PROTEASES AS BIOMARKERS IN WOUND HEALING

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ABSTRACT

Introduction: Wound healing complications could sometimes cause a transformation of acute wounds in chronic wound. Pathophysiology studies revealed that the physiological process of wound healing may be disrupted by high levels of proteases and/or an imbalance between the protease and their inhibitors. In recent decades, research has reported sustained concerns about the role of proteases as biomarkers present in the healing process, in order to develop new evaluation methods. It was suggested that measurement of the systemic and local response to injury using serum inflammatory biomarkers as indicators, may allow accelerated intervention for wound closure and treatment. Objective: The main aim of the study was to explore the literature that refers on protease role as biomarkers in predicting wound healing progress. The secondary aim was to establish the relevance of this knowledge for practice area. Material and methods: A systematic literature review using the PubMed database was conducted to identify relevant articles. The articles were selected in accordance with preset criteria and were then critically evaluated. Additionally, a search was conducted to identify possibly relevant information about the topics of interest. Results: We identified no eligible studies based on our inclusion criteria. After we performed the additional search, 47 papers with relevant information about the topics of interest were identified 7,8 as possibly relevant for practice area. Conclusions: Future study of protease activity could lead to innovative solutions for the development of diagnostic tests in the field of wound healing.

Key Words: proteases, biomarkers, wound healing

INTRODUCTION

Wounds are one of the most common reasons for which patients of all ages require health care.1-6 It is well-known that neglected wounds may lead to the installation of multiple disabilities, with negative impact on patients’ daily activities.7,8

Even if the wound itself is not a direct cause of death, lack of proper care and treatment allows potentially severe complications to develop. Delayed healing, infections and wound dehiscence, can cause a transformation of acute wounds in chronic wounds, which from an economic point of view is important, due to increase in healthcare costs.

In the last decades, research in the field of wound healing pathophysiology revealed a number of key factors important for wound progress evaluation. Proteases are one of those factors; they are present in the inflammatory stage of wound healing and are supposed to influence local mechanisms.5-18 Wound healing could be impaired by a persistent inflammatory state which is characterised by ongoing proteolysis and degradation, an increased level of proteases and
an imbalance in protease/protease-inhibitor levels. During tissue formation, proteases are released in wound area and orchestrate a complex interaction with extra cellular matrix (ECM) components. Is generally accepted that for a proper wound healing, enzymatic remodelling of the wound environment (especially extra cellular matrix) is an important requirement.

Multiple studies confirmed the hypothesis that during healing, the proteases level is variable, in relation of healing stage. Previous reports support this hypothesis by providing data which shows higher levels of proteases in exudates from chronic wounds (especially matrix metalloproteases - MMPs) compared with acute wounds and this persistently high level of MMPs contributes to the chronic character of the wound which results in healing delay or failure.

Although proteases are important for facilitating the tissue repair, it seems that an increased concentration has a negative effect on wound healing. Routine assessments performed by clinicians seem not to be sufficient to reveal essential elements that impede wound healing. Developing new methods to measure some biomarkers related to this process was a concern for researchers in recent decades. It was suggested that measuring the systemic and local response to injury using serum inflammatory biomarkers as indicator, will allow for a more controlled treatment that can accelerate wound closure and treatment.

**STUDY DESIGN**

The main aim of the study was to explore the literature that describes the role of proteases as biomarkers in predicting wound healing progress. The secondary aim was to establish the relevance of this knowledge for wound treatment.

**MATERIAL AND METHODS**

**Criteria for Selecting Studies**

The following studies were included in the review: control trials, meta-analysis and randomized controlled trials that have investigated the role of proteases as biomarkers of wound healing. For the purpose of this study we search for any protease that was investigated in relation with its role as predictor in wound healing process. We chose studies on any population sample, involving any type of intervention in the wound area, with no restrictions related to year of publication were selected. To be included in our selection, each study had to analyze healing in acute or chronic wound. We considered trials if they reported both of the following outcomes:

- Assessment of wound healing progress using objective methods;
- Assessment of at least one protease as a predictive biomarker in wound healing.

**Search Methods**

To identify the articles, we carried out a systematic literature using an online database (PubMed) and the following key words: (((wound) AND protease) AND wound healing) AND biomarker. The search strategy was limited to the following type of articles: clinical trial, meta-analysis, randomized control trial. The bibliographies of all retrieved and relevant publications, identified by these strategies, were searched for further studies, accessing databases CINAHL Plus with Full Text, Embase, Medline, and Web of science. Additionally, we performed a search in CINAHL Plus with Full Text, Embase, Medline, and Web of science applying the “snowball method” which involves searching references from the papers already selected, for possibly relevant information about the topics of interest.

