

Cellular events and biomarkers of wound healing

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ABSTRACT

Researchers have identified several of the cellular events associated with wound healing. Platelets, neutrophils, macrophages, and fibroblasts primarily contribute to the process. They release cytokines including interleukins (ILs) and TNF- α , and growth factors, of which platelet-derived growth factor (PDGF) is perhaps the most important. The cytokines and growth factors manipulate the inflammatory phase of healing. Cytokines are chemotactic for white cells and fibroblasts, while the growth factors initiate fibroblast and keratinocyte proliferation. Inflammation is followed by the proliferation of fibroblasts, which lay down the extracellular matrix. Simultaneously, various white cells and other connective tissue cells release both the matrix metalloproteinases (MMPs) and the tissue inhibitors of these metalloproteinases (TIMPs). MMPs remove damaged structural proteins such as collagen, while the fibroblasts lay down fresh extracellular matrix proteins. Fluid collected from acute, healing wounds contains growth factors, and stimulates fibroblast proliferation, but fluid collected from chronic, nonhealing wounds does not. Fibroblasts from chronic wounds do not respond to chronic wound fluid, probably because the fibroblasts of these wounds have lost the receptors that respond to cytokines and growth factors. Nonhealing wounds contain high levels of IL1, IL6, and MMPs, and an abnormally high MMP/TIMP ratio. Clinical examination of wounds inconsistently predicts which wounds will heal when procedures like secondary closure are planned. Surgeons therefore hope that these chemicals can be used as biomarkers of wounds which have impaired ability to heal. There is also evidence that the application of growth factors like PDGF will help the healing of chronic, nonhealing wounds.

KEY WORDS

Cytokines; growth factors; matrix metalloproteinases; platelet-derived growth factor; wound healing

In the last 30 or so years, researchers have identified several of the cellular and biochemical events associated with wound healing. The process is becoming clearer, with the understanding of the cells and chemicals that help wounds to heal, and of those that inhibit healing. Investigators are trying to analyze the chemicals in chronic wounds in order to determine their condition and fitness for closure. A major advance is the clinical application of some of these chemicals to improve outcomes in wound healing.

Access this article online	
Quick Response Code: 	Website: www.ijps.org
	DOI: 10.4103/0970-0358.101282

In this paper we look at the biology of normal and abnormal healing, see if wounds analysis can predict poor healing, and review some literature on the clinical applications of this knowledge.

CELLULAR EVENTS ASSOCIATED WITH NORMAL WOUND HEALING

Wounds heal in four overlapping phases: haemostasis, inflammation, proliferation and remodelling.^[1,2]

Phase I: Haemostasis

Haemostasis results from the activation of platelets, which initiate the coagulation cascade. Platelets also release substances that initiate and influence wound healing. One of these is platelet-derived growth factor (PDGF), a protein that can be isolated from platelets using chromatography techniques.^[3] Other factors, produced by platelets and other cells, include the transforming growth factors (TGFs), the fibroblast growth factors (FGFs), and vascular endothelial growth factor (VEGF).

Phase II: Inflammation

Inflammation begins within 24 hours, and lasts for 2 weeks or more. Inflammatory cells secrete enzymes and various mediators that result in the classical hallmarks of inflammation: pain, redness, warmth, and swelling. While several other cells are involved in the process, in terms of healing the key players are the neutrophils, macrophages, and the T-lymphocytes.^[2]

Neutrophils are the first cells to respond to the platelet products.^[2] From the circulation they reach the affected area in response to the chemotatic properties of some of the mediators. Here they marginate, adhere to vascular endothelial cells and subsequently migrate out to the extravascular space, with the help of cell adhesion molecules (CAMs). Fibroblasts too carry CAMs, which also function as receptors for cell-cell interaction.^[4] Deficiency of adhesion molecules delays healing.^[5] Elastase and collagenase, released by neutrophils, help their migration through capillary walls into the extracellular spaces of the wound for phagocytosis. The enzymes also lyse and remove damaged structural proteins. Elastase can destroy some of the growth factors.^[6] In addition, neutrophils produce TNF- α and IL-1 that will recruit fibroblasts and epithelial cells.

