

## PERSPECTIVE ARTICLES

## Molecular dissection of abnormal wound healing processes resulting in keloid disease

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### ABSTRACT

Keloids are locally aggressive scars that typically invade into healthy surrounding skin and cause both physical and psychosocial distress to the patient. These pathological scars occur following minimal skin trauma after a variety of causes including burns and trauma. Although the pathogenesis of keloid disease is not well understood, it is considered to be the end product of an abnormal healing process. The aim of this review was to investigate the molecular and cellular pathobiology of keloid disease in relation to the normal wound healing process. The molecular aberrances in keloids that correlate with the molecular mechanisms in normal wound healing can be categorized into three groups: (1) extracellular matrix proteins and their degradation, (2) cytokines and growth factors, and (3) apoptotic pathways. With respect to cellular involvements, fibroblasts are the most well-studied cell population. However, it is unclear whether the fibroblast is the causative cell; they are modulated by other cell populations in wound repair, such as keratinocytes and macrophages. This review presents a detailed account of individual phases of the healing process and how they may potentially be implicated in aberrant raised scar formation, which may help in clarifying the mechanisms involved in keloid disease pathogenesis.

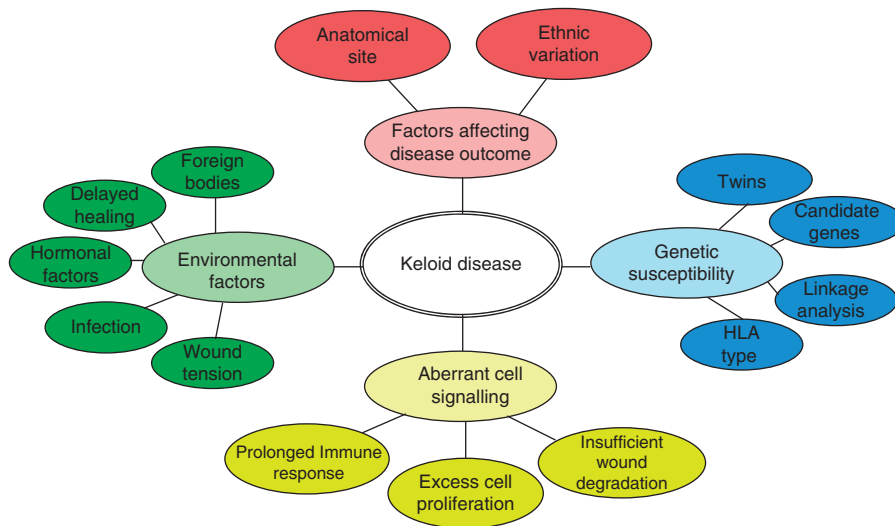
Keloid scars are raised dermal scars that form in response to an abnormal healing process.<sup>1,2</sup> They are unique to humans and appear to be common in darkly pigmented individuals.<sup>1,2</sup> In 2000, it was reported that about 11 million people were affected by keloid disease annually in the developed world alone.<sup>3</sup> Keloid scars are not only aesthetically displeasing but can be functionally disabling with intense pruritus and pain, and may lead to psychosocial distress.<sup>1,3</sup>

While fetal scarless wound repair represents one end of the healing spectrum, keloids can be said to represent the other end of this wide spectrum.<sup>4</sup> Even though the pathogenesis of keloid scarring is not well understood, it is suggested that it may result from a multifaceted abnormal wound healing process.<sup>5,6</sup> It was demonstrated in non-keloid forming healthy individuals that dermal scarring occurs following a certain critical depth in cutaneous wounding.<sup>7</sup> Keloids scarring however, is described to occur following any degree or form of skin trauma.<sup>8</sup> Although some keloid cases have been reported to arise “spontaneously,”<sup>9–11</sup> minor skin trauma, such as insect bites or acne, may have occurred in these cases without being noticed previously by the affected individual.<sup>12</sup> Mustoe et al.<sup>13</sup> have classified clinical scars into the following categories: normal mature scar, immature scar, linear hypertrophic scar (HS), widespread HS, minor keloid, and major keloid. Of these categories, major keloids are described as a most challenging clinical problem as they are often highly resistant to any treatment.<sup>13</sup> Both keloids and HSs are raised scars that are considered to result from ab-

normal wound healing processes.<sup>14</sup> However, unlike HSs, which do not extend beyond the confines of the original wound, keloid scars are locally aggressive, continually grow, and invade the surrounding normal skin.<sup>3</sup> Surgical removal of keloids has a high recurrence rate and a worse scar may recur following excisional surgery, in particular without the use of adjuvant therapy.<sup>15</sup>

It has been suggested that keloid scarring is caused by an inability to stop the wound healing process.<sup>5</sup> The events occurring during the wound healing process can be classified into three distinct, yet temporal overlapping phases: the inflammatory, proliferative, and scar maturation phase.<sup>16</sup> Keloid formation is often considered to be the result of a prolonged proliferative and a delayed remodeling phase.<sup>6</sup> In addition, there has been a theory that keloid formation is due to an abnormal response to inflammation by fibroblasts.<sup>17</sup> Nonetheless, the excessive scarring in keloids is certainly shown to be caused by increased proliferation and an excess collagen deposition by fibroblasts.<sup>18–21</sup>

Several etiological factors for keloids have been proposed in the past (Figure 1), which include: genetic predisposition,<sup>22</sup> hormonal, and endocrine factors,<sup>8,23</sup> the presence of foreign bodies in the wound site,<sup>24,25</sup> infection,<sup>26</sup> tension present in the local skin environment,<sup>27,28</sup> delayed healing,<sup>29</sup> prolonged excessive inflammation,<sup>30,31</sup> and abnormal epithelial–mesenchymal interactions.<sup>32</sup> Apart from genetic predisposition, which appears to play an important role in keloid development, there is inadequate scientific evidence to support the majority of these theories.



**Figure 1.** Possible causative factors in keloid pathogenesis. A number of factors have been implicated in the etiology of keloid disease, including environmental factors, genetic susceptibility, and aberrant cell signalling, as well as those that affect disease outcome.

Considerable attention has been placed on determining the genetic variations that may contribute to keloid susceptibilities (reviewed by Brown and Bayat<sup>33</sup>). Investigations in biological pathways and whole genome expression studies have also been carried out in order to determine the causative genes in keloid developments, from which several wound healing-related genes have been identified, but none has been confirmed and verified as the causative factor in keloid formation.<sup>34–38</sup>

The aim of this review was to describe the different stages of wound healing and correlate them with the current molecular understanding in the pathology of keloids, with respect to events, cell types, cytokines, and growth factors involved.

