</td>
<td>~600</td>
</tr>
<tr>
<td>MMP14 (Membrane type 1 MMP)</td>
<td>45 ± 3</td>
<td>~600</td>
</tr>
<tr>
<td>Neutrophil elastase</td>
<td>157 ± 37</td>
<td>~1740</td>
</tr>
</tbody>
</table>

*Errors represent standard errors from triplicate experiments. Concentration in brackets indicates the enzyme concentration used in the respective assay.
†Errors represent standard errors from duplicate experiments.

Each of the MMPs tested (Figure 2B). Extracts were tested in triplicate against each MMP.

**Neutrophil elastase inhibition assay**

Aqueous extracts of OFM and ORC/C were prepared by incubating approximately 30–100 mg of sample per ml PBS for 1 hour at 37°C with shaking. Extracts were concentrated on a 10 kDa MW centrifugal concentrator (Amicon; Merck Millipore, Billerica, MA). The OFM and ORC/C extracts were serially diluted in NE assay buffer (100 mM Hepes, 500 mM NaCl, 0.05% Tween 20, pH 7.2) over a final concentration range of 8–1740 μg/ml and 12 to 1800 μg/ml, respectively. Inhibition of the NE was quantified using a colorimetric NE kit (Enzo Life Sciences), according to the manufacturer’s instructions. Diluted solutions of the extracts (20 μl), purified human NE (0–02 μU/μl, 10 μl) and NE assay buffer (60 μl) were pre-incubated for 30 minutes at 37°C, and then colorimetric substrate MeOSuc-Ala-Ala-Pro-Val-pNA (10 μl, 1 mM) (28) was added. Production of the colorimetric cleavage product was quantified at 405 nm every 1 minute for 10 minutes at 37°C. The rate of the reaction (Abs/min) was determined from the slope of a plot of absorbance versus time. Percent activity was determined from the observed rate relative to the rate of an untreated control. Sigmoidal dose–response curves were plotted (SigmaPlot, v11.0) of percent activity versus extract concentration and the half-maximal IC50 derived. Extracts were tested in duplicate.

**Results**

**MMP solid state assay**

Protease inhibition of intact OFM was assessed according to the approach of Cullen et al. (14) using MMP8 and MMP9 as a representative collagenase and gelatinase, respectively. In the presence of either intact OFM or ORC/C, the residual activity of MMP8 was reduced relative to the untreated control at all time points sampled (P < 0.05), but only OFM displayed a time-dependent decrease in activity (Figure 1A). There was no statistical difference between the activity of intact OFM and ORC/C against MMP8 (P > 0.05), except following a 360-minute incubation (P = 0.02). The activity of intact ORC/C was increased relative to OFM when assayed against the gelatinase, MMP9 (P < 0.001) (Figure 1B), and both intact OFM and ORC/C demonstrated significant activity relative to the untreated control at all time points (P < 0.05). Like MMP8, the inhibitory activity of OFM against MMP9 was time-dependent.

The time-dependent decrease in the activity of MMP8 and MMP9 in the presence of OFM suggested aqueous soluble components were responsible for the observed effect, rather than direct adsorption of proteases to the surface of the intact biomaterial. This was supported by the observed correlation between the residual activity of the OFM-treated enzyme and the corresponding protein concentration following incubation of the biomaterial in solution (Figure 1C).

**Protease inhibition**

Dose–response curves of percentage enzyme activity versus protein concentration (Figure 2B) were determined using aqueous extracts of both OFM and ORC/C. Inhibitory activity against a range of proteases (Table 1 and Figure 2C) were expressed as an IC50, where lower concentrations represent more potent enzyme inhibition. The IC50 was estimated when a complete sigmoidal dose–response could not be calculated due the concentration limits (e.g. ORC/C, Figure 3). Extracts were prepared by incubating either OFM or ORC/C in PBS at 37°C for 1 hour. The incubation time used to prepare the extract (e.g. 1 or 16 hours) was shown to have little effect on the bioactivity of the extracts (data not shown) suggesting that the composition and diversity of solubilised components were not significantly altered by extending the incubation period. OFM extracts inhibited all MMPs tested in this study, including collagenases, stromelysins and gelatinases. Inhibitory IC50 ranged from 23 ± 5 to 115 ± 14 μg/ml, with the most potent inhibition being determined against MMP2 (IC50 = 23 ± 5 μg/ml) and MMP12 (IC50 = 24 ± 1 μg/ml). The weakest inhibition was recorded against MMP1 (IC50 = 115 ± 14 μg/ml). The bioactivity of ORC/C extracts were more variable across the MMPs surveyed, with similar inhibitory potency to OFM determined only for the gelatinases, MMP2 and MMP9 (IC50 = 31 ± 6 and 55 ± 7 μg/ml, respectively). ORC/C extracts were only modest inhibitors of MMP1, MMP3, MMP8, MMP12, MMP13 and MMP14 with IC50 estimated to be approximately 600 μg/ml or greater. The OFM and ORC/C extracts were concentrated using a 10-kDa MW concentrator prior to assaying against NE to derive full dose response curves. The OFM extracts inhibited NE in a dose-dependent manner and were approximately 10-fold more potent than the ORC/C extracts (IC50 = 157 ± 37 and ~1740 μg/ml, respectively).
Ovine forestomach matrix inhibits matrix metalloproteinases and neutrophil elastase

