Mechanisms, Potential Therapies, and the Role of TGF-β in the Formation of Scars

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Abstract
Scarring is the inevitable outcome of wound healing. This review looks at some of the underlying mechanisms of this complex process with the aim of identifying targets for therapeutic manipulation that could result in reduced scarring or even scarless wound repair. Fetal wounds are shown to heal without scars primarily due to low levels of TGF-β1 and TGF-β2 and high levels of TGF-β3 as compared to adult wounds which heal with scars. Abnormal excessive scarring in keloid and hypertrophic scars are also attributed to TGF-β. Clinical manipulation of TGF-β ratios showed promise as a therapeutic means of controlling scar formation. The effect of the COX enzyme and PGE2 levels remains controversial and more research is needed to understand the exact roles these molecules play in the wound healing process before they can be exploited in a clinical setting.

Introduction

Scarring, both as a component and complication of, wound healing affects millions of US citizens every year at a cost of more than 7 billion dollars (Wilgus, et al. 2003). In addition to esthetic concerns, scar formation is also implicated clinically in fibrotic diseases that some reports suggest are responsible for up to 45% of deaths in the industrial world (Wynn, 2008). Despite such a heavy toll on the healthcare system, current treatment options are limited. Further research is needed to understand the underlying causes and mechanisms of scar formation so that effective treatments and even methods of prevention can be developed.

Cellular response to tissue damage and the consequent formation of fibrotic tissue is a complex process that is still not very well understood. Current paradigms include the contribution of such varied cells as epithelial cells, macrophages, T-cells and, of course, fibroblasts. Initial response to tissue wounds involves the rapid recruitment of clotting factors and immune inflammatory moderators to stem blood loss and reduce potential infection by pathogenic microbes. Later, the relatively extended process of wound healing begins. Injured epithelial cells undergo a process of reprogramming, initiation, and propagation of mesenchymal pathways that includes the ubiquitous TGF-β related pathways. Macrophages also contribute to the inflammatory response and secrete profibrotic cytokines as well as growth factors, including TGF-β, that recruit and activate fibroblasts. Fibroblasts accumulate and ultimately produce and deposit new extracellular matrix and collagen that replaces normal tissue architecture. It is this deposition that characterizes scar formation and clinical fibrosis.

Interestingly, the development of scar tissue differs greatly between adult and embryonic wounds. Contrary to the normal fibrotic result in adult wounds, fetal wounds have been shown to exhibit scarless healing. Many cellular and molecular differences, including differences in key inflammatory mediators and various cytokines and growth factors, have been studied and documented to attempt to explain this phenomenon.

In addition to expected scarring as a result of injury, there exist two clinical examples of atypical wound healing that result in abnormal scar formation. The etiology of hypertrophic scars and keloids is not well-understood, and although important differences between them have been detailed, both clearly involve the deposition of excessive amounts of collagen even as compared to normal scarring.

Such anomalous results, from the scarless healing of fetal wounds to the excessive scarring of keloid and hypertrophic scars, may serve as paradigms for understanding the mechanisms behind the wound healing and scarring processes and the ability to prevent undesired outcomes.

Methods

Literature searches were chiefly performed using the PubMed database. After relevant literature was found, related articles were accessed using the Related Citations feature on the PubMed website. Additionally, references in these articles were retrieved and then served as additional points of reference for further research. Review articles relevant to the research topic were helpful in providing references to source material. The Touro College Online Library and Google Scholar proved to be valuable for finding and accessing the necessary relevant articles. Moreover, conversation with plastic surgeons led to additional searches for source material on the website of the Plastic and Reconstructive Surgery journal.

Discussion

The formation of scar tissue in the process of wound healing is both a positive construct that effectively patches breaks in damaged tissue and a negative consequence that can have dire effects. Scar tissue production is intended to increase the tensile the strength of wounds but never surpasses 70% of the strength of the original undamaged tissue (Wilgus, et al. 2003). Scar tissue also can impede tissue function, restricting mobility in joints and impairing normal growth in the case of external injuries involving the skin. The issue is even more problematic in the case of scar deposition as a result of internal organ damage. In such cases, scars can dramatically reduce function of vital organs as with

victims of myocardial infarctions whereby normal heart tissue is replaced with nonfunctional scar tissue. However, the problem of scar formation is perhaps greatest with respect to its role in devastating fibrotic diseases where scar tissue often times fatally overtakes normal tissue function.