**Search Outcome**

The first search identified 8 studies. We assessed their titles and abstracts for potentially relevant research. 7 articles were relevant for our work: 3 randomized control trials (RCT) and 4 clinical trials. We didn't found any Meta-analysis.

**Data Abstraction and Quality Appraisal**

Full text versions of articles were extracted and data was summarized into a pre-prepared form. Studies were appraised critically for the quality assessment in terms of eligibility criteria, using an assessment which incorporated study design, participant's selection, intervention and outcomes measured. Following the detailed review of the papers, none of the studies have met the criteria; the reasons for this are outlined in Table 1.

**Synthesis**

Structured narrative summary of extracted reviews were performed. The low number of selected studies and heterogeneous data didn't allow us to perform a meta-analysis.

**RESULTS**

We identified no eligible studies based on our inclusion criteria. After we performed an additionally search, we found 47 papers with relevant information about proteases’ role in wound healing.
Those proteases are more or less implicated in wound healing. The major research focus was on enzymes represented by metalloendopeptidases and serine endopeptidases.29

From the metalloendopeptidases family, the sub-family with the most influence in the wound healing process is the matrix metalloproteinases (MMPs), which consists of over 20 proteases.30 MMPs are not usually found in normal tissue or are presented as pro-proteases at low levels and are release in different physiological and pathological remodelling processes (eg tissue repair, inflammation, tumour invasion, embryonic development) by inflammatory cells, keratinocytes, and fibroblasts. They play an important role in tissue repair and remodelling, angiogenesis and in excessive destruction of connective tissue during inflammatory disorders.31 MMPs are able to cleave practically all essentially ECM molecules and various other substrates including other proteases, growth factors and cytokines, acting often as activators.31,32 According to Nagase et al, MMPs can be divided.

**DISCUSSION**

Wound healing is a complex physiological process consists of overlapping stages: inflammatory phase (the wound is debrided and bioburden is controlled), reconstructive phase (appearing granulation tissue, reepithelialization and wound contraction occurs), and remodeling and scar formation phase.26 These wound healing phases involve complex successions of chemical events at cellular level controlled temporally and spatially by enzymes generated by the body. This is a vital phenomenon for wound healing process.27

The literature mentions more than 100 enzymes involved in the wound healing process. Those enzymes are known as proteinase, protease, proteolytic enzymes.28 The most investigated protease families in relation with their contribution to wound healing are: aspartanic acid endopeptidase, brinolase (or brinase), cysteine endopeptidases, metalloendopeptidases, serine endopeptidases and streptokinase. Those proteases
into subgroups related to their substrate specificity and structural similarity for example: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and the group of other MMPs.\textsuperscript{31,33,34}

Serine proteases have an important role in inflammatory process. Researchers investigating chronic wounds found elevated levels of the following serine proteases: neutrophil elastase (human leukocyte elastase), urokinase-type plasminogen activator, possibly cathepsin, and plasmin.\textsuperscript{29}

For normal wound healing, which requires a balance between degradation of the ECM and synthesis and deposits of protein components of the granulation tissue, proteases activity has to be controlled by specific inhibitors called tissue inhibitors of matrix metalloproteinases (TIMPs), which can attach themselves to MMP membranes and inactivate them.\textsuperscript{35} All TIMPs bind all MMPs and inhibit them through different mechanisms. Ladwig et al., in a study of a large population of patients with chronic wounds, suggests a strong correlation between good healing and low ratios of MMP-9/TIMP-122. Subsequent data supports the hypothesis that excessive proteolytic activity, in particular an imbalance of MMPs and their natural inhibitors, TIMPs, may be part of the pathogenesis of venous leg ulceration and impaired wound healing.\textsuperscript{35-37}

Other studies have confirmed the hypothesis that during healing, the proteases level is variable and is influenced by the healing stage.\textsuperscript{9,21,38} Significant correlations were also found between the amount of proteases and modification in wound status.\textsuperscript{28,44} Studies on proteases are controversial, according to Meyer et al, who interestingly found that MMP activities were elevated in healing and non-healing ulcers, and that the total MMP levels did not differ between the groups.\textsuperscript{44}