**METHOD**

This review is based on the scientific and clinical experience of the authors in this field, their previous and current research activities as well as a comprehensive search and identification of appropriate literature. Relevant articles were identified by carrying out a systemic search on scientific electronic search engines, including PubMed and Scopus. Key terms used for the searches included: “keloid,” “wound healing,” “wound repair,” “inflammation,” and “angiogenesis.” Following this, further

searches were carried out using a combination of key words: “keloid” together with each key cell type or key molecules involved during wound healing (identified using wound healing reviews obtained from earlier searches).

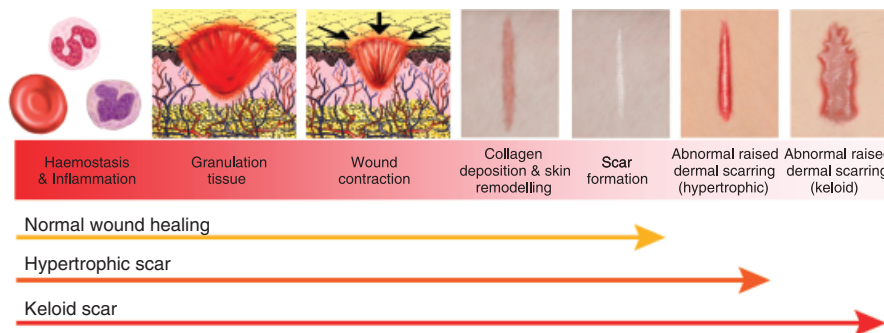
**Cutaneous wound healing**

Wound healing is a complex process with multiple steps involving: hemostasis, inflammation, neovascularization, fibroplasia, contraction, and remodeling. These processes are commonly summarized into three histologically and functionally distinct phases that overlap temporally: (1) inflammatory, (2) proliferative, and (3) maturation phase<sup>16</sup> (Figures 2 and 3). Some authors have suggested that keloid scar formation may be related to an abnormal response to inflammation, while others emphasize a rather more prolonged proliferative phase.<sup>30,39</sup>

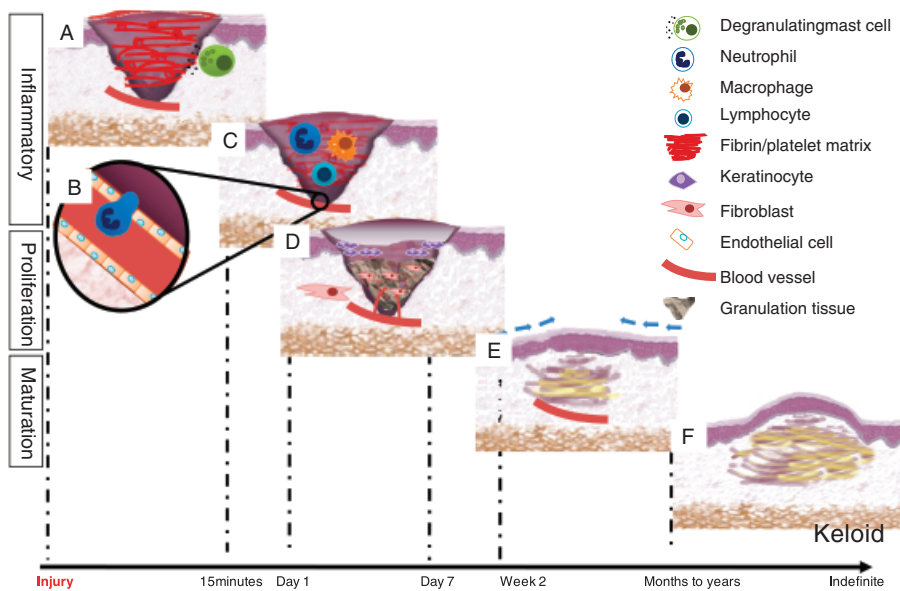
**Inflammatory phase**

**Hemostasis**

Hemostasis is the process that responds to injury to stop blood loss by plugging the wound through vasoconstriction and the formation of blood clot.<sup>40</sup> In response to the outflow of blood following wounding, the injured vessels



**Figure 2.** Spectrum of normal to abnormal wound healing resulting in keloid scar formation. Wound healing is a complex series of events that involves an inflammatory phase, fibroblastic phase, and a remodeling phase. Dysregulation of this process may cause a number of abnormal scars.



**Figure 3.** Classic phases of wound healing. Wound healing processes have been classified into three broad phases, inflammatory phase, proliferation phase, and maturation phase. During (A) the early inflammatory phase, together with mast cells, fibrin, and platelet, the two major components in blood clot, a number of mitogens and chemotactic factors (B) that increase the permeability of the blood vessel and recruit inflammatory cells released. (C) Neutrophils arrive at the wound site first, followed by monocytes that transform into macrophages and T lymphocytes. These cells produce a number of factors that attract the migration of neighboring keratinocytes and fibroblasts, promoting (D) the proliferation phase, during which re-epithelialization, neovascularization, and granulation tissue formation occurs.

(E) The maturation of the scar involves the replacement of disorganized type III collagen with type I collagen, which can take from months to years. (F) Keloids are scars that continually grow beyond the boundaries of the original wound. It is unclear which event of wound healing, or whether multiple events, become aberrant and lead to the formation of keloids.

undergo constriction and platelets aggregate at the site of injury, leading to the formation of blood clots through the coagulation system.<sup>41</sup> As well as acting as a barrier to microbial invasion, blood clots act as a provisional matrix for cell migration and wound repair, and a reservoir for growth factors and cytokines.<sup>41</sup>

Blood clots predominantly consist of fibrin, but other extracellular matrix (ECM) proteins, such as fibronectin, vitronectin, and thrombospondin, are also present.<sup>41</sup> Possible involvements of fibrin and fibronectin in keloid pathogenesis have both been suggested previously.<sup>42,43</sup> Through the proteolytic cleavage of fibrinogen by thrombin, fibrin is produced and forms cross links with each other.<sup>44</sup> Fibrin binds to platelets, and together they adhere to the subendothelium through integrin.<sup>45</sup> Fibrin assists several events in wound healing; it is able to bind to integrin CD11b/CD18 on monocytes and neutrophils during the inflammatory phase, to the fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) in neovascularization, and to the insulin-like growth factor-I (IGF-I) in stromal cell proliferation.<sup>46,47</sup> As well as thrombin up-regulation,<sup>38</sup> altered fibrin degradation have been demonstrated in keloids fibroblasts due to their high levels of plasminogen activator inhibitor-1 (PAI-1) and low levels of urokinase (urokinase-type plasminogen activator [uPA]).<sup>43,48</sup> The importance of fibrin was showed by the abnormal wound healing in fibrinogen-deficient mice.<sup>49</sup> PAI-1 and fibrin have been indicated in fibrosis as PAI-1-deficient mice show reduced fibrosis post-pulmonary injury.<sup>50</sup>

Shortly following the formation of the blood clot, the degradation of fibrin and the activation of the complement system take place, releasing several chemotactic agents and cytokines, which stimulate the chemotaxis of immune cells and initiate the inflammatory phase.<sup>40</sup>

## Inflammation

A variety of chemokines and vasoactive mediators are produced at the site of injury by the products of complement activation, platelet aggregation, degranulation, and bacterial degradation.<sup>51</sup> Upon injury, resident mast cells also degranulate and release histamine, bradykinins, and leukotrienes. These events lead to the recruitment of immune cells. Table 1 summarizes the major cell types, cytokines, chemokines, and growth factors that are involved in this phase, and whether the growth factors or cytokines involved have been implicated to be dysregulated in keloids.

