Wound Diagnostics: Can a Single Molecular Marker Concur With An Expert's Multi-Factorial Assessment Of Wound Healing Status?

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Background

The healing of acute wounds is a well documented process regulated by a variety of bio-molecules. Wounds which become stalled or stuck within this normal process possess a potentially destructive imbalance in some of these essential molecules, such as pro-inflammatory cytokines and matrix metallo-proteases (MMP's) which may be dominant or absent at the wrong time (1).

Tests to determine an imbalance of key indicator bio-molecules can be used to assess wound status. However, the assay of multiple bio-markers requires time, equipment and experienced operators, incurring significant cost. Despite a pressing need for diagnostics, it is impractical to asses chronic wounds with these existing methods as part of the normal care regime. Cost effective technology is needed.

This poster describes a novel 'Healing Status Indicator' that works by measuring a unique synthetic peptide indicator molecule, designed to be degraded by the action of MMP 8 & 9 in the same way that key bio-molecules are degraded in the wound.

Aims

The overall aim was to develop a healing status indicator to determine if a wound is responding favourably to treatment, so as to guide care and give confidence in new treatment options. To be effective it had to provide the highest level of diagnostic accuracy, irrespective of the carer's expertise.

A further aim was to determine if a novel rapid protease test could give the same diagnostic conclusion as an expert's multifactorial clinical judgment.

Peptide Modelling

A range of peptide ligands were designed and manufactured in-house to include a protease sequence optimized for cleavage by MMP's 8 & 9, and also recognized by a tracer antibody. The primary peptide sequence was modelled with the Molecular Mechanics function on Chem Draw 3D, as shown for peptide 38 in figure 1. A biotin molecule was linked to the N terminus to enable sensitive detection in affinity binding assays. The P1' residue is marked with an arrow to show the enzyme cleavage point.

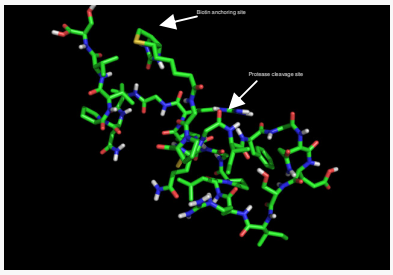

Figure 1. Assay molecule 38 modeled as a folded structure

Assay Design

Reference affinity binding assays were developed to evaluate and confirm the performance of the peptide ligands. In one of these, a microplate system was used to detect the non-digested peptide ligand. High MMP activity rapidly cleaved the ligand to yield peptide fragments that couldn't be recognised by the tracer antibody. This event is detected as loss in assay signal, proportional to the protease activity (figure 2). Figures 3 & 4 show the relative sensitivities of two of the peptides to different MMP's. Sequences with a bias for MMP 8 & 9 were further developed in the ultra simple lateral flow assays which are appropriate for point of care (POC) use.

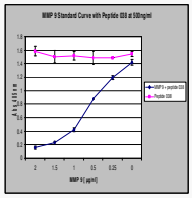

Figure 2. Standard curve showing digestion of Peptide 38

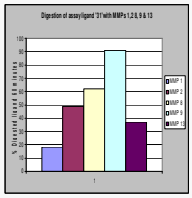

Figure 3. Peptide 31 shows a clear bias for MMP's 8 & 9

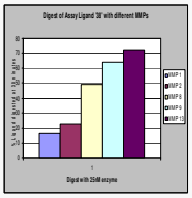

Figure 4. Peptide 38 shows a slightly different bias with most sensitivity to MMP's 13, 9 & 8

Collection and testing of wound samples

Samples were collected from wounds of different aetiologies at the Toronto Wound Healing Centre's, Canada. Wound fluid was collected with flocculated swabs using the 'Z' technique. At the time of collection the clinician assessed the status of the wound, based on the normal procedures of the clinic. Samples were stored at -80°C and shipped to the UK packed in dry ice. Twenty samples were selected randomly and tested blind. They were extracted into 100µl of buffer and assayed for total MMP 8 & 9, and TIMP-1 using commercial kits (from R&D systems). They were also tested with the Mologic lateral flow assay, where 10µl of wound extract was incubated for 15 minutes with peptide ligand 31 prior to testing in the device. Once the enzyme step was complete, the rapid assay took a further 2 minutes to run. The results are shown in table 1 and figures 5 & 6.