Macrophages enter the wound, and participate in the phagocytic process. In addition, macrophages release growth factors and cytokines that help bring in the

proliferative phase of healing. These factors include PDGF, TGF- β , β -FGF, TNF- α , interleukin 1 (IL-1), and IL-6.

Lymphocytes are the last cells to infiltrate wounds,^[6] but are important in the production of IL-2, which helps recruit fibroblasts.

Phase III: Proliferation

Proliferation itself consists of the following three phases: fibroplasia, granulation, and epithelialization. It effectively begins with fibroblast migration into the wound, a process initiated primarily by the PDGF that has been released by platelets and macrophages.^[2] PDGF stimulates fibroblastic proliferation, chemotaxis, and collagenase production.

Fibroblasts lay down structural proteins such as collagen [Table 1^[7]]. They also produce the matrix metalloproteinases (MMPs). These proteolytic enzymes facilitate fibroblast movement within the matrix. Later the fibroblasts decrease their proteolytic activity and start to lay down structural proteins. This step is regulated by two growth factors. One of these is TGF- β , also secreted by both platelets and macrophages. The other is connective tissue growth factor (CTGF), secreted by the fibroblasts themselves.^[8] Collagen synthesis involves the hydroxylation of proline, and is affected by vitamin C deficiency.

Angiogenesis replaces damaged vasculature with granulation tissue. Epidermal cells, fibroblasts, vascular endothelial cells, and macrophages contribute to angiogenesis by the production of β FGF, TGF- β and VEGF. The proliferative effects of VEGF are regulated by hypoxia, which stimulates VEGF-induced angiogenesis,^[2] using adenosine as an intermediary. In fact, adenosine, acting via A_{2A} receptors, is now considered a potent regulator of the early stages of wound healing.^[9]

Epithelialization proceeds with the proliferation and migration of the epithelial cells, and is helped by EGF, keratinocyte growth factors (KGFs) and TGF- α . The cells and the extracellular matrix interact closely and continually, stimulating each other.^[10]

Table 1: Main structural proteins in the extracellular matrix

Collagens
Laminins
Fibronectin
Vitronectin
Matricellular proteins: thrombospondins, Tenascins, SPARC (Secreted Protein Acidic and Rich in Cysteine)

Phase IV: Remodelling

Remodelling is part of the resolution stage of healing. Inflammatory cells leave, and cells that release the growth factors become fewer. Fibroblasts continue to lay down collagen, even as they too begin to decrease in number.^[4] Remodelling takes place by further covalent cross-linking of collagen molecules. In a well-healed wound the final tensile strength may be as high as 80% of that possessed by normal tissue.^[2]

Table 2 lists some of the main cytokines, growth factors, and enzymes involved in the healing process.^[1,4,11,12]

CELLULAR EVENTS ASSOCIATED WITH ABNORMAL WOUND HEALING

Several cellular changes occur in chronic, nonhealing wounds that may differentiate them from acute or healthy wounds. Research that looks into these events is more often experimental than clinical. The evidence from such research indicates that fibroblasts in acute, healthy wounds show more activity, and respond well to inflammatory stimuli.

In comparison, fibroblasts in unhealthy wounds respond poorly to inflammatory stimuli. There is also increased proteolytic activity that destroys the extracellular matrix.^[2]

FIBROBLAST AND EPITHELIAL CELL ACTIVITY IN HEALTHY AND UNHEALTHY (CHRONIC) WOUNDS

In the chronic, unhealthy wound, fibroblast function is adversely affected. Is this because the environment is unfavourable, or is this because the fibroblasts themselves are damaged? The evidence suggests that both factors play a role.

Secretions from healthy wounds stimulate DNA synthesis in fibroblast cultures.^[13] On the other hand, secretions from chronic, nonhealing wounds (e.g., leg ulcers) inhibit the same fibroblasts.^[14] Interestingly, Bucalo *et al.*^[15] found that denaturing the chronic wound fluid contents by heating removed the inhibitory effect, and allowed fibroblasts to flourish again.