Neutrophils are the first inflammatory cells to arrive and their primary role appears to be killing microbes.<sup>52</sup> Neutrophils also provide a source of proinflammatory cytokines.<sup>53</sup> Monocytes enter the wound bed and develop into activated macrophages, which have a dual role—the phagocytosis of any remaining cell debris and the orchestration of new-tissue formation.<sup>54</sup> Macrophages secrete numerous growth factors and cytokines that act on fibroblast, endothelial cells (EC), and keratinocytes, including: platelet-derived growth factor (PDGF), transforming growth factor (TGF)- $\alpha$ , TGF- $\beta$ , FGF-2, VEGF, and IGF-I.<sup>54</sup> In addition to their involvements in the inflammatory phase, some of these growth factors and cytokines are also involved in the proliferative phase and have also been implicated to be abnormal in keloids.<sup>55–58</sup>

There has been conflicting evidence regarding the importance and the benefit of inflammatory cells in wound healing.<sup>52</sup> Simpson and Ross<sup>59</sup> observed in the guinea pig model that, provided the conditions are kept sterile, wound healing is not dependent on neutrophils. However, while earlier studies suggest that macrophages are essential for wound repair, recent studies demonstrate normal, or improved in

**Table 1.** Association between cytokines, growth factors, and the major cell types that contribute to the inflammatory phase

Cells	Main function	Main growth factors/ cytokines they release	Recruited/activated by
Platelets	Formation of blood clot Cytokine secretion	<b>EGF</b> , <i>FGF-2</i> , <i>IL-1</i> , <b>TGF-<math>\beta</math></b> , <b>IGF-I</b> , <b>PDGF</b> , <b>TNF-<math>\alpha</math></b>	<b>Fibrin</b>
Mast cells	Cytokine secretion Matrix production	<b>TGF-<math>\beta</math></b> , <b>TNF-<math>\alpha</math></b> , <i>IL-4</i> , <i>IL-13</i> , tryptase, <b>histamine</b>	Complements pathway, direct injury
Neutrophils	Remove cellular debris, foreign particles, and bacteria Degradation of matrix Macrophage activation Neovascularization	<i>IL-1</i> , <b>VEGF</b> , <b>TNF-<math>\alpha</math></b> , <b>IL-6</b> , antimicrobial substances, proteases	<b>PDGF</b> , <b>IFN-<math>\gamma</math></b> , <i>IL-8</i> , <i>C5a</i> , growth-related oncogene- $\alpha$ , MCP-1, bacterial products
Monocytes/ macrophages	Phagocytosis of neutrophils and fragments of tissue degradation Reepithelialization ECM deposition ECM remodeling Neovascularization	<i>FGF-2</i> , <b>TGF-<math>\beta</math></b> , <b>VEGF</b> , <b>PDGF</b> , <i>TGF-<math>\alpha</math></i>	<b>TGF-<math>\beta</math></b> , <b>VEGF</b> , <b>TNF-<math>\alpha</math></b> , <b>IGF-I</b> , <b>PDGF</b> , <i>TGF-<math>\alpha</math></i>

Cytokines or growth factors that have been investigated in keloid scarring are indicated as follow; bold indicates genes that have been implicated to show dysregulated expression level or response in keloids; italics indicate no significant difference in expression level or response was observed in keloids. Please refer to Table 2 for more details.

EGF, epidermal growth factor; FGF, fibroblast growth factor; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; MCP, monocyte chemoattractant protein; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

some cases, healing in knockout mice models for macrophages, neutrophils or platelets.<sup>60–63</sup> Mice that essentially lack both neutrophils and macrophages appear to undergo wound repair without fibrosis, resulting in scarless healing like those in embryonic wound healing.<sup>64,65</sup>

Significant mast cell numbers in the keloid margin and abnormal mast cell distribution in keloid dermis have been described.<sup>66,67</sup> Higher numbers of macrophages and lymphocytes have also been reported in keloids.<sup>30</sup> Moreover, while normal acute wound healing showed an initial high CD4:CD8 T lymphocyte ratio that decreases as the wound heals,<sup>68</sup> the ratio is low in chronic wounds and high in keloids when compared with normal skin.<sup>30</sup> However, to date, there has been no conclusive evidence that elucidates the role of inflammatory cells in keloids formation.

Various growth factors, cytokines, and ECM, which are involved in the inflammatory phase, contribute to the regulation of cellular proliferation and fibrosis, which has led many researchers to believe that a dysregulated inflammatory phase may play a crucial role in the development of keloid scars.<sup>30,69–71</sup> Many studies have looked into the levels of expression of growth factors and cytokines (Table 2 and Figure 4). Dysregulated levels of cytokines have been found in the peripheral blood mononuclear cell fraction obtained from keloid patients, including up-regulation of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, and interferon (IFN)- $\beta$ , and the down-regulation of IFN- $\alpha$ , IFN- $\gamma$ , and TNF- $\beta$ .<sup>71</sup> In normal wound healing, IFN- $\gamma$  normally antagonizes the fibrotic effects of TGF- $\beta$  by reducing procollagen levels<sup>72</sup> and increasing collagenase

synthesis.<sup>73</sup> Intralesional injection of recombinant IFN- $\gamma$  induces several epidermal and dermal changes in keloids lesions, such as thinning of suprapapillary plates, diminished quantity of thickened collagen bundles, reduced number of active fibroblasts, and increased number of inflammatory cells.<sup>74</sup> IL-6 pathway has been suggested to play a major role in keloid pathogenesis through an auto-crine manner through microarray analysis and keloid fibroblasts responses to electron beam irradiation.<sup>75</sup> Besides the up-regulation of IL-6 observed in keloids,<sup>76,77</sup> an addition of IL-6 peptides has a significantly higher impact on several downstream targets of the IL-6 signaling pathway in keloid fibroblast than in normal fibroblasts.<sup>76</sup> IL-6-deficient mice show significantly delayed wound healing that is accompanied by reduced amount of inflammatory response, granulation tissue formation, and reepithelialization.<sup>78</sup>

Other important components of the wound healing response are the ECM proteins such as fibronectin and fibrinogen, which form a provisional matrix through which cells can migrate during the repair process. Keloids are characterized by an abnormal ECM and have elevated levels of fibronectin,<sup>42,79</sup> type I collagen,<sup>80,81</sup> elastin in deep dermis,<sup>82</sup> as well as reduced degradation of fibrin.<sup>43</sup> Controversial results have been reported regarding levels of hyaluronic acid in keloids.<sup>83,84</sup> These elevated levels may be a result of higher expression levels during the initial inflammatory stage of the wound healing response or may be due to a lack of matrix degradation in the later fibroproliferative stages of the wound healing response.