It is well known that in acute wound healing, proteases reach a peak level during inflammation assisting with wound debridement and the cleaning of necrotic tissue, slough, foreign bodies and bacteria.\textsuperscript{45} During the phase of reconstruction and remodeling, proteases digest extracellular matrix and facilitate tissue repair and tissue synthesis and as a result their activity decreases progressively. Exploring the pattern of collagenase activity (MMP-1, MMP-8) in the wound fluid of healthy volunteers, Nwomeh et al\textsuperscript{46} showed peak levels of MMP-8 on day 4 and peak levels of MMP-1 on day 7, with important statistical difference among all the time points examined (100-fold to 200-fold). Study provides evidence that human dermal wound healing involves in extracellular matrix degradation enzymes from MMP family, MMP-8 being one of the most involved collagenase.

Interestingly, although most of the research shows that the biochemical environment of the healing wound is different from that of a non-healing wound, Subramanian et al found that acute graft fluid having a profile very similar to that of chronic venous leg ulcer fluid.\textsuperscript{37}

Chronic wounds were found to have abnormal elevated protease levels which are not controlled by the physiological mechanisms of the body, thus impairing healing. High level of proteases is important because their excessive activity destroys growth factors and their receptors, inhibits angiogenesis, and breaks down granulation tissue, resulting in tissue damage. Therefore, the degradation of growth factors by proteases leads to wound healing delay caused by continuous tissue destruction. Although proteases are important for facilitating the tissue repair, it seems that an increased concentration has a negative effect on wound healing.\textsuperscript{46} Indeed, research found that exudates from chronic wounds have higher levels of inflammatory cytokines, elevated levels of proteases (especially matrix metalloproteases-MMPs), low levels of growth factor activity compared with acute wounds and this persistently high level of MMPs contributes to the wound chronic character and the healing delay or failure.\textsuperscript{9,11,13,15,17,22,49-55}

Trengove et al noted significant differences in a study performed on chronic wounds (leg ulcers with different etiology and pressure ulcers) versus acute surgical wounds. They found that MMPs from wound fluid measured in chronic wounds was elevated by 30 comparing with acute wounds (p<0.001) accompanied by an inverse correlation on tissue inhibitor of metalloproteases (p=0.02, r=-0.78).\textsuperscript{53}

In particular, MMP-2 and MMP-9, are found predominantly associated with the inflammatory infiltrate, where is presumed to be involved in tissue degradation.\textsuperscript{56-58} In fluids from 56 pressure ulcers, Ladwig et al reported about 8-fold higher MMP-9 activity than MMP-2 activity in the pressure ulcer wound fluids; this agrees with the findings of Yager et al who reported an average level of pro MMP-9 activity about 50-fold higher than the average level of pro MMP-2 activity in the chronic wound fluids.\textsuperscript{11,22} Moor et al found 7-fold excess of MMP-9 in wound fluid (documented in long-standing venous ulcers that had showed resistance to standard therapy) compared to tissue biopsy samples, with 73% in the activated form and nearly equal levels of MMP-8.\textsuperscript{59} Several studies have demonstrated increased MMP-2, MMP-8 and TIMP expression and activity in chronic venous ulcers.\textsuperscript{51,52,60}

In a research that compared MMP-9 and MMP-2 levels in wound edges biopsies from healthy patients with acute partial-thickness wounds with...
patients with venous leg ulcer and chronic wounds of different etiologies, Miraskitskij et al found that the overall activity of gelatinases MMP-9 (p = 0.814) and MMP-2 (p = 0.742) is not increased in chronic wounds compared to normally healing wound tissues. They revealed that chronic nonhealing wounds may not be caused by excessive gelatinase activity, but are distinguished from healing wounds by an unfavorable distribution and persistence of MMP-961. Although MMP-2 activity remains unclear in venous leg ulcers, pathologic exams showed that its presence delays healing through excessive degradation of basement membrane, which predisposes to loss of epidermal integrity. Beidler et al found MMP-8 and MMP-9 is elevated in ulcer tissues, with significant reduction associated with healing. Based on these findings, they concluded that reduction in the proinflammatory environment and ulcer healing is associated with resolution of specific elevated levels of protease expression. A study published in 2005 emphasized a linear correlation between plasma MMP-9 levels and the severity of ischemia in patients with varying degrees of peripheral arterial occlusive disease.

