

# The role of growth factors, cytokines and proteases in wound management

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## Abstract

The wound healing process involves a series of cellular and biochemical events that ultimately lead to tissue repair and regeneration. These events are classically defined as haemostasis, inflammation, proliferation, epithelisation, maturation and remodelling of the scar tissue. Of particular importance to the healing process is the involvement of endogenous growth factors, cytokines and proteases at each of these events. Current knowledge of these at each stage of healing, as well as the biochemical nature of wounds, suggests that there is great potential for analogues of these agents to improve the quality and healing time of a wound, particularly a chronic wound, when they are applied exogenously.

However, many products containing growth factors, cytokines or protease inhibitors are yet to reach market availability. This is mainly due to formulation problems (such as stability), safety concerns, pharmacological aspects (such as effective doses and growth factor combinations) and difficulties with developing well-controlled clinical trials to prove their efficacy.

However, the concept of employing exogenous growth factors, cytokines and protease inhibitors to promote optimal wound healing is only in its infancy. And, while further research into the pharmacology, toxicology and formulation of design and development is still warranted, the American Food and Drug Administration's approval of the growth factor based product, Regranex<sup>®</sup>, should indicate that the role of these substances in wound management seems promising.

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## Introduction

A sound knowledge of the healing process and the factors that influence it is vital for the successful management of any type of wound. Before the 1960s, there was very little insight into the molecular and physiological processes that occur during wound healing. Clinicians once assumed a passive, and somewhat naive, role when treating a wound. This is best illustrated by the renowned French surgeon, Paré, who stated "I dressed the wound and God healed it"<sup>1</sup>.

During this era, the fundamental aim of wound management was to keep the wound as dry as possible in order to prevent

bacterial infection. Landmark studies performed in the early 1960s led to the discovery that scab formation impeded epithelial cell migration within the wound and subsequently prolonged healing time<sup>2,3</sup>. More importantly, occlusive dressings maintained a moist wound environment, accelerated epithelisation and reduced healing time.

It was not until the 1970s that wound healing was described as a dynamic process, entailing a number of phases that involve complex interactions between different cell types<sup>4,5</sup>. With the advent of recombinant DNA technology, researchers have recently been able to identify and study the regulation of these phases on a molecular level. These discoveries have heralded exciting new concepts in wound management. The most notable pertain to the importance of cytokines, growth factors and proteases in tissue repair and regeneration.

## Growth factor classification and mechanism of action

The term cytokines refers to a heterogeneous group of proteins that exert their actions on target cells via specific interactions with cell surface receptors<sup>6,7</sup>. Substances

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included in this group are interferons, interleukins, tumour necrosis factors, colony stimulating factors and growth factors<sup>8</sup>. Growth factors were initially discovered because of their ability to stimulate mitosis of quiescent cells<sup>6</sup>. They are classified in terms of families, based upon structural and functional similarities and their interacting receptors.

Growth factors are synthesised and released by many cell types. Once a growth factor is released, it may:

- Act on the same cell that produced it (autocrine stimulation).
- Act on cells that are adjacent to the producer cell (paracrine stimulation).
- Enter the circulation to be transported to cells that are distant from the producer cell (endocrine stimulation)<sup>9,10</sup>.

To our present knowledge, there are five families of growth factors involved in wound healing (Table 1). They exert a very powerful influence on the tissue repair process through a number of actions<sup>6,13</sup>. In particular, they:

- Attract other cells into the wound area (chemotaxis).
- Induce cell proliferation (mitosis).
- Stimulate the formation of new blood vessels (angiogenesis).
- Regulate the synthesis and degradation of the extracellular matrix (ECM).

## The wound healing process

To appreciate the importance of the functions of the cytokines, an understanding of the wound healing process is essential. Injury to the skin triggers an organised cascade of cellular and biochemical events that ultimately lead to the restoration of the skin's integrity. The wound healing process is classically defined as a series of continuous, and sometimes overlapping, events. These are haemostasis, inflammation, proliferation, epithelisation, maturation and remodelling of the scar tissue<sup>12</sup>.