**Table 2.** Molecules that have a role in wound healing and have been investigated in the context of keloid pathogenesis

Molecule	Major function in wound healing	In keloids
<i>Cytokines</i>		
TGF- $\beta$ 1	ECM synthesis and remodeling Macrophages recruitment Fibroblasts motility Fibroplasia	Increased; <sup>108,58</sup> no difference (PBMC) <sup>155</sup>
TGF- $\beta$ 2	Myofibroblast transformation ECM synthesis and remodeling Macrophages recruitment Fibroblasts motility Fibroplasia	Increased <sup>32,58</sup>
TGF- $\beta$ 3	Myofibroblast transformation Antiscarring effects	No difference <sup>58</sup>
TNF $\alpha$	Expression of growth factors Macrophages recruitment Reepithelization	Increased <sup>71</sup> (PBMC)
IFN- $\gamma$	Decrease collagen synthesis in fibroblasts Neutrophils recruitment Decrease collagen synthesis in fibroblasts	Decreased <sup>71</sup> (PBMC)
IL-1	Expression of growth factors Reepithelialization	No difference <sup>71</sup> (PBMC)
IL-6	Reepithelialization Keratinocyte proliferation Neutrophil recruitment	Increased <sup>71,75-77</sup> (PBMC)
<i>Growth factors</i>		
EGF	Cell motility Reepithelialization Fibroplasia	Enhanced response <sup>101,102</sup> ; no significantly different response; <sup>55</sup> reduced response <sup>103</sup>
PDGF	Fibroblast proliferation and motility Recruitment and activation of macrophage Reepithelialization Matrix formation and remodeling Myofibroblast transformation	Enhanced fibroblast response <sup>55</sup>
PDGF-receptors	Fibroblast proliferation	Up-regulated by TGF- $\beta$ in keloids <sup>100</sup>
VEGF	Angiogenesis Vascular permeability (assists inflammatory cell recruitment) Granulation tissue formation	Increased; <sup>95-97</sup> not increased <sup>98</sup>
CTGF	Fibroplasia ECM synthesis Neovascularization	Increased <sup>38,92</sup>
IGF-I	Reepithelialization Granulation tissue formation ECM synthesis	(see receptor)
IGF-I-receptor	Reepithelialization Granulation tissue formation	Increased <sup>116,117</sup>
<i>Other molecules</i>		
Histamine	Inflammatory cell recruitment	Increased <sup>157</sup>
p53	Apoptosis	Increased <sup>148</sup>
PAI-1	ECM remodeling	Increased <sup>43</sup>
uPA	ECM remodeling	Decreased <sup>43,128</sup>

**Table 2.** Continued.

Molecule	Major function in wound healing	In keloids
uPA receptor	ECM remodeling	Increased <sup>128</sup>
MMP-1	ECM remodeling	Increased <sup>81,131</sup>
MMP-2	ECM remodeling	Increased <sup>81</sup>
MMP-3	ECM remodeling	Increased <sup>129</sup>
MMP-8	ECM remodeling	Decreased <sup>131</sup>
MMP-13	ECM remodeling	Increased <sup>131</sup>
MMP-19	ECM remodeling	Increased <sup>37</sup>
TIMP-1	ECM remodeling	Increased <sup>81</sup>

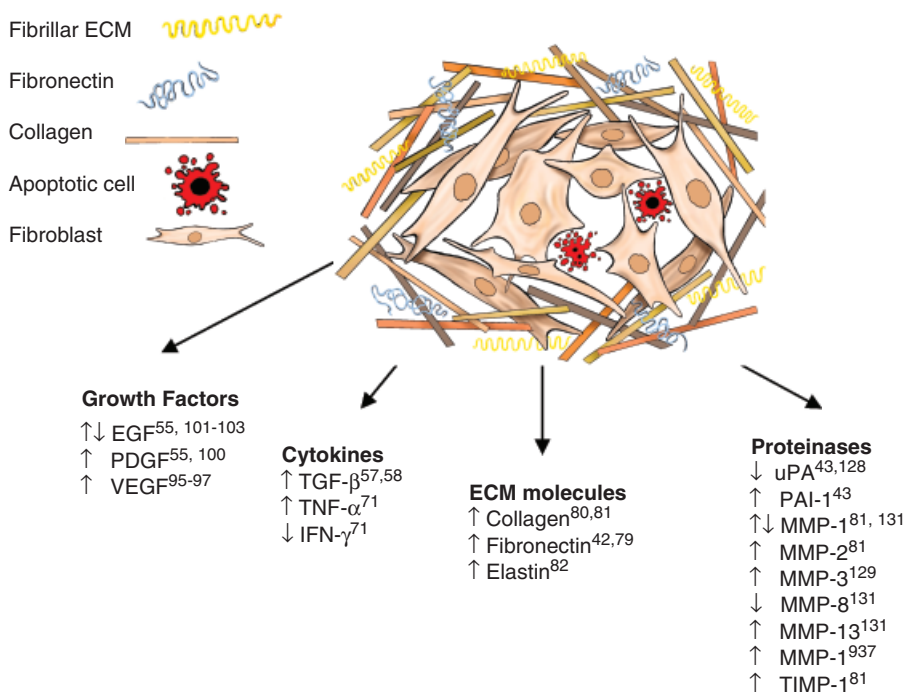
When compared with normal fibroblasts, a number of studies have revealed that keloid fibroblasts have altered expression of a number of molecules. Differences in the level of cytokines have also been studied on peripheral blood mononuclear cell (PBMC) fractions.

CTGF, connective tissue growth factor; ECM, extracellular matrix; EGF, epidermal growth factor; IFN, interferon; IGF-I, insulin like; IL, interleukin; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor growth factor-1; TGF- $\beta$ , transforming growth factor- $\beta$ ; TIMP, tissue inhibitor of matrix metalloproteinase; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.