Table 2: Partial list of cytokines and their actions. Note that most of the molecules exist in several forms (e.g. IL-1 exists as at least two subtypes)

<i>Name</i>	<i>Abbreviation</i>	<i>Class</i>	<i>Produced by</i>	<i>Action</i>
Interleukins 1, 6, 8	IL1, IL6, IL8	Cytokines	Macrophages, keratinocytes	Proinflammatory; recruit fibroblasts and keratinocytes
Interleukin 2	IL2	Cytokine	T lymphocytes	Recruits fibroblasts
Interleukin 4	IL4	Cytokine	T lymphocytes	Inhibits TNF, IL1, IL6, inhibits fibroblast proliferation
Tumor necrosis factor alpha	TNF- α	Cytokine	Macrophages	Proinflammatory; helps collagen synthesis
Epidermal growth factor	EGF	Growth factor	Platelets, macrophages, keratinocytes	Promotes keratinocyte and fibroblast proliferation, keratinocyte migration, and granulation tissue formation
Fibroblast growth factors acidic and basic	FGF-a and b	Growth factors	Endothelial cells, fibroblasts, macrophages, T lymphocytes	Cause angiogenesis, fibroblast chemotaxis and proliferation
Keratinocyte growth factors 1 and 2	KGF	Growth factors	Fibroblasts	Stimulate keratinocyte division and differentiation
Platelet derived growth factors (PDGF exists in several forms: AA, BB, AB, others)	PDGF	Growth factor	Platelets, macrophages; also fibroblasts, endothelial cells	Cause neutrophil and fibroblast chemotaxis; fibroblast proliferation, and synthesis of matrix proteins, metalloproteinases, stimulates angiogenesis
Transforming growth factors (alpha and beta)	TGF- α, β	Growth factors	Platelets, macrophages, fibroblasts, keratinocytes, T lymphocytes	Cause fibroblast and keratinocyte chemotaxis, angiogenesis; upregulates TIMP; inhibits production of MMPs and keratinocyte proliferation, induces TGF β production
Vascular endothelial growth factors (a family of peptides)	VEGF	Growth factors	Endothelial cells, keratinocytes, platelets, macrophages, fibroblasts	Cause angiogenesis (mitogenic for endothelial cells). Expression increased in the presence of hypoxia
Tissue inhibitor of metalloproteinase	TIMP	Enzyme	Most mesenchymal cells	Inhibits MMPs
Matrix metalloproteinases	MMPs	Enzymes	Monocytes, macrophages, endothelial cells	Degrade the extracellular matrix

Again, the fibroblasts from chronic wounds themselves show poorer responses to growth factors than do fibroblasts from acute wounds, indicating that the fibroblasts of chronic wounds are themselves affected.^[16,17] Loots and colleagues^[18] measured the response of fibroblasts from chronic ulcers to various growth factors, and compared them to fibroblasts from nonlesional controls. Fibroblasts from chronic ulcers responded poorly, and needed higher doses of growth factors for the same response as healthy fibroblasts. The authors wondered if damaged receptors could be a factor in the poor response, but mentioned in their discussion that such receptor changes had been sought by others and not found. It is worth mentioning that epithelial cells from chronic ulcers, like the fibroblasts, also show poor response to growth factors. However, in epithelial cells it has been possible to show a defective expression of receptors for TGF- β .^[19]

If fibroblasts become nonresponsive, one would expect that cytokine secretion, representing neutrophil and macrophage activity, would increase in an attempt to cause a fibroblast response. That this indeed does occur was shown by Trengove *et al.*^[20] who reported that levels of the proinflammatory cytokines IL-1 and TNF- α were higher in nonhealing wounds compared to healing wounds. The levels fell significantly when healing began to occur.