Scars are essentially large deposits of extracellular matrix and collagen that are deposited by activated fibroblasts in response to tissue damage. After an injury, inflammatory mediators are immediately recruited and activated. Numerous cell types are involved in this response which includes the production and excretion of various growth factors and cytokines such as the well-studied TGF-β, IL-6, IL-8 and others.

Tissue injury involves disruption of capillaries and immediately triggers activation of platelets which begin a clotting cascade. Inflammatory events follow with recruitment of neutrophils which secrete a variety of growth factors and cytokines. Monocytes arrive to facilitate changes in the wound matrix and macrophages release factors that activate fibroblasts to begin adding hyaluronate and glycoproteins such as fibronectin to the extracellular matrix (ECM). Gradually, these fibroblasts shift to producing proteoglycans and collagen which are then deposited at the wound site. Over the course of several days, macrophages and fibroblasts work to remodel the matrix and thus convert the granulation tissue into scar tissue (Harty, 2003).

Studies comparing scarless fetal wounds to adult wounds have found significant differences in the levels of proinflammatory cytokines such as IL-6 and IL-8. Liechty et al. (1998) grafted adult and fetal skin subcutaneously into SCID mice, wounded the site and subsequently excised the wound after varying lengths of time. The presence of IL-8 was confirmed using reverse transcription polymerase chain reaction (RT-PCR). Their results showed that fetal fibroblasts produced significantly less IL-8 at baseline (50 +/- 6 pg/mL versus 450 +/- 115 pg/mL, $P < 0.001$). IL-8 mRNA was detected in unstimulated adult fibroblasts but not in fetal fibroblasts and much less IL-8 mRNA was detected in stimulated fetal fibroblasts than in adult fibroblasts. IL-8 mRNA was detected 4 hours after wounding in both fetal and adult wounds but by 12 hours no IL-8 mRNA was detected in fetal wounds, whereas IL-8 mRNA persisted for up to 72 hours in adult wounds.

In a similar experiment, Liechty et al. (2000a) excised incisional wounds and examined them for IL-6 mRNA quantification by RT-PCR. As with IL-8, fetal fibroblasts produced less IL-6 protein and mRNA at all points examined ($P < 0.01$ vs adult). IL-6 mRNA was detected 4 hours after wounding in both fetal and adult wounds. By 12 hours there was no IL-6 mRNA in the fetal wounds but the adult wounds had IL-6 mRNA persisting to 72 hours. In this experiment, another set of grafts had 5 micrograms of IL-6 injected at wounding and the wound subsequently harvested at 7 days for analysis. The wounds with administered IL-6 resulted in scar formation. Since these cytokines are responsible for the recruitment and activation of various leukocytes including macrophages, the diminished inflammatory response by fetal tissues may explain the lack of cellular recruitment and inflammation in fetal wound healing and further suggests that the inflammatory response plays a key role in the process of scar formation.

One well studied growth factor, TGF-β, exists as three known isoforms and is involved in many aspects of wound healing, from the immediate inflammatory response to matrix deposition (Adzick & Lorenz, 1994). In response to tissue injury, macrophages release TGF-β which stimulates the deposition of collagen and other matrix components by fibroblasts, thus implicating it in the etiology of fibrosis. Specifically, the TGF-β1 and TGF-β2 isoforms have been directly implicated in the fibrotic response and formation of scar tissue. Whitby and Ferguson (1991) detected both TGF-β1 and TGF-β2 isoforms with immunolocalization in adult wounds but not in fetal wounds. In a pioneer study, Shah et al. (1992) studied the effect of reduced levels of TGF-β in adult rodent wounds by using various neutralizing agents. The results of that experiment indicated inhibited fibrosis and scar formation. In another similar experiment, Shah et al. (1994) injected a polyclonal neutralizing antibody to TGF-β1, 2 into full-thickness cutaneous wounds of adult rodents just prior to wounding or within 24 hours of wounding and repeated daily for two days post-wounding. The effect was successful reduction in scarring. Numerous similar studies have been performed that confirm these results with more or less comparable data (Singer, et al. 2009; Shah, et al. 2000). Conversely, up-regulation of these TGF-β isoforms via exogenous application resulted in excessive scar formation in a number of animal models (Lin, et al. 1995; Wang, et al. 1999; Lanning, et al. 1999). Thus direct evidence exists that increased levels of TGF-β1 and TGF-β2 play an important role in the fibrotic response and suggests that manipulation of TGF-β levels may provide an efficient strategy of reducing scar formation.