**Proliferative phase**

Several mitogens and chemoattractants released by the inflammatory cells are important in the proliferative phase, which occur largely in parallel to the inflammatory phase. However, the importance of the contribution by the inflammatory cells to the proliferative phase is unclear. While Leibovich and Ross<sup>62</sup> have demonstrated delayed healing in guinea pigs depleted of macrophages through drug administration, however, Dovi et al.<sup>60</sup> have shown accelerated reepithelialization in neutrophil-depleted mice, and Martin et al.<sup>65</sup> have shown normal wound repair time course and less scarring in mice defect in raising an inflam-

matory response. The sources of cytokines and growth factors involved in the proliferative phase are from both the inflammatory cells and the local cell population, including EC, keratinocytes, fibroblasts, and dendritic epidermal T cells. Some of these growth factors, such as FGF-2 and hepatocyte growth factor (HGF), are chemotactic factors for mesenchymal stem cells.<sup>85,86</sup> Moon et al.<sup>87</sup> have isolated a population of mesenchymal-like stem cells from scalp keloids, demonstrating several mesenchymal stem cell marker proteins and multipotent characteristics. The authors suggest that the fibroblasts-like cells in keloids may be multipotent cells that are at a



**Figure 4.** Molecular alterations in keloids. Keloid fibroblasts display aberrant expression levels or altered responses for several molecules involved in wound healing, including growth factors, cytokines, extracellular matrix molecules (ECM), proteinases, and other factors often associated with malignant tumors. ECM, extracellular matrix; EGF, epidermal growth factor; IFN- $\gamma$ , interferon- $\gamma$ ; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; TGF- $\beta$ , transforming growth factor- $\beta$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TIMP-1, tissue inhibitor of metalloproteinase 1; uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.

proliferative state.<sup>87</sup> Complementary to this, a study by Akino et al.<sup>88</sup> demonstrated that keloid-derived fibroblasts induce higher mesenchymal stem cell migration toward themselves than normal fibroblasts.

The first visible event of the proliferative phase is reepithelialization, which is characterized by the migration and proliferation of keratinocytes from the epidermis at the wound edge that occurs 1–2 days post-wounding.<sup>51</sup> Other major events during the proliferative phase include neovascularization, collagen deposition, granulation tissue formation, and wound contraction.<sup>89</sup> Although keloids are traditionally thought of as a dermal disease involving fibroblasts, there is increasing evidence suggesting keratinocyte involvements.<sup>84,90</sup> Increased proliferation has been observed in fibroblasts that have been co-cultured (without direct contact) with keloid keratinocytes, which suggests an involvement of keloid keratinocytes by acting on the fibroblasts through a paracrine fashion.<sup>90–92</sup>

Another key event in the proliferative phase is neovascularization, which contributes to the provisional granulation tissue formation and supplements nutrients and oxygen at the wound site.<sup>93</sup> Active neovascularization has been implicated in keloid disease.<sup>94</sup> VEGF plays a significant role in neovascularization and both have been linked to keloids and malignant diseases.<sup>51,94</sup> The level of VEGF in keloids has been disputed but generally it is thought to be up-regulated in keloid scars compared with normally healing scars.<sup>95–98</sup> Higher levels of VEGF in burns patients have also been associated with scars with a more disorganized, hypercellular, higher levels of keratinization, and thickened epidermis.<sup>99</sup>

PDGF is another growth factor that plays an important role in the proliferative phase. Keloid fibroblasts were found to be more responsive to all three isoforms of PDGF than fibroblasts from normal skin, which appears to be mediated by four to five times higher levels of PDGF- $\alpha$  receptors in keloids than in normal skin fibroblasts.<sup>55</sup> The expression of the PDGF- $\alpha$  receptor was found to be up-regulated by TGF- $\beta$ 1 in keloid fibroblasts but not in normal skin and normal scar fibroblasts.<sup>100</sup> However, increased neovascularization may occur as a consequence of the increased scar mass rather than a causative factor in keloid formation.

Contradictory results have been reported on the responsiveness of keloid fibroblasts to epidermal growth factor (EGF).<sup>55,101–103</sup> While some studies showed enhanced response to EGF in keloid fibroblasts,<sup>101,102</sup> one reported no enhanced response,<sup>55</sup> and one reported diminished response.<sup>103</sup> Connective tissue growth factor (CTGF) is an important growth factor that is involved in the proliferative phase of wound healing. It is produced by fibroblasts, is involved in reepithelialization, granulation tissue formation, and ECM production and remodeling.<sup>104,105</sup> Elevated levels of CTGF have been observed in the basal layer of keloid epidermis and keloid tissue extract.<sup>92</sup> When normal or keloid fibroblasts are co-cultured with keloid keratinocytes, there is a higher level of 12 kDa secretory CTGF and a lower level of basal endogenous 38 kDa CTGF.<sup>92</sup> Gene expression level for CTGF has also been showed to be up-regulated in keloid fibroblasts.<sup>38</sup>

CTGF has been showed to modulate TGF- $\beta$  signaling, a growth factor that takes part in several events at various

time points throughout wound healing.<sup>104</sup> Events that TGF- $\beta$  is involved in include recruitment of inflammatory cells, inhibiting the proteolytic environment created by the inflammatory cells, promoting the migration of keratinocytes, inhibiting keratinocyte proliferation, angiogenesis, myofibroblast transformation, promoting collagen production by fibroblasts, and granulation tissue formation.<sup>106</sup> TGF- $\beta$  stimulates type I collagen transcription and inhibits collagenase transcription in fibroblasts.<sup>107</sup> TGF- $\beta$  is overproduced by keloid tissue,<sup>57,58</sup> and thus the excess collagen present in keloid scars may result from overexpression of TGF- $\beta$  and decreased collagen degradation. TGF- $\beta$  mRNA and proteins expression have been detected in areas active in types I and type IV collagen expression, which include microvascular EC.<sup>108,109</sup> It has been suggested that initialization of fibrosis may involve microvascular EC expressing TGF- $\beta$ 1 and activating adjacent fibroblasts at the periphery to overexpress TGF- $\beta$ 1 and types I and IV collagen.<sup>2,108,109</sup> On the other hand, SMAD 6 and 7, which are involved in the termination of the TGF- $\beta$  signal, are down-regulated in keloid fibroblasts.<sup>110</sup> There are three highly conserved isoforms of TGF- $\beta$  in the human body designated TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3. TGF- $\beta$ 1 and TGF- $\beta$ 2 have been associated with scar formation and fibrotic conditions, whereas TGF- $\beta$ 3 is associated with reduced scarring and fibrosis.<sup>111</sup> Unlike wound healing in adults, fetal wounds exhibit a reduced inflammatory response and heal without scars.<sup>112</sup> Induction of inflammatory responses in the fetus induce an adult-like healing response.<sup>113</sup> Adult wound sites contain high levels of TGF- $\beta$ 1 and TGF- $\beta$ 2, whereas TGF- $\beta$ 1 is expressed transiently and at low levels after injury in the embryo and TGF- $\beta$ 3 is the predominant isoform found at the wound site.<sup>114</sup> Thus, endogenous expression of TGF- $\beta$ 1 and TGF- $\beta$ 2 and low levels of TGF- $\beta$ 3 may contribute to keloid development.