Proteolytic activity in unhealthy wounds

Protease (e.g., collagenase) activity, which should fall as the fibroblasts start to lay down collagen, is higher in chronic wounds than acute. Studies show that the levels of MMPs, neutrophil elastase, and cathepsin-G remain high in chronic wounds.^[2,21,22] Tissue inhibitors of metalloproteases (TIMPs), which inhibit MMP function, tend to have lower levels in chronic wounds.^[23] The overall result of these changes is to increase the enzyme levels, and thus to reduce the proper deposition of matrix proteins. The proteases can also degrade and deplete growth factors like EGF, TGF1- α , and PDGF, molecules that are stable in fresh and clean injuries. Other proteases, not ordinarily involved in wound healing, also increase in fluid secretions of chronic wounds.^[2]

BIOMARKERS OF HEALING AND NONHEALING WOUNDS

Surgeons would love to be able to tell if a wound is fit enough for an attempt at closure. The badly infected wounds are obviously unfit, and of course the fresh, clean

wounds are obviously fit. The confusion regarding fitness for closure arises in wounds that have enough infection to affect healing, but not enough to produce clinical signs of infection.

Some workers believe that wounds should be classified in terms of colonization by bacteria: sterile, contaminated, colonized, critically colonized, and infected. "Critically colonized" wounds are those without clinical evidence of infection, but with enough bacteria to affect healing.^[24] They appear healthy and fit for closure, but will become overtly infected afterwards. These are the wounds which are most likely to be associated with incorrect surgical judgment, and which need the help of the laboratory.

Can biomarkers identify the "critically colonized" wounds: the ones in which healing has been affected? The answer is "probably". The biomarkers that hold most potential are cytokines and proteases.^[25]

Cytokine levels

Cytokine levels are higher in nonhealing wounds than in healing wounds, especially the levels of IL-1, IL-6, and TNF- α .^[2] Trengove *et al.*^[20] found that median levels of IL-1 were 9200 U/ml (range 1300-48000) in nonhealing wounds, compared to 2700 (400-14000) U/ml in healing wounds ($P = 0.003$). Similarly, the levels of IL-6 and TNF- α were also significantly higher in nonhealing wounds. Beidler *et al.*^[26] reported very high values for IL-8 in chronic wounds. Unfortunately, the wound and serum cytokine levels are so variable that it is difficult to use their levels as reliable markers of poor healing.^[27] Harris *et al.*,^[28] for example, did not find cytokine levels in the two types of wounds to be significantly different. Forsberg *et al.*^[29] measured cytokine levels in severe military wounds, and tried to predict which wounds would fail to heal: there was almost no correlation.

Protease levels

Protease levels are more likely to prove reliable as biomarkers of poor healing. Utz *et al.*^[30] studied 38 wounds in 25 patients, and collected samples during successive wound debridements. They stated that serum MMP-2 and MMP-7 were statistically predictive of wound healing outcome ($p < 0.001$): the higher the level, the lower the chance of successful healing. Snyder *et al.*^[22] reported the findings of a consensus panel, which concluded that the wound levels of elastase, metalloproteinases, and, in particular, MMP-9/TIMP ratios could prove good prognosticators of wound behaviour.^[23]

Gene expression

Gene expression analysis may be an option in the future. Asada and coworkers^[31] reported a fascinating experimental study in which they showed changes on gene expression analysis. Using reverse transcription polymerase chain reaction on wound fluid in rats, they showed that virulence factor bacterial genes were more likely to be expressed in the presence of invasive infection rather than mere colonization of wounds. Several host housekeeping genes (genes constitutively expressed to perform the cell's basic functions^[32]), in contrast, were more likely to be expressed in healthier wounds, but not in wounds that were frankly infected. They suggested that gene expression analysis could help establish the status of the wound.

Other substances

Moor *et al.*^[33] reported that wound fluid myeloperoxidase levels were associated with nonhealing and infected wounds. Studies are also on to determine if wound analysis for volatile organic compounds (e.g., various esters, alcohols, and organic compounds) may help in prognostication.^[34] Chronic wounds also contain higher quantities of reactive oxygen species.^[6] Forsberg *et al.*^[29] found that healing was invariably successful in wounds with procalcitonin levels lower than 220 pg/ml. This suggests that procalcitonin may have potential as a marker of healing.