Results Summary

Table 1.Results from reference assays, the Mologic lateral flow and the expert assessment

Status	Patient	Total MMP 9[µg]	Total MMP 8[µg]	TIMP-1 [ng]	Lateral flow	Category A,B or C
H	72	1.59	0	7.7	-	A
H	74	0.134	0	<0.15	-	C
H	75	0.176	0.37	<0.15	-	C
H	78	0.639	0.264	3	-	A
H	81	1.4	0.44	12.8	-	A
H	87	0.3	0.09	<0.15	-	C
H	88	0.085	0.07	<0.15	-	C
H	86	>5	3.523	22.7	++++	B
H	89	1.5	0.642	5.8	++	B
H	90	>5	2.085	81.7	+++	B
NH	71	>5	3.301	149	+++	B
NH	73	>5	9.75	218	++	B
NH	76	1.914	3.915	167	++++	B
NH	79	>5	11.28	759	++++	B
NH	80	>5	16.3	249	++++	B
NH	82	>5	2.94	196	++++	B
NH	85	>5	4.45	43.9	++++	B
NH	77	2.51	0.452	16.2	-	A
NH	83	0.8	0.16	7.5	-	A
Infected	84	1.7	0.426	3.3	-	A

The level of total MMP's 8 & 9 concentrations measured by the commercial laboratory assays was categorised as A= low to moderate 8/High 9; B=High 8/High 9; C=Low 8/Low 9. All of the samples found to be high in protease by lateral flow belonged to category B. Seven out of nine wounds judged by the expert as non-healing were detected as having high MMP 8 & 9 activity. Of the healing wounds, 3 out of 10 were found to have high MMP 8 & 9 activity resulting in a positive test.

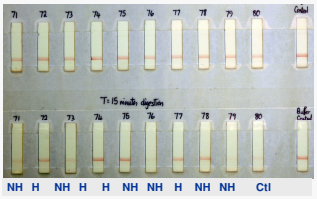

Figure 5. Samples 71- 80 with assay peptide at time 0 and after 15 minutes incubation.

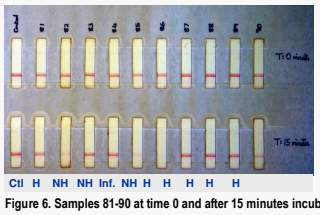

Figure 6. Samples 81-90 at time 0 and after 15 minutes incubation

Interpretation of the lateral flow test result

If no test line developed, then the MMP 8/9 activity was high. In this condition the wound is expected to be non healing. If the test line appeared, then the aggregate MMP 8/9 activity is normal and the wound was expected to be healing. In normal use the test will also include a control line that always forms when a device functions correctly.

Conclusion

The study hypothesis was that wounds could be diagnosed as non-healing if the rapid assay couldn't detect the peptide ligand after exposure to wound fluid. Conversely, a wound could be diagnosed as being on a healing trajectory by the presence of a test line. In other words, a healing state was hypothesised to correlate with low levels of MMP 8 & 9 activity, as detected in this system, and vice-versa.

In order to be relevant to nursing practice the hypothesis was tested to determine whether or not a user of this prototype test could, with reasonable accuracy, ascertain the healing status of wounds by observing the appearance of a test line, without even seeing the wound. If so, then the test may approach the diagnostic acumen of a skilled, experienced clinician using a concerted set of observations. This might seem an impossible objective. It was, then, surprising that the correlation between the test and the expert's opinion was good, with a test sensitivity of 77.7% and specificity of 80%. The randomly chosen wounds would inevitably have been on a continuum between healing and non-healing, with some on the borderline between the two states. Thus, many of the samples must be impossible to assign with certainty to one or the other state.

The evidence in the literature indicates that chronic non healing wounds accumulate elevated MMP activity, which declines as the wound progresses to a healing state (2, 3). Our findings support this, as highly elevated MMP 8/9 activity was found in most of the non-healing wounds. Both of these enzymes are released from the granules of neutrophils in the inflammatory response , so it is likely that the test was ultimately discerning the predominance of activated neutrophil aggression. This accords well with the understanding of leukocyte impact on the healing process (4). So, in answer to the question posed in the title, "Yes, it appears that ....."

Refs. 1. BA Mast and GS Schultz. Wound Rep Reg, 4. 411-420,1996; 2. Trengrove et al. Wound Rep Reg, 7. 442-452 1999. 3. Ladwig et al. Wound Rep Reg, 10. 26-37 2002. 4. R. Diegelmann and M. Evans. Frontiers in bioscience 9,283-289 2004.