Daian et al.<sup>115</sup> have proposed that IGF-I may enhance fibrosis in keloids through TGF- $\beta$ 1 post receptor signaling. Higher expression of IGF-I receptors (IGF-IR) has been reported in keloid tissues and fibroblasts.<sup>116,117</sup> Studies on keloid fibroblasts have suggested that the aberrant IGF-I/IGF-IR in keloid fibroblasts may account for their proliferative,<sup>118</sup> antiapoptotic,<sup>116</sup> invasive,<sup>119</sup> and excess ECM production<sup>118</sup> characteristics.

During the fibroproliferative phase of wound healing, fibroblasts proliferate and form a new ECM composed of granulation tissue, which replaces the fibrin clot. The ECM provides structural support and acts as a skeleton for cells, connective tissue, and other components to adhere to and grow. As wound healing progresses, a proportion of the wound fibroblasts differentiate into myofibroblasts, which express  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). This conversion is triggered by growth factors such as TGF- $\beta$ 1, which has a strong positive effect on the expression of  $\alpha$ -SMA.<sup>120</sup> These myofibroblasts establish a grip on the wound edges and contract themselves, making the wound smaller. As keloids are typically excluded from palms and soles, the level of  $\alpha$ -SMA was compared in keloids and palmar fibroblasts.<sup>18</sup> Collagen I,  $\alpha$ -SMA, and thrombospondin-1 (TSP-1) were found at higher levels in keloid than in palmar fibroblast.<sup>18</sup> These differences were suggested to result from the aberrances in TGF- $\beta$ 1 or the TGF $\beta$ 1-activator, TSP-1 levels.<sup>18</sup>

## Maturation phase

During the maturation phase, the newly laid down matrix is constantly turned over by enzymes such as collagenases and elastases, until a steady state is reached where the dermal defect has been reconstituted.<sup>121</sup> Remodeling of the ECM is the key event in scar maturation. Three major groups of lytic enzymes that degrade the ECM include PA-plasmin, matrix metalloproteinases (MMPs), and a disintegrin and metalloproteinases (ADAMs). PA converts plasminogen into plasmin, which breaks down fibrin.<sup>122</sup> PA also activates procollagenase into collagenase which breaks down collagen.<sup>123</sup> These events allow the immature type III collagen of the early wound to be replaced by mature type I collagen. During remodeling, the predominance of type III collagen in ECM gradually become converted type I collagen, which strengthens the scar.<sup>124</sup> In keloid scars, the type I/III collagen ratio is approximately 17, which is significantly higher than the ratio of around 6 observed in normal scars.<sup>80</sup>

There is increasing evidence to suggest that the final maturation phase of wound healing may be insufficient in keloid scars, resulting in an imbalance between the synthesis and degradation of the ECM, which results in an excess accumulation of collagen within the wound.<sup>48,91,125</sup> During normal wound healing, interferon- $\gamma$  (IFN- $\gamma$ ) has been shown to inhibit proliferation of fibroblasts and production of ECM macromolecules.<sup>126</sup> IFN- $\gamma$  down-regulates collagen synthesis by reducing mRNA levels of types I, II, and III procollagen<sup>72</sup> and increases collagenase synthesis.<sup>73</sup> Reduced production of IFN- $\gamma$  has been reported in patients with keloids,<sup>71</sup> which may partly explain the reduced ECM degradation reported in keloid scars. Keloid fibroblasts appear to exhibit a decreased capacity for fibrinolysis and fibrin clot degradation.<sup>43</sup> Early studies suggest that collagen production is normal in keloid fibroblasts and that keloid fibroblasts showed lower levels of degradation of procollagen peptides.<sup>91,127</sup>

A number of enzymes responsible for degrading the ECM have differential expression in keloids including: uPA, MMP-2, MMP-3, and MMP-13.<sup>128-131</sup> These enzymes are believed to be involved in the expansion of keloids beyond the wound margins in part through the degradation of the ECM.<sup>128-131</sup>

## Abnormal regulation of apoptosis

Apoptosis is an important event during the wound healing process.<sup>132</sup> Upon completion of their tasks, cell populations responsible for the previous events, such as inflammatory cells, are gradually removed from the wounds as the next events commence.<sup>132</sup> A mature wound is relatively acellular and avascular.<sup>132</sup>

There has been evidence indicating that the excessive scar formation in keloids may be a result of reduced apoptosis and the extra fibroblast activities may lead to an imbalance between collagen synthesis and degradation.<sup>18-21</sup> Keloid lesions were found to have lower rates of apoptosis (22% decrease) than normal skin<sup>20</sup> and keloid fibroblasts were found to be refractory to apoptosis,<sup>133</sup> which may be in part due to decreased expression of apoptosis-associated genes.<sup>134</sup> Akasaka et al.<sup>21</sup> looked at specific regions of the keloid tumor and demonstrated that

a subpopulation of keloid fibroblasts had reduced cell survival and increased apoptotic cell death.<sup>21</sup> The highest distribution of apoptotic cells was found in the peripheral, hypercellular areas of keloids, rather than in the central hypocellular regions,<sup>21</sup> which is in keeping with the apoptotic distribution reported by Appleton et al.<sup>135</sup>

One group of investigators analyzed the expression of apoptotic genes in keloids and normal skin using microarray studies.<sup>136</sup> Gene expression profiles of fibroblasts from different sites of keloid scars were characterized using Affymetrix microarrays covering the whole human genome.<sup>37</sup> This study revealed that 79 genes were up-regulated and 26 genes were down-regulated. The apoptosis-inducing gene, ADAM12, was up-regulated in the regressing keloid center, while the caspase activation inhibitor (AVEN) was found to be up-regulated at the active margin of keloids.<sup>37</sup>