Tissue bacterial levels

Another biological marker that may be predictive of healing in both chronic and acute wounds is the tissue bacterial level.^[35] High tissue levels of bacteria inhibit or impair all the processes of wound healing and prevent satisfactory repair of wound^[36,37]

Table 3 lists biomarkers that may have potential in predicting nonhealing wounds.^[20,22,23,29-31,33,34]

Table 3: Biomarkers of nonhealing. The following substances have potential as possible biomarkers of wound healing

MMP levels (raised in nonhealing wounds)
MMP/TIMP ratios (raised in nonhealing wounds)
Gene expression analysis: increased expression of bacterial housekeeping genes, decreased expression of host housekeeping genes
Cytokine levels (raised in nonhealing wounds)
Procalcitonin levels (raised in nonhealing wounds)
Myeloperoxidase levels in wound fluid (raised in nonhealing wounds)
Reactive oxygen species (raised in nonhealing wounds)

APPLICATION: THE CLINICAL USE OF GROWTH FACTORS

Over the last 20 years, researchers have investigated the results of topical application of growth factors on wounds. They have conducted animal and human studies, on both acute and chronic wounds. In an attempt to recreate an experimental "nonhealing" environment, some workers have used the setting of diabetes, ischaemia, and irradiation.

Animal studies

In the laboratory animal PDGF healed wounds in significantly less time and with less scarring compared to controls.^[38] Other compounds that have been used with good results include EGF,^[38] adenosine A_{2A} receptor agonists,^[39-41] fibrin sealant,^[42] and even fibronectin.^[43]

PDGF was superior to controls when applied topically to acute wounds in diabetic rats,^[44] and in rats with impaired healing due to steroid administration.^[45] Acute but ischaemic wounds also show accelerated epithelialization following application of PDGF.^[46] The factor also shows benefit in irradiated wounds.^[47]

Why do chronic ulcers respond worse to growth factors than even ischaemic and irradiated ulcers? One possible reason is that in chronic wounds, the cells (e.g., fibroblasts) fail to respond, perhaps because of a reduction of appropriate receptors. If this is true, then this poor response should be limited to chronically infected wounds, but not to acute wounds with ischaemia or irradiation. The acute irradiated wounds and acute ischaemic wounds showed improved healing when growth factors were applied, perhaps because they were not chronic, and the serum had not yet acquired the presence of inhibitors of growth factors.

If cellular response to growth factors is weaker in chronic wounds than in acute, the resultant impairment in healing should remain only until the environment remained unfavourable. After good wound care, the tissues should once again become responsive to growth factors. This in fact was shown in a recent study. In the first week of therapy chronically infected wounds behaved poorly, whether or not growth factors were administered. However, after a week of good wound care, the lesions in the growth factor group showed improved healing as compared to controls.^[48]

PDGF genes, delivered in plasmids,^[39] viruses,^[49] or in fibroblasts^[50] have also resulted in improvement in markers of healing.

Although not all studies confirm that growth factors improve healing,^[51,52] there is nevertheless considerable evidence that small but significant changes take place in angiogenesis, wound contraction, and epithelialization in experimental animals.

Clinical studies on acute wounds

PDGF exists as dimers of A and B chains.^[53] As compared to PDGF-AA and PDGF-AB, PDGF-BB is more active^[38] and is approved by the US Food and Drug Administration for application to wounds to promote healing.^[54] In the last 20 years, several studies have shown that PDGF applied to acute wounds results in earlier angiogenesis and complete healing. Ehrlich and Freedman^[55] applied topical PDGF to acute wounds behind the ear in patients undergoing elective surgery. Treated wounds healed significantly faster (16 days vs 20 days) and by epithelialization rather than by contraction in a small study of four patients.

Clinical studies on chronic wounds

In contrast, the use of growth factors in chronic wounds is limited. Fernández-Montequín *et al.*^[56] used an intralesional epidermal growth factor-based formulation (Heberprot-P) in chronic diabetic foot ulcers. They showed that treatment was well tolerated and safe, but, lacking a control group, could not show improvement in healing.