Haemostasis occurs immediately after injury and involves the exposure of subendothelial collagen to platelets located in the intravascular space. Thrombin, formed by the exposed collagen, activates the platelets; this leads to a number of events such as:

- Activation of the coagulation cascade<sup>14</sup>. This eventually leads to the formation of a fibrin clot that acts as scaffolding for other cells that later enter the wound<sup>15</sup>.
- Activation of the complement system<sup>11</sup>.
- Platelet degranulation: this releases an array of cytokines, growth factors and vasoactive substances from the platelet

$\alpha$ -granules. Such substances include PDGF, TGF- $\beta$ , FGF, EGF, platelet-derived angiogenesis factor, serotonin, bradykinin, platelet-activating factor, thromboxane A<sub>2</sub>, platelet factor IV, prostaglandins and histamine<sup>11,12</sup>. These platelet releasates initiate the early events of wound healing.

Inflammation begins immediately after injury and may continue for up to 6 days<sup>16</sup>. Growth factors released from the platelets diffuse into tissues surrounding the wound and chemotactically draw inflammatory cells into the injured area. Neutrophils are the first inflammatory cells to enter the wound, followed by monocytes which later differentiate into macrophages<sup>17,18</sup>. Following chemotaxis, local mediators activate the inflammatory cells.

Activated neutrophils release a number of lysosomal enzymes (such as elastase, neutral proteases and collagenase) which proteolytically remove damaged components of ECM and aid in host defence<sup>1,19,20</sup>. Macrophage activation leads to a series of processes that are vital to tissue repair<sup>21</sup>. As seen in Figure 1, growth factors and cytokines mediate many of these important functions.

Regulatory mechanisms ensure that growth factors and cytokines are present within the wound for sustained periods. For instance, lymphocytes are activated by macrophages to produce cytokines such as interferon- $\gamma$  (INF- $\gamma$ ) that act back on macrophages and monocytes (in a paracrine manner) to release other cytokines such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 (IL-1)<sup>22</sup>.

The proliferation phase is characterised by the formation of the ECM and the beginning of angiogenesis<sup>23</sup>. The primary cells involved in this phase are fibroblasts and endothelial cells<sup>22</sup>. They proliferate in response to growth factors and cytokines that are released from macrophages, platelets or mesenchymal cells, or have been stored in the fibrin clot. In addition to chemotactically drawing fibroblasts into the wound, PDGF, FGF and EGF induce fibroblast activation and proliferation<sup>24</sup>. TGF- $\beta$ , INF- $\gamma$ , IL-1 and TNF- $\alpha$  either stimulate or reduce fibroblast proliferation, depending on their concentrations in the wound<sup>22</sup>.

During the first 2-3 days post-injury, fibroblast activity predominantly involves migration and proliferation. After this time, fibroblasts release collagen in response to macrophage released growth factors, hypoxia and by-products of anaerobic metabolism<sup>12</sup>. They also produce glycosaminoglycans<sup>12</sup>. These (mainly hyaluronic acid, chondroitin-4-sulphate, dermatan

sulphate and heparin sulphate) form an amorphous gel in which collagen fibres deposit and aggregate. The combination of fibronectin and collagen forms the ECM which is essential for the development of granulation tissue that eventually fills the wound<sup>23</sup>.

Angiogenesis accompanies fibroblast proliferation and allows nutrients and healing factors to enter the wound space<sup>12</sup>. It is