In diabetic foot ulcers a high level of MMP-1 seems essential to wound healing but the wound is impaired by excessive activity of MMP-8 and MMP-9. Muller et al found a significant correlation between a high ratio of MMP-1/TIMP-1 and good healing (r=0.65, p=0.008), assuming that this could be a predictor of wound healing in diabetic foot ulcers.

Elevated levels of various serine proteinases have also been found at chronic wound sites (animal model and venous ulcers), particularly at those of neutrophil origin, including cathepsin G, urokinase-type plasminogen activator, and particularly neutrophil elastase. Herouy et al found that the expression and activity of urokinase-type plasminogen activator, a fibrin-independent plasminogen activator, and its receptor, the urokinase-type plasminogen activator receptor, which potentiates the activity of MMP-2, were also elevated in venous ulcers. Neutrophil elastase was found elevated 30- to 1300-fold in chronic wound fluid compared to plasma. In agreement with earlier reports, data analysis performed by Tarlton et al showed statistically significant differences for pro-MMP-9 (p < 0.001), neutrophil elastase (p < 0.005) and activated matrix metalloproteinase-9 (p < 0.05). This research shows the potential role of markers of collagen’s biochemistry as predictors of healing in venous ulcers, especially MMP-9 and neutrophil elastase.

The findings of all these studies lead to the hypothesis that chronic wounds are a result of metabolic imbalances in the wound that generates excessive release of proteases and trigger inflammatory mechanisms, which trapped the wound in the inflammatory phase of repair. This inflammatory environment changes the physiology of wound healing and have to be reversed to an acute nature environment for achieve success in healing. Usually this achievement is obtained due debridement, process which remove foreign bodies, infection, necroses, biofilm and not least, excessive exudates with proteases from wound bed.

Researchers have explored many hypotheses in order to address the challenge of proteases and to develop sophisticated devices, materials and methods that improve wound healing.

Exploring the hypothesis that a triangular relationship exists among the protease/anti-protease profile at the wound surface, angiogenesis and re-epithelialisation, Caulfield et al (2008) suggest that VAC therapy could create a low protease level environment by continuously removing the wound fluid and proteases (in the fluid) from the wound. Comparing this intervention with a local treatment involving hydrocolloid dressing that creates a high protease level environment by keeping the exudates in contact with the wound, it was demonstrated that VAC therapy treatment is associated with a statistically significant decrease of neutrophil elastase level (p<0.001), protease-inhibitor level (p<0.001), angiogenic factor VEGF (p<0.001). The results obtained by Caulfield et al are similar with previous studies. It was stated that even though exudates are beneficial for wound healing 48 because they ensure a moist environment, their excessive and persistent presence in wound can cause a higher concentration of proteases and wound healing failure.

An interesting study performed by Beidler et al investigating the effects of sustained high compression bandaging on patients with pathogenesis of chronic venous insufficiency ulcers found that MMP-1, -2, -3, -8, -9, -12, and -13 protein levels were elevated in ulcer tissue compared with healthy tissue before intervention, with higher values for MMP-8 and -9. Proteases MMP-3, -8, and -9 significantly decreased following treatments and this reduction was associated with significantly higher rates of ulcer healing after 4 weeks.

Even iodine use became controversial, as showed in an in-vitro research, where manifest cellular toxicity and inhibited normal wound healing, and some animal trials demonstrated that low doses of iodine could significantly inhibit purified plasmin and neutrophil elastase activity (p<0.001) in wound fluid.

Biopsies performed on nonhealing wounds or chronic leg ulcers after different topical application
(mitogenic bovine whey extract, oak bark extract) show that these products were able to modulate the expression of MMP-2, -9, and TIMP-2. Testing the feasibility of using the level of MMP-2 as an indicator of wound healing in patients with nonhealing wounds in transition from chronic-inert to active healing, Karim et al found that levels of MMPs in wound biopsies are correlated with the severity of wound healing impairment and are affected as result of unbalanced processes extracellular matrix. In addition, monitoring the expression of urokinase plasminogen activator and matrix metalloproteinase-9 appears to be a useful biomarker to determine the status of wound healing. 

Proteases are important for healing, however, in many models of wound healing, lower levels of inflammation are associated with faster healing and less scarring and it started to be clear that greater levels of proteases from chronic wounds indicate an uncontrolled proteolysis which is a characteristic of delayed healing. Exudates from wounds that were healing well (mastectomy and colectomy patients) expressed maximal levels of MMP-9 at 24 h, followed by a significant decline by 48 h. Absence of the significant decline in the second postoperative day indicated by a persistent elevation of MMP-9 expression, was later associated with infected and chronic wounds. Measurement of MMP-9 in postoperative wound fluids, therefore, could provide an early indicator of impaired healing, which may be evaluated non-invasively within 48 h of closure.