The culpability of TGF-β in the formation of scar tissue is further supported by studies that have investigated the cellular and molecular differences abnormal scar formation such as keloid and hypertrophic scars. Experimental evidence suggests that upregulation of TGF-β is necessary for the excessive scarring characteristic of these pathologies (Lee, et al. 1999; Campaner, et al. 2006). Wang et al. (2000) compared the levels of TGF-β1 mRNA in hypertrophic scar tissue and normal skin and found that hypertrophic scar tissues expressed five-fold more TGF-β1 mRNA than normal skin per unit of wet weight. TGF-β1 mRNA expression in 5 pairs of fibroblast cultures derived from hypertrophic scar tissue also was found to be significantly elevated as compared to those derived from normal cells (116 +/- 6 vs. 97

+/- 7, p = 0.017). Additionally, Schmid (1998) demonstrated by immunohistochemistry and in situ hybridization, that hypertrophic scars exhibit unresolved persistent overexpression of TGF-β receptors by fibroblasts leading to overproduction of matrix protein and subsequent fibrosis.

Table 1

TGF-β Isoform	Scarless Fetal Healing	Scar-forming Adult Healing
TGF-β1	low/absent	high
TGF-β2	low/absent	high
TGF-β3	high	low

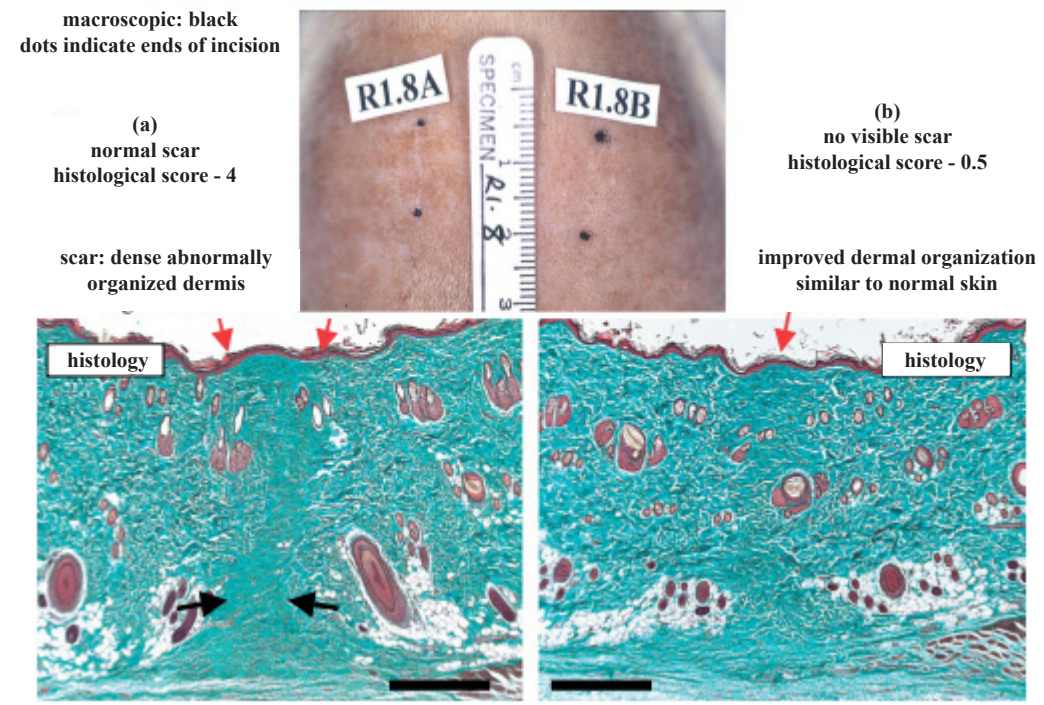
Summary of relative TGF-β isoform differences in scarless fetal wound healing and scar-forming adult wound healing. (Ferguson, O'Kane, 2004).

However, although the TGF-β1 and TGF-β2 isoforms have been implicated in scar formation, a third isoform has been shown to reduce scarring. Whitby and Ferguson (1991) demonstrated that fetal wounds express low levels of TGF-β1 and TGF-β2 but high levels of the third isoform, TGF-β3 (Table 1).