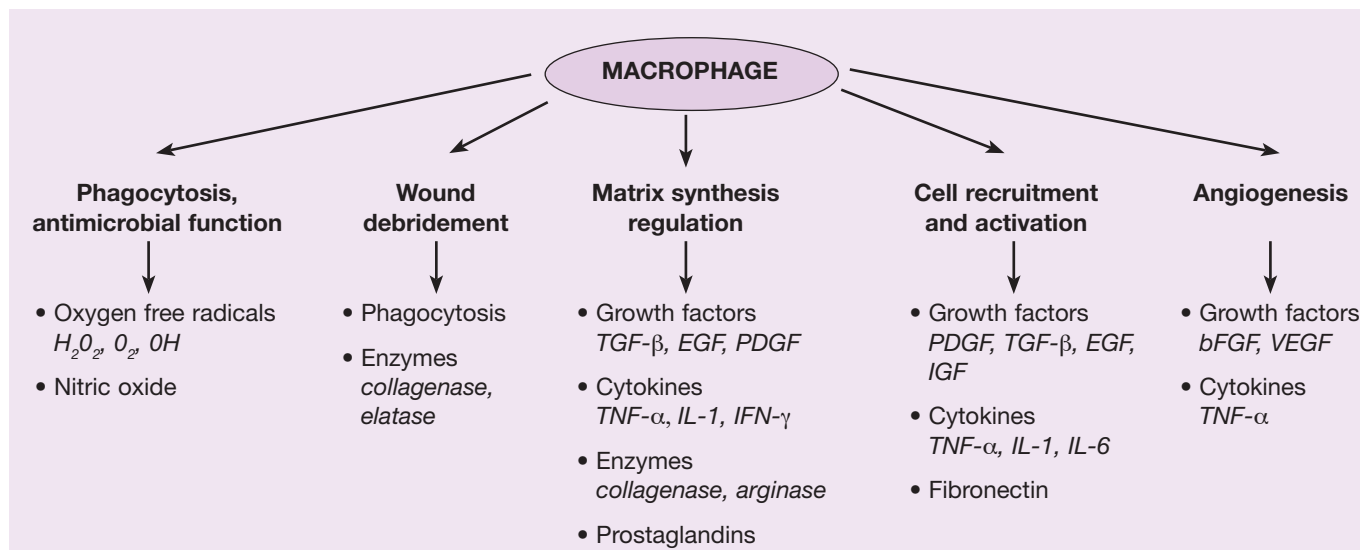
also essential for the growth of granulation tissue. The principle growth factors that regulate angiogenesis are bFGF, released by damaged endothelial cells and macrophages, and VEGF which is released by keratinocytes and macrophages<sup>25</sup>.

Fibroblasts also release IGF-1, bFGF, TGF- $\beta$ , PDGF and KGF. Endothelial cells produce VEGF, bFGF and PDGF and keratinocytes secrete TGF- $\beta$ , TGF- $\alpha$  and IL-1 $\beta$ . These factors

**Table 1. Growth factor families involved in wound repair, cell sources and biological actions<sup>6, 11, 12</sup>.**

Growth factor	Cell source	Principle action(s) in wound healing
<b>EGF family</b>		
EGF	Platelets	Stimulates proliferation of epithelial cells, fibroblasts and vascular endothelial cells
TGF- $\alpha$	Platelets Macrophages Keratinocytes Eosinophils	Similar to EGF, more potent inducer of angiogenesis
Hb-EGF	Macrophages	Mitogenic for keratinocytes
Amphiregulin	Keratinocytes	Mitogenic for some cells, inhibits others. Role in wound healing not yet established
<b>TGF-<math>\beta</math> family</b>		
TGF- $\beta_{1-3}$ (There are five subunits of TGF- $\beta$ , however only TGF- $\beta_{1-3}$ are found in mammalian cells)	Macrophages Lymphocytes Fibroblasts Keratinocytes Platelets	Inhibits proliferation of many cell types in vitro, including keratinocytes, endothelial cells and macrophages. May inhibit or stimulate fibroblasts.
<b>PDGF family</b>		
PDGF	Fibroblasts Vascular endothelial cells Vascular smooth muscle cells	Attracts fibroblasts, smooth muscle cells, monocytes and neutrophils into the wound
VEGF	Pituitary cells Macrophages Keratinocytes	Mitogen for vascular endothelial cells, stimulates angiogenesis
<b>IGF family</b>		
IGF- I	Fibroblasts Macrophages Platelets	May promote migration of endothelial cells into the wound. Mitogenic for fibroblasts.
<b>FGF Family</b>		
aFGF and bFGF (The IGF family includes IGF- I and IGF- II. IGF- I usually represents this family)	Macrophages Neural tissue Fibroblasts Astrocytes Endothelial cells Bone cells Smooth muscle	Mitogens for tissues of mesenchymal and neural origin
KGF	Fibroblasts	Mitogen for epithelial cells