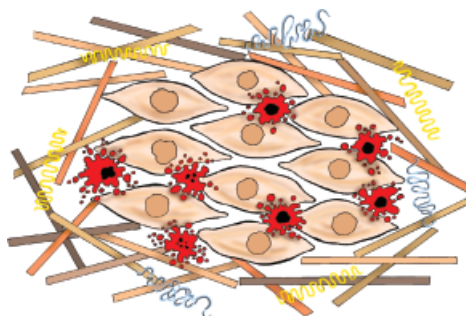
TGF- $\beta$ 1 may account for the resistance keloid fibroblasts present against Fas-mediated and staurosporine-induced apoptosis because the addition of anti-TGF- $\beta$ 1 antibodies abrogated this characteristic.<sup>133</sup> Similarly, ADAM12, which is regulated by TGF- $\beta$  pathway, is overexpressed in keloids.<sup>37</sup> ADAM12 has also been proposed to be a potential biomarker in several lines of cancer, including breast, liver, and bladder cancers.<sup>137-139</sup> However, TGF- $\beta$ 2, which has also been implicated in the etiology of keloid disease was not found to contribute to this apoptotic resistance.<sup>133</sup>

Caspases are a group of cysteine proteases involved in apoptosis. Caspases 2, 3, 6, 8, 9, and 14 have been studied in keloid disease.<sup>140-142</sup> Higher levels of caspase 3 and 9 and no difference in the level of caspase 8 were reported in keloid fibroblasts compared with normal fibroblasts.<sup>140,141</sup> When the normal skin of keloid-prone patients was studied, a higher level of caspase 6 and a lower level of caspase 14 expression were seen.<sup>142</sup> Interestingly, caspase 14 expression is mainly confined to epidermal keratinocytes and thought to play a role in keratinocyte differentiation<sup>142</sup> Nassiri et al.<sup>142</sup> recently postulated that the innate decrease of caspase 14 in normal skin of keloid-prone patients might be responsible for the lack of an inhibitory signal for proliferation; it was shown that nonhealing burn wounds, which lack the epidermis, show excess collagen production and often result in HSs.<sup>142,143</sup>

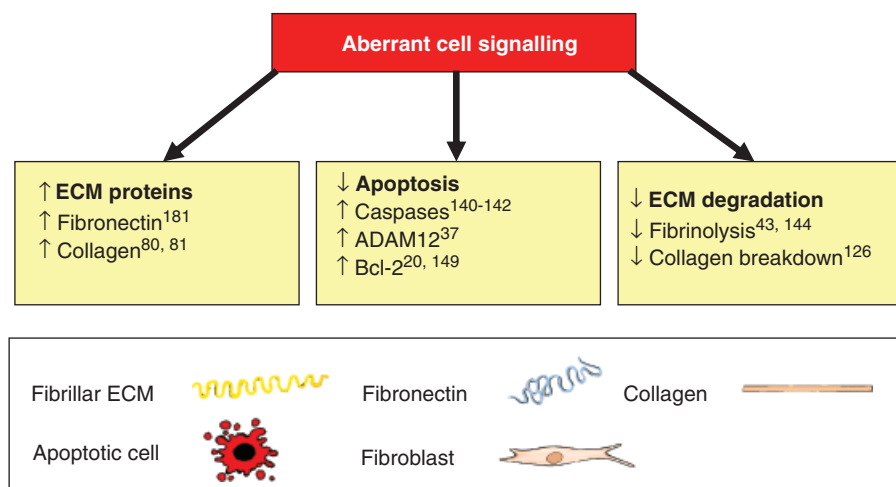
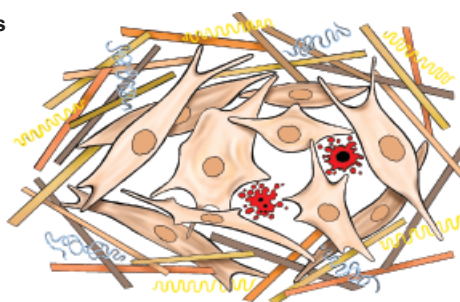
Bcl-2 family proteins are a group of important proapoptotic and antiapoptotic proteins involved in the apoptosis pathway.<sup>144</sup> While Bcl-2 and Bcl-x protect mitochondrial integrity, Bax promotes apoptosis through the release of cytochrom c.<sup>145,146</sup> Bcl-2 and Bcl-x are up-regulated in several types of tumor, suppressing the apoptosis of transformed cells.<sup>147</sup> Conflicting data exist regarding the expression of Bcl-2 in keloids vs. normal skin. Chodon et al.<sup>133</sup> found no difference in the level of expression of Bcl-2 or Bax in normal fibroblasts and keloid fibroblasts. Lu et al.<sup>148</sup> compared the level of expression of Bcl-2 between central and peripheral regions of keloid fibroblasts and also detected no difference between the two areas. In contrast, Ladin et al.<sup>20</sup> reported increased Bcl-2 expression in the hypercellular, peripheral areas of keloids and Teofoli et al.<sup>149</sup> reported intense Bcl-2 staining in keloids, with little staining in normal skin. Discrepancies in results may reflect the small sample number used in some of these studies or may be due to genetic differences



**A Normal fibroblasts**



**B Keloid Fibroblasts**



**Figure 5.** (A) Aberrant cell signaling in keloid fibroblasts. Normal fibroblasts are located in the dermis and within the extracellular matrix (ECM), which consists of type I collagen and fibronectin. These cells undergo apoptosis at a normal rate (cells shown in red). (B) Keloid fibroblasts proliferate quicker than normal fibroblasts and produce excess ECM proteins such as type I collagen. Keloid fibroblasts are also thought to undergo apoptosis at a reduced rate. ADAM12, a disintegrin, and metalloproteinase domain 12; Bcl-2, B-cell CLL/lymphoma 2; ECM, extracellular matrix.

between patients. Ideally a large study focusing on more members of the Bcl-2 family would be more informative as the relative expression of the proapoptotic vs. antiapoptotic proteins could be directly compared.