This implied that the ratio of TGF-β isoforms in wounded tissue may be the key to understanding the mechanism of wound healing and whether or not a scar forms. Shah et al. (1995) confirmed this hypothesis when they demonstrated that pan-neutralization of all three known TGF-β isoforms in linear incisions made on rats did not improve scarring, while exogenous application of TGF-β3 resulted in marked improvement in scarring and even scarless healing. Numerous studies have exploited this therapeutic approach of increasing the relative ratio of TGF-β3 to TGF-β1 and TGF-β2 with novel pharmaceutical agents (Ferguson, O'Kane, 2004) (Figure 1).

The first clinical application of human recombinant TGF-β3, avotermin (Juvista; Renovo, UK) administered as an intradermal injection at the time of surgery, had shown promise in reducing scarring in controlled, double-blind, randomized phase I/II clinical studies (Durani, et al. 2008). So et al. (2011) studied sixty patients (35 men and 25 women; age, 19 to 78 years; 53 Caucasians; scar length, 5 to 21 cm) who received intradermal

Figure 1



(a) Placebo-treated incisional rat wound compared with (b) scarless healing in TGF-β3-treated wound 84 days post-wounding (Ferguson, O'Kane, 2004).

avotermin (200 ng/100 µl/linear cm wound margin) and placebo to wounds immediately after scar revision surgery and again 24 hours later. Primary efficacy was measured as a total scar score derived from a visual analogue scale and scored by a lay panel from standardized photographs from 1 to 7 months following treatment. Profilometry showed a greater reduction in scar surface area from baseline with avotermin treatment compared with placebo and histologic analysis showed collagen organization that more closely resembled normal skin. However, an earlier study of dermal ulcers on the ears of female rabbits had demonstrated only that exogenous topical TGF-β3 had accelerated wound healing but with no improvement in scar morphology and prominence. Wu et al. (1997) showed that the use of TGF-β3 (0.3-0.75 microgram per wound) increased granulation tissue formation by more than 100% ($P < .005$) but no significant difference in the hypertrophic index was noted as compared with controls. In fact, phase III trials of avotermin failed to meet primary and secondary endpoints, suggesting that the difference between scarless and scar-forming wound healing is perhaps more complex than simply altering the ratio of TGF-β isoforms (Penn, et al. 2012) .

Interestingly, a number of studies have demonstrated that even in fetal wounds, exogenously applied TGF-β can induce fibrosis. McCallion and Ferguson (1996) applied exogenous TGF-β1 to fetal wounds and observed a profound fibrotic response with scar formation. Krummel et al. (1988) showed that the addition of TGF-β to polyvinyl alcohol sponges implanted in fetal rabbits produced fibrosis. Sullivan et al. (1995) also examined human scarless fetal wounds transplanted subcutaneously into adult nude mice. Immunohistochemistry performed on the wounds did not show TGF-β staining. In a second part of the study, a slow-release disk with varying amounts of TGF-β1 was placed beneath the fetal skin graft at the time of wounding. Marked scarring in these fetal grafts was observed fourteen days post-wounding with the size of the scar proportional to the amount of TGF-β1 applied. These data suggest that the cellular mechanism of scar formation characteristic of adult wound healing is present in fetal wounds. The absence of scar formation in fetal wounds thus implies active suppression of these inflammatory mediators that have been implicated in scar formation.

The concept of immune inflammatory suppression as the mechanism of scarless healing has been investigated further by testing with the known anti-inflammatory cytokine IL-10. IL-10 deactivates macrophages and inhibits expression of both IL-6 and IL-8. Liechty et al. (2000b) hypothesized that the diminished levels of IL-6 and IL-8 they had revealed in a previous study were in fact due to the effects of IL-10. They developed a new syngeneic murine model of fetal wound repair in which 15-day-gestation skin from either normal or transgenic IL-10 knockout mice was grafted to the back of the same strain adult mice. Incisions were made in the grafts after 5 days, which were then harvested at 1 week and analyzed for inflammatory response and scar formation. The normal fetal skin grafts showed minimal inflammation and were histologically consistent with scarless healing. The IL-10 knockout fetal skin grafts, in contrast, displayed significant inflammation and scar formation. Thus, the anti-inflammatory effects of IL-10 are necessary for active suppression of the inflammatory response leading to the observed paucity of macrophages, less TGF-β release, and consequently, less scarring.