EGF = epidermal growth factor; TGF $\alpha$ = transforming growth factor alpha; Hb-EGF = heparin binding epidermal growth factor; VEGF = vascular endothelial growth factor; TGF $\beta$ = transforming growth factor beta; IGF = insulin-like growth factor; PDGF = platelet derived growth factor; FGF = fibroblast growth factor; aFGF = acidic FGF; bFGF = basic FGF, KGF= keratinocyte growth factor

Figure 1. The role of the macrophage in wound healing<sup>22</sup>.

continue to stimulate cell proliferation, ECM synthesis and angiogenesis throughout the proliferative phase<sup>26</sup>.

The maturation phase usually begins 3 weeks after injury and can take up to 2 years to complete<sup>1</sup>. Unlike uninjured skin, the arrangement of newly formed collagen fibres in the wound is random and disorganised. The remodelling of collagen fibres into a more organised lattice structure gradually increases the tensile strength of the scar tissue, though this never exceeds 80 per cent of the strength of intact skin<sup>27</sup>.

There are 13 different types of collagen found in the human body<sup>12</sup>. The predominant types of collagen found in intact skin are Type I and Type III. Granulation tissue is composed of a higher Type III collagen content compared with normal, intact skin, and the amount of Type III collagen is much less in scar tissue<sup>22</sup>. Collagen is made of three polypeptide chains, each of which forms a helix. The three chains combine to form tropocollagen, the basic collagen unit. The collagen units combine to form fibrils, which aggregate and become collagen fibres. Remodelling of the ECM involves a balance between collagen synthesis and degradation.

Matrix metalloproteinases (MMPs) contribute to the degradation of the ECM<sup>28, 29</sup>. MMPs are zinc dependent endopeptidases that cleave most macromolecules within the ECM. There are nine members of the MMP family, four of which are involved in wound repair<sup>28</sup>. These are interstitial collagenases, stromelysins, gelatinases and membrane type metalloproteinases.

The enzymes share a number of structural and functional similarities; however, they differ in terms of substrate

specificity<sup>29</sup>. Collagen fibrils, which are resistant to proteolytic degradation by most enzymes, are susceptible to collagenases. Collagenases degrade the helical structure of Type I collagen, which renders the molecule more susceptible to degradation by other proteases. After the initial cleavage by collagenase, gelatinases degrade the molecule into smaller fragments. Stromelysins degrade ECM components such as fibronectin, proteoglycans, laminin, elastin and collagen Types IV, V, IX and X. Membrane type metalloproteinases may play important roles in the proteolytic activation of gelatinase precursors<sup>20</sup>.

Macrophages and neutrophils release gelatinases and stromelysins that aid the diffusion of inflammatory cells into the wound during the inflammatory phase. Elastase and gelatinase, released by neutrophils, also remove debris from the ECM. Vascular endothelial cells release MMPs that aid the migration of angiogenic cytokines (VEGF, TGF- $\alpha$ , TGF- $\beta$ , TNF- $\alpha$ , aFGF and bFGF) into the wound<sup>20</sup>.

## Chronic wounds

Chronic, non-healing wounds contain higher concentrations of proteases (such as MMPs) compared with acute wounds<sup>13, 26</sup>. Chronic wounds also contain lower levels of growth factors and cytokines<sup>20, 30</sup>. High levels of proteolytic activity in chronic wounds may lead to the degradation of endogenous growth factors as well as exogenously applied growth factors<sup>31</sup>. One proposed method of reducing the proteolytic degradation of growth factors and cytokines is to inhibit MMP activity<sup>26</sup>.

The activities of MMPs in the wound environment are determined by several factors<sup>20, 28, 29</sup>. In particular, tissue

inhibitors of metalloproteinases (TIMPs) are specific proteins that tightly control MMP activity<sup>29</sup>. Hence, an alternative for treating chronic wounds may be the application of an exogenous growth factor in combination with a protease inhibitor<sup>32</sup>.