The p53 tumor-suppressor gene is central to many anticancer mechanisms in the body and can induce growth arrest, apoptosis, and cellular senescence.<sup>150</sup> If the p53 gene is damaged, tumor suppression is severely reduced as cells with damaged DNA are not destroyed. Ladin et al.<sup>20</sup> found that 18 of 20 keloids showed overexpression of p53 protein and Saed et al.<sup>151</sup> reported mutations in the p53 gene in seven patients with keloids. Conversely, Teofoli et al.<sup>149</sup> and Chipev et al.<sup>18</sup> found no overexpression of p53

in keloids. Lu et al.<sup>148</sup> studied these different cell populations and found that the central keloid fibroblasts, which mostly distribute to the G0–G1 phase of the cell cycle, exhibited high expression of the p53 protein, while low p53 protein expression was detected in most peripheral keloid fibroblasts, which mostly distribute to the proliferative phases of the cell cycle.<sup>148</sup> Mutations in the p53 gene or low expression of the p53 protein may be the reason why most of the peripheral keloid fibroblasts are in the proliferative phases of the cell cycle.<sup>148</sup> Interestingly, p53 has been found to be up-regulated in other types of pathological scars including HSs.<sup>152</sup> The levels of p53 protein were higher in the order of keloid, red HS, white, and hard

HS.<sup>152</sup> In the atrophic white scar group, the level of p53 was almost the same as that of the control.<sup>152</sup>

## DISCUSSION

It appears that a number of events, transcripts, and proteins involved in various phases of wound healing are altered in keloid scarring. Several major cell populations involved in wound healing have been suggested to be altered or responsible for the differential molecular events and activities in keloids; these include mast cells,<sup>69,71</sup> macrophages,<sup>30</sup> lymphocytes,<sup>30</sup> keratinocytes,<sup>90,92</sup> and fibroblasts.<sup>36</sup> Out of all the cell types found in wound healing, the fibroblasts have received the most attention in keloid research to date. This is not surprising considering that keloid disease is of reticular dermal origin where fibroblasts reside as the predominant cell type involved in excessive collagen deposition. Keloid fibroblasts proliferate and migrate at a faster rate than normal skin fibroblasts.<sup>153</sup> Although abnormalities of several immune cells have been reported by some,<sup>30,69,71</sup> there have been few studies that have investigated the role of immune cells in keloid pathology. In addition, lack of an established in vivo animal model for keloids has not helped in further elucidation of its pathology. There have been several recent in vitro studies that have investigated keloid keratinocytes and suggest keratinocyte contribution in keloid pathogenesis through their interactions with fibroblasts.<sup>90,92</sup> The contribution of mesenchymal stem cells in keloid pathogenesis has also been suggested.<sup>87,88</sup>

Several growth factors, cytokines, signaling molecules, and proteases involved in wound healing have been found to show aberrant expression or response to activation (Table 2). However, it is currently unclear whether these molecular differences observed in keloids are causative factors or the consequences of other events. The molecular alteration can be categorized into three groups: (1) ECM proteins and their degradation, (2) cytokines and growth factors, (3) apoptotic pathways growth factors, and (4) apoptotic pathways. The main molecular alterations that are associated with ECM proteins and degradation include: fibronectin,<sup>79</sup> elastin,<sup>82</sup> type I collagen,<sup>80</sup> PAI-1,<sup>43</sup> uPA,<sup>43,128</sup> MMP-1,<sup>81</sup> MMP-2,<sup>81</sup> MMP-3,<sup>129</sup> MMP-8,<sup>131</sup> MMP-13,<sup>131</sup> MMP-19,<sup>37</sup> and TIMP-1.<sup>81</sup> Numerous cytokines and growth factors have been implicated to show aberrant levels or abnormal responses to activation in peripheral blood mononuclear cell fraction, cultured keloid fibroblasts or keloid tissues including: TNF- $\alpha$ ,<sup>71</sup> IL-6,<sup>75,77</sup> IFN- $\beta$ ,<sup>71</sup> IFN- $\gamma$ ,<sup>71</sup> TNF- $\beta$ ,<sup>71</sup> VEGF,<sup>95,96,98</sup> PDGF,<sup>55</sup> CTGF,<sup>92</sup> TGF- $\beta$ ,<sup>57,58</sup> IGF-I,<sup>114,115,117,118</sup> and EGF.<sup>55,101</sup> Lastly, several genes involved in apoptotic pathways have been implicated to be abnormal in keloids; these include ADAM12,<sup>37</sup> AVEN,<sup>37</sup> several caspases,<sup>140–142</sup> Bcl-2,<sup>20,133,148,149</sup> and p53. Of these molecular abnormalities, large number of research focuses around type I collagen, as excessive type I collagen is a feature in keloids. The importance of the TGF- $\beta$  and the IL-6 pathways has been proposed in several studies.<sup>57,58,75,76,108,154</sup> The excess deposition of collagen observed in keloids may occur through the up-regulation of TGF- $\beta$  and the down-regulation of IFN- $\gamma$  in keloid fibroblasts.<sup>71,107</sup> IFN- $\gamma$  has been used as treatment for keloid.<sup>74</sup> TGF- $\beta$ 1 stimulates collagen

transcription and inhibits collagenase transcription in fibroblasts,<sup>107</sup> thus resulting in excess scar formation.

Some molecular abnormalities reported in keloids by various investigators appear to have been contradictory, including reports on the role of TGF- $\beta$ ,<sup>58,71,155</sup> EGF,<sup>55,101–103</sup> VEGF,<sup>95,96,98</sup> and MMP-1.<sup>81,131</sup> These differences may in part be due to different experimental conditions and disease heterogeneity leading to a variety of findings. Firstly, keloids are composed of a heterogeneous population of cells with different properties, as showed by studies demonstrating the potential roles of keratinocytes and mesenchymal stem cells in keloids.<sup>87,88,90,92</sup> In addition, these discrepancies may in part arise due to differences in the patient age, ethnicity, family history, age of keloids, the anatomical locations of the keloids, and whether the keloid samples investigated had undergone any previous treatments.<sup>156</sup> These information are often not included in the reported studies. Also, use of samples from different lesional sites within the keloid, such as margin or center, would also have an impact on the outcome of the investigations, as demonstrated by Seifert et al.<sup>37</sup> The peripheral regions are often termed as the “proliferative” or “invasive” regions of the keloid, while the central areas have been described as “mature” and hypocellular.<sup>20</sup> The majority of studies have not distinguished between these regions and have tested a heterogeneous population of cells. In general, the majority of research conducted to date suggests that inadequate apoptosis accounts for the high collagen synthesis and reduced degradation seen in keloids (Figure 5). However, it is likely that the majority of work in this area will need to be repeated to take into account these different cell populations and confirm these findings.

It is postulated that keloid disease is a complex polygenic disorder whose progression is influenced by aberrant cell signaling pathways, possibly as a result of genetic predisposition and environmental factors. The current molecular understanding of keloid pathogenesis suggests that several stages of wound healing, from the inflammatory phase to the maturation phase, may be altered in keloids. Further research into other cell types, such as inflammatory cells, keratinocytes and mesenchymal stem cells, and their interaction with fibroblasts may help our understanding in the pathogenesis of keloids. Clarification of the different lesional sites and degree of maturation of keloid scars may also be of particular importance to understanding the disease mechanism, as it is possible that they are at different stages of wound healing. Further research into the cellular and molecular mechanisms involved in the abnormal wound healing of keloids, may be important for devising improved strategies in clinical management of keloid disease.<sup>157</sup>

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