The impact of IL-10-induced immune suppression is a key factor in scarless wound healing. Yamamoto et al. (2001) performed Northern blot analysis to show that IL-10 differentially regulates collagen deposition by downregulation of TGF-β1-induced collagen mRNA. In a phase II randomized controlled clinical trial, Kieran et al. (2013) demonstrated that exogenous IL-10 applied to human incisional wounds reduced scarring and generally improved scar appearance.

In a recent study, Wise et al. (2014) studied the effects of a purified IL-10 homolog derived from orf virus (ovIL-10). Orf virus infections induce persistent skin lesions that are reminiscent of sustained wound healing, yet surprisingly resolve with minimal scarring (Gurel 2002), and genetic studies have discovered that orf virus encodes for a variety of factors that allow it to subvert host immune responses, including an IL-10 homolog (Fleming 2007). Wise et al. made excisional wounds in mice and divided them into four groups. The first three groups were treated with either recombinant murine IL-10 (mIL-10) or ovIL-10 in a buffered saline solution (PBS), or PBS alone, while the fourth group received no treatment. The wounds were biopsied at varying times and analyzed. Histological analysis revealed that wounds treated with ovIL-10 exhibited accelerated healing as evident by increased wound reepithelialization as compared to controls. Reduction in visible scarring was also evident and quantitative PCR confirmed decreased levels of key proinflammtory mediators. Thus supporting evidence exists that limiting the inflammatory response in wound healing can improve the quality of wound repair.

TGF-β is an upstream regulator of the cyclooxygenase (COX) pathway (Wilgus, et al. 2004). COX enzymes catalyze the conversion of membrane phospholipid-derived arachidonic acid into prostaglandins which are known mediators of the inflammatory response. Western blot analysis and immunohistochemistry of biopsied hypertrophic and keloid scar lesions revealed significant overexpression of COX-1 and COX-2 isoforms, respectively, in comparison to normal dermal tissue which further suggests a significant role these enzymes play in the pathogenesis of these abnormal scarring processes (Rossiello, et al. 2009). Indeed, TGF-β was shown to induce prostaglandin production in cultured

fibroblasts via COX-1 overexpression (Diaz, et al. 1998).

The significance of COXs in the etiology of pathological scarring such as keloids and hypertrophic scars has led to the investigation of its role in wound healing in general. Wilgus et al. (2003) examined the role of COX inhibitors in reducing scar formation. Celecoxib belongs to the family of nonsteroidal anti-inflammatory drugs (NSAIDs) which target the COX enzymes. Specifically, celecoxib selectively targets COX-2 and prevents its enzymatic activity and subsequent production of prostaglandins including prostaglandin E2 (PGE2). Wilgus et al. made full-thickness incisions in mice and closed the wounds with stainless steel wound clips to mimic surgical wounds. The staples were removed after 5 days. The wounds were treated topically immediately after wounding and for up to 14 days daily with either 200 µl of the vehicle control (K-Y Jelly; Ortho Pharmaceutical Corp., Raritan, NJ) or with 1 mg of celecoxib capsules (Celebrex®, Searle, St. Louis, MO) dissolved in 200 µl of vehicle such that 200 µl contained 1 mg of the drug.

Skin sections were excised and analyzed. Immunohistochemical analysis using an antibody specific for the neutrophil surface marker Ly-6G, revealed a stark contrast between vehicle-treated and celcoxib-treated wounds. While a major inflammatory response, manifested as a massive infiltration of neutrophils, was observed in the vehicle-treated wounds in comparison to unwounded tissue, wounds treated with celecoxib were virtually devoid of neutrophils.

Additionally, Biotrak enzyme immunoassays (EIA; Amersham-Pharmacia, Piscataway, NJ) were used to quantify the concentration of PGE2. An approximately 50% decrease in wound PGE2 levels was observed in wounds treated with celecoxib ($p < 0.05$). Levels of TGF-β1 were examined by Western blot analysis which revealed significantly lower levels in celecoxib-treated wounds compared to vehicle-treated wounds. These data were substantiated by the observation of reduced scarring later on.