### Pharmaceutical considerations

Most products containing growth factors or cytokines either are under investigation or still in pre-clinical stages of development<sup>33</sup>. This is mainly due to formulation difficulties, pre-clinical concerns (such as toxicology and pharmacology) and problems with clinical trial development<sup>34</sup> such as selecting a suitable patient population according to a defined criteria and ensuring that response variables are kept to a minimum.

Several factors contribute to the formulation of an effective growth factor based product. Most growth factors require prolonged exposure to wound cells in order to exert a significant effect on the healing process<sup>26</sup>. As growth factors generally have short half-lives, they need to be incorporated into a delivery system that will retain their bioactivity<sup>35</sup>. For example, a single application of EGF formulated in multilamellar liposomes (that released EGF over 5 days) was found to produce a 200 per cent increase in the tensile strength of surgical wounds compared to control. On the other hand, a single application of EGF in saline failed to produce significantly different results compared with control<sup>36</sup>.

Questions remain about the doses that are required to achieve optimal healing. The usual concentration of exogenously applied growth factors *in vivo* ranges from 10-1,000mg/ml<sup>26</sup>. This is much higher than the concentrations that are needed to stimulate DNA synthesis and the migration of wound cells *in vitro*, possibly because of diffusion from the wound area and degradation by proteinases<sup>26</sup>.

A common opinion is that a cocktail of growth factors may be more effective than individual growth factors<sup>26, 37</sup>. For example, the application of single growth factors (PDGF, IGF-1, EGF and FGF) had little or no effect on epithelisation or the formation of granulation tissue in partial thickness porcine wounds. Conversely, a combination of PDGF and IGF-1 caused a dramatic increase in connective tissue regeneration and epithelisation<sup>38</sup>.

It may be that certain combinations of growth factors may act synergistically to accelerate wound healing<sup>37, 38</sup>. For instance, the application of a growth factor that stimulates the formation of granulation tissue combined with one that enhances epithelisation. However, for some wounds, therapy

with a single growth factor may be sufficient to overcome healing impairment<sup>37</sup>. Therefore, growth factor combinations may only be necessary for treating specific types of wounds. This concept may also influence the decision to use either autologous growth factors (a combination of factors derived from human platelets, such as platelet derived wound healing formula<sup>39</sup>) or a single, recombinant growth factor.

Specific interactions between growth factors and their target cell receptor sites are also paramount to the effectiveness of these agents<sup>6</sup>. Many growth factors exist as different isomers. A prime example is PDGF which exists as three isoforms, PDGF-AA, PDGF-BB, PDGF-AB. As seen in Table 2, PDGF receptors have different affinities for the three isomers<sup>6</sup>. Hence, the response of a PDGF target cell depends upon the type of PDGF receptor(s) the cell expresses and the isoform of PDGF presented to it.

There are number of safety issues surrounding the use of exogenously applied growth factors. As they are protein drugs, they are likely to cause immunogenicity, especially with repeated application. It has been hypothesised that neoplastic transformation may occur via the unregulated expression of growth factors<sup>40</sup>, thus raising fears about the carcinogenic potential of exogenously applied agents. The prolonged exposure of growth factors to wound cells could induce excessive tissue proliferation, leading to pathologies such as atherosclerosis<sup>41</sup> or hypertrophic scarring<sup>12, 42</sup>.

The concentration of TGF- $\beta$  has been experimentally reduced in murine wounds by means of a neutralising antibody (NA) to this growth factor<sup>43</sup>. The NA treated wounds healed without scar tissue formation, whereas control wounds (not treated with the antibody) healed with scarring. TGF- $\beta$  has also been implicated in the pathogenesis of fibrotic disorders and kidney disease<sup>44</sup>.