Discussion
Inhibition of tissue proteases by biomaterials has traditionally been demonstrated using solid-state assays, whereby the intact samples are incubated in enzyme solutions and then the residual enzyme activity quantified following removal of the sample (14–19,22,29). In the case of soluble or partially soluble biomaterials, such as OFM and ORC/C, reductions...
in enzyme activity using this approach can be accounted for either by (i) surface adsorption of the enzyme to the biomaterial; or (ii) dissolution of soluble inhibitory components. The correlation between the concentration of soluble proteinaceous OFM components and reduction in enzyme activity (Figure 1C) suggests that solubilised matrix components contribute to the inhibitory effect of OFM. By determining the IC50-value of these soluble components, a direct comparison to the inhibitory effect of OFM. In contrast, ORC/C are absorbed into the wound bed over time, releasing soluble components through hydrolysis and proteolysis. The IC50 values were based on the protein concentration of the extracts, and therefore did not take into account any soluble ORC present in solution. Therefore, the IC50s determined for ORC/C may be significantly higher if total solubilised material (i.e. collagen and ORC) was taken into account.

Extracts of OFM had broad spectrum activity against a range of tissue proteases, whereas the activity of ORC/C was limited to the gelatinases, MMP2 and MMP9 (Table 1 and Figure 2C). The greater relative bioactivity of OFM versus ORC/C is most likely encoded in the composition of the biomaterials. OFM is relatively heterogeneous, containing a range of structural, adhesion and signalling molecules that constitute native ECM in tissues (25). In addition to the collagenous major components of OFM, there is the potential for a variety of secondary macromolecules to contribute to the broad spectrum activity of OFM. In contrast, ORC/C comprises only acid solubilised and denatured bovine collagen I and ORC (15). It has been proposed that ORC/C acts as a sacrificial substrate in the presence of proteases (15) and, therefore, given the composition of ORC/C, the gelatinases (MMP2 and MMP9) would be most susceptible to inhibition, through sacrificial means. The results of this study support this notion as ORC/C only inhibited the gelatinases (Table 1 and Figure 2C). Interestingly, it has been shown in previous studies that unlike OFM, extracts of the dECM-based biomaterial small intestinal submucosa (SIS) do not inhibit MMP1, MMP2 or MMP9, but rather SIS attenuates protease activity through the non selective mechanism of surface adsorption (17).

While it is recognised that tissue proteases play an important role in the state of chronic wounds there is still no literature consensus as to which proteases are most important in contributing to chronicity and, therefore, should be targeted for therapeutic intervention (30,31). Various reports have identified elevated concentrations or expression of MMP1 (11), MMP2 (16,32), MMP3 (33), MMP8 (34,35), MMP9 (32,36), MMP13 (16,33, 37) and elastase (16) in chronic wound fluid and/or wound tissues. In this study, target proteases were selected based on those known to be in abundance in chronic wounds, as well as MMP12 and MMP14 that are known to play a role in cutaneous wound repair (30). OFM was shown to inhibit all classes of proteases included in the panel and we propose that this broad spectrum activity potentially provides a significant clinical advantage over inhibitory activity against a single protease class. For example, ECM collagens type I, II and III are known to be sequentially degraded, first by the action of collagenases MMP-8, MMP-1 and MMP-13, respectively, which cleave the collagen fibrils to initiate degradation and second, by the gelatinases (MMP2 and MMP8) that act exclusively on the cleaved fibril (38). Initiation of the proteolytic cascade additionally requires activation of the collagenases themselves, either through autolyse of the pro-enzymes or via MMP-mediated proteolysis. For example, stromelysin-1 (MMP-3) is known to activate both MMP13 and the gelatinases leading to collagen III degradation (38). Therefore, while the gelatinases may be in higher abundance in chronic wounds, relative to the collagenases (31), biomaterials that are capable of inhibiting both the upstream (i.e. stromelysins, collagenases) and downstream (i.e. gelatinases) proteases are more likely to halt collagen proteolysis relative to the agents that exclusively inhibit the downstream gelatinases. The notion of broad spectrum activity against a variety of protease classes can be likened to the combinatorial therapies that have been successfully used in the treatment of HIV infection and cancer. This is the first report of soluble components of a dECM-based biomaterial being inhibitory towards a range of tissue proteases and suggests that OFM has the potential to modulate the hyper-proteolytic environment of chronic wounds. Additional clinical studies are warranted to validate our understanding of the inhibitory activity of OFM and how this effect translates into the resolution of chronicity and subsequent healing outcomes.

Acknowledgements
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