The evidence of this research can be helpful in the development of effective wound management protocols in terms of diagnostic and treatment initiation; however, according to Liu et al, all the data surrounding MMPs and TIMPs remains slightly conflicting, presenting all the potential bias facing wound research: small sample size, inconsistent methodology, not well defined outcomes.

**CLINICAL RELEVANCE**

Upon their cellular activation, proteases are involved in many physiological processes in the extra- and peri-cellular space at the wound level. Over the years, studies have assessed various aspects of their activity in wound healing, trying to elucidate their role. The role of proteases as biomarkers in wound healing was recognized for many years as a significant one. Toft et al. suggested that those components of inflammatory response are correlated with the magnitude of the tissue damage, some interventions, and therefore reconstructive surgery, should be postponed until the inflammatory response is normalized. Evaluating the wound microenvironment is a challenge due to multiple factors that contribute to this process. Selecting a molecule as prognostic biomarker is very hard because it is difficult to analyze the complexity of its interaction in the wound, to understand its pathogenesis, and to find rapid laboratory-based tests or point of care measurement.

Sophisticated analytical methodology used in research accompanied by a wealth of technology exceeds generally the requirements of clinical wound healing and emphasis has been laid on the need to identify “robust measurement techniques which can be applied to such a heterogeneous system.”

Herouy et al. suggested that understanding the molecular pathway of matrix degradation by proteolytic enzymes of matrix metalloproteinases in venous ulcers may facilitate finding potential therapeutic strategies that can assist in managing patients with advanced complications of chronic venous insufficiency.

Many of the crucial cellular responses of early wound healing, such as inflammatory infiltration, angiogenesis and re-epithelialization, are the result of the matrix MMPs action; therefore, by measuring wound proteases as biomarker, clinicians may be able to predict wound outcomes for difficult-to-heal wounds.

Although proteases can be considered as objective indicators in assessing the prognosis of wound repair, MMPs present in wound exudate or biopsy tissue may be less reliable in clinical work because of their inconsistent prognostic value among different methods of analysis, wound fluid collection, or bacterial contamination.

Such tests are time consuming and relatively expensive, which limits the use of these markers in routine wound assessment in clinical settings. From another point of view, routine assessments consisting of wound size measuring, macroscopic exudate evaluation, type of wound tissue, microbial load, do not seem to be sufficient for an objective evaluation of wound healing progress.

Research conducted in recent years reflects that an appropriate approach on wound healing pathophysiology could conduct to find new advanced modalities in molecular mediators control for healing.

The use of reliable diagnostic tests that indicate the presence and level of proteases in the healing environment can play an important role in therapy initiation and in establishing an effective treatment to prevent delays in wound healing. Investigating the current literature, Hahm et al didn’t found any available biological assays for wound physiology evaluation, with further implication in management of complex
soft-tissue wounds. Future research has to be done in order to establish clear physiologic landmarks related to wound care process in order to develop valuable tools for protease measurement. These instruments need to be cost-effective and easy to use in all circumstances where wound care is involved. Their existence would add more value in decisions making for an effective wound diagnostic and treatment through determining the cause of a wound, assessing its status, predicting healing and helping identify any complications that may contribute to healing delay.

CONCLUSION

Normal wound healing is the result of balanced tissue degradation and synthesis. Controlled degradation of extracellular matrix in damaged tissue and controlled formation of the new tissue is essential for the healing processes.

There is poor and contradictory evidence available in the literature that refers to the role of proteases as biomarkers in predicting wound healing progress and how this knowledge it is relevant of for clinical practice. Consequently, no firm recommendations for how to use this inconsistent information in wound healing evaluation can be done. There is a need for further studies in this area with quality methodology, adequate sample size and clear end points.

We can conclude that wound fluid, wound biopsy, or blood plasma MMP levels could reflect wound status and can be used as a possible prognostic indicator. Understanding this area better and implementing long-term program, with specific targets, will assist the development of evaluation and therapeutic strategies. It is clear that proteases are essential in the healing process and an increasing knowledge on their activity and role in altering the wound environment is of great clinical value for the development of innovative solutions for cost-effective diagnostic tests.

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