A number of *in vivo* animal studies have been conducted to assess the systemic toxicity, sensitisation, local irritation and

**Table 2. PDGF receptor-isomer interactions.**

PDGF isomer	PDGF receptor type	
	PDGF- $\alpha$	PDGF- $\beta$
PDGF-AA	✓	✗
PDGF-AB	✓	–
PDGF-BB	✓	✓

✓ High binding affinity (receptor recognises both A and B sub-units)

– Low binding affinity (receptor only recognises B sub-unit)

✗ Negligible receptor-isomer binding

genotoxic potential of recombinant PDGF-BB (becaplermin)<sup>45</sup>. The results of these studies indicate that there is little potential for becaplermin to elicit clinically significant adverse clinical effects; however, clinical studies are of course warranted to substantiate the safety of becaplermin.

Two clinical studies have shown negligible systemic absorption of becaplermin following daily topical administration of 100mg/g gel to patients with full thickness, lower extremity diabetic ulcers for 14 days<sup>41</sup>. Six blinded clinical studies, involving patients with full thickness, lower extremity diabetic ulcers, have been conducted to compare the safety of various concentrations (30, 100 and 300mg/g) of becaplermin gel with either a placebo gel or good ulcer care alone<sup>41</sup>. The incidence and nature of ulcer related adverse effects were similar across all treatment groups, as was the incidence of serious adverse events, cardiovascular events and mortality.

Another major obstacle for pharmaceutical companies is proving the efficacy of growth factor based products. The United States Food and Drug Administration (FDA) requires proof that these agents are not only safe but will substantially improve wound healing<sup>46</sup>. For more than a decade, clinical trials have been conducted to assess healing of chronic wounds in response to exogenously applied growth factors<sup>47</sup>.

Although the results of some of these studies seem promising, the deductions drawn from the majority of studies, regarding the efficacy of growth factors in wound healing, are somewhat disappointing. This is mostly due to the difficulties in controlling clinical trials of this nature. Firstly, it is difficult to compare tissue repair in clinical terms, as all wounds are different. Chronic wound patients are particularly undesirable subjects as the underlying aetiologies of chronic wounds are often difficult to control. The quality and time of wound healing also depends upon a number of variables, such as infection, dehiscence, vascular supply and tissue oxygenation<sup>1,16</sup>.

At present, Regranex<sup>®</sup> is the only growth factor based product that has been approved by the FDA. It contains 100mg/g of becaplermin in an aqueous based gel. Its indication is to promote healing of full thickness, lower extremity, neuropathic diabetic ulcers that have adequate blood supply<sup>48,49</sup>.

The safety and clinical efficacy of becaplermin gel has been evaluated over a 20 week period in two multicentre, randomised, double blind, placebo controlled clinical trials<sup>50,51</sup>. One of the studies found the incidence of complete healing of

diabetic ulcers treated with 30mg/g becaplermin gel was significantly higher compared with those receiving a placebo gel. The other study found that, compared with a placebo gel, 100mg/g becaplermin gel significantly increased the incidence of complete ulcer closure and decreased the time to achieve closure.

Two other clinical studies have compared 100mg/g becaplermin gel with good ulcer care alone<sup>49</sup>. The results of one study did not show a significant difference between treatments. The other study was not powered for statistical evaluation, though the incidences of complete wound healing were 44 per cent for patients who received the becaplermin gel and 22 per cent for those who received good ulcer care alone.

## Conclusion

Exogenously applied growth factors, cytokines and protease inhibitors have the potential to improve both the quality and healing time of chronic wounds. The question as to whether there is a need for these agents in the management of chronic wounds has been answered in terms of their importance in the healing process and the biochemical nature of chronic wounds.

However, many of the concerns regarding their efficacy and safety are still warranted and better-controlled clinical trials need to be conducted. The clinical development and FDA approval of Regranex<sup>®</sup> for diabetic ulcers should be a precedent for future clinical trials that evaluate the use of other growth factor based products on other types of wounds. However, it should also be recognised that statistically significant results (in terms of wound healing quality and time) generated from nonclinical and clinical studies with Regranex<sup>®</sup> may not necessarily entail clinical significance.

The clinical effectiveness and safety of this product will only be determined with its continued use. The efficacy of these agents will also depend on the selection of an appropriate formulation and a suitable growth factor (or combination of growth factors). In the future, it might be possible that these agents will be used to target specific deficiencies in particular types of wounds in order to achieve optimal therapeutic responses with minimal adverse effects.

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