Platelet-derived growth factor

Kurtz *et al.*^[57] applied PDGF to the wounds of ten patients who had nonhealing wounds three months after abdominoperineal resection for inflammatory bowel disease. Six of the 10 wounds healed after an average of average 80 days. This report shows promise, but in the absence of controls does not confirm the value of PDGF in chronic wounds.

Unfortunately, controlled studies are rare. Shackelford *et al.*^[58] conducted a double-blind randomized placebo-controlled trial in patients with surgical wound separation. They randomized ten patients to treatment with 0.01% recombinant human PDGF gel, and eleven to control groups. Wounds of patients receiving placebo closed 54 days after operation; treated wounds closed in 35 days ($P = 0.05$). In their discussion they commented that wound closure rates were inferior to operative secondary closure. Nevertheless, this report provides strong evidence of the positive influence of PDGF in this setting of separated (but not chronically infected) wounds.

Recombinant PDGF (becaplermin, marketed as Regranex[®]), approved in the United States for specific wounds, significantly improved the incidence of complete healing in chronic pressure ulcers.^[59] In this study the ulcers were of over 4-week duration, and underwent debridement before drug application. On the other hand, Chan and coworkers were unable to show any benefits with PDGF on burn wounds in diabetic mice.^[51]

Other growth factors

Experience with factors other than PDGF is limited, since few are approved for clinical use. bFGF, approved for clinical use in Japan, showed reduced scarring in a prospective study of 230 cases.^[60] In contrast, Richard *et al.*,^[61] in a prospective randomized trial, found no benefit with bFGF in chronic ulcers. Tsang *et al.*, in a randomized trial,^[62] showed significantly improved healing in chronic diabetic foot ulcers using recombinant EGF, as did a phase III trial from India.^[63]

Bao and colleagues^[12] reviewed the literature and stated that VEGF gene transfer (e.g., by VEGF-expressing plasmids) may be used for chronic wounds, though at present the evidence is limited.

Personalized wound care

Personalized treatment is now evolving. Tests that detect individual biologic differences may allow individualized, targeted patient management. With the identification of biomarkers, biologic differences can be identified to aid in individualizing treatment. These concepts have recently been applied to predict individualized wound care in combat wounds.^[64-67]

Table 4 lists growth factors that may have uses in clinical practice.^[12,56-60]

Verdict on clinical usage

Costs

Growth factor therapy is somewhat expensive. For the management of a foot ulcer, a patient needs one to two tubes of recombinant human PDGF at about \$25 per tube.^[68] Langer and Rogowski^[69] conducted a systematic survey of the costs involved; admitting that there were weaknesses in the studies they reviewed, they concluded

Table 4: Potentially useful growth factors

PDGF (now approved for clinical use by the FDA)
EGF
VEGF

that the overall costs of therapy with PDGF resulted in some small but measurable saving.

Should growth factors be used clinically?

As of today there is very little level 1 evidence (i.e., by randomized controlled trials, Shackelford *et al.*^[58] being an exception here) to prove the value of growth factor therapy for acute or chronic wounds. Acute wounds respond well to PDGF, but heal well without help. Chronic wounds need help, but respond poorly to the factor. Nevertheless, bearing in mind the scanty evidence, there is still enough to warrant the use of PDGF in selected cases. It would be well to warn the patient that the results may be less than spectacular.

The surgeon should be aware of the caveats. First, and foremost, growth factors are a small adjunct to good wound care, not a replacement.^[54] Second, in patients whose wounds are suitable for secondary closure or cover, these options result in quicker healing. Third, there are several biological therapies: cultured skin, allografts, xenografts, and skin substitutes. They are not part of this review, but should be considered in the appropriate setting.^[70] Finally, texts of plastic surgery have begun recommending that PDGF should be “considered” in wounds not responsive to initial comprehensive therapy,^[70] therefore today there is certainly a small but definite place for this form of pharmacotherapy in chronic wounds.

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How to cite this article: Mohd. Yussof SJ, Omar E, Pai DR, Sood S. Cellular events and biomarkers of wound healing. *Indian J Plast Surg* 2012;45:220-8.

Source of Support: Nil, **Conflict of Interest:** None declared.