Wilgus et al. (2003) also reported that PGE2 promotes fibroblast proliferation and collagen synthesis consistent with known findings (Lupulescu 1975) as well as their data showing decreased scar tissue deposition in treatment with celecoxib. This seems to be corroborated by the fact that lower levels of the proinflammatory fibrogenic TGF-β1 were detected. A similar result was presented by Miyajima et al. (2001) who also showed the ability of COX-2 inhibitors to reduce TGF-β levels and thus fibrosis.

The demonstrated effect of treatment with topical celecoxib in reducing scar formation in adult wound healing led Wilgus et al. (2004) to examine the role of COX-2 in scarless fetal wound repair. Incisions were made in the dorsum of fetal mice at either 15 (E15) or 18 (E18) days after the detection of a vaginal plug using microsurgical scissors. These time points represent the ages at which either scarless or fibrotic healing occurs. Normal E15 and E18 skin were harvested as controls along with the wounded samples at varying times for examination. Immunohistochemical staining at 24 hours post-wounding demonstrated COX-2 protein expression only in wounded E18 skin but not in E15 skin. Western blot was also used to detect COX-2 protein and the levels were analyzed by image analysis software. Levels of COX-2 protein as well as PGE2 were higher in wounds introduced at E18 compared to those at E15, implying that COX-2 is involved in the process of scar tissue formation.

It is thought that one mechanism by which PGE2 contributes to scar deposition is by increasing the rate of fibroblast proliferation. Indeed, Wilgus et al. showed that exogenous PGE2 applied to fetal fibroblast cultures resulted in statistically significant ($p < 0.05$ compared to untreated cells) dose dependent increased proliferation.

However, conflicting data suggest that fibroblasts from other organs do not respond in the same way to increased PGE2 levels. For example, PGE2 reduces proliferation and collagen synthesis by lung fibroblasts. This suggests that PGE2 can have varied effects perhaps resulting from differences in the expression or activity of various PGE2 receptors (Wilgus 2004).

Thus, the mechanism of PGE2-induced scarring could involve a number of plausible options. PGE2 could be contributing to the inflammatory response which has already been implicated in scar formation. Alternatively, PGE2 could be promoting scar deposition through increases in the profibrotic TGF-β. Lastly, PGE2 could be directly encouraging scar formation by stimulating fibroblast proliferation.

Of note, however, is that although keloid and hypertrophic scars were found to express high levels of COX proteins, Yeh et al. (2006) found that fibroblasts derived from keloid patients exhibited diminished capacity to produce PGE2, in line with the studies that identified PGE2 as an antifibrotic agent. In this experiment, fibroblast cultures were stimulated using the proinflammatory cytokine IL-1 which is known to activate fibroblasts and induce the formation/release of COX pathway products such as PGE2 (Elias, et al. 1987). The effects of PGE2 on the synthesis of collagen were determined using enzyme-linked immunosorbant assay (ELISA) kits. According to these data, the excessive deposition of collagen characteristic of keloids may in fact be due to decreased levels of PGE2 rather than increased.

The conflicting and perhaps confounding data surrounding PGE2 and its effect on fibroblast proliferation and collagen synthesis

necessitate further investigation into the exact mechanisms by which this enzyme affects the healing process. Such contradictory data does not now lend to sufficient understanding that can lead to exploitation and development of therapeutic clinical applications in the treatment or prevention of scarring. In fact, some have suggested that the use of NSAIDs and COX-2 inhibitors may even exacerbate wound scarring because of their ability to decrease PGE2 production (Su, et al. 2010).

Until such time as the role of PGE2 and its effect on wound healing becomes better understood, a more promising approach to treatment and/or prevention of scarring would seem to be manipulation of the various TGF-β isoforms, which perhaps play a more direct role in the process of scar formation .

Already, clinical trials that manipulated the ratio of TGF-β isoforms have proven efficacy in the controlling scarring, although not without some failure. Further investigation into the exact role the various isoforms of TGF-β play in the process of wound healing can hopefully lead to the development of better targeted therapies to reduce scarring and perhaps even cure the numerous fibrotic diseases.

Conclusion

In this review, the underlying mechanisms of scar formation in wound healing were explored. The various TGF-β isoforms (and their ratio) seem to play a vital role by regulating the inflammatory response although the process is undoubtedly more complex. The role of COX and PGE2 remains unclear with contradictory evidence pointing either to a reduction in scarring or perhaps exacerbation of the problem. The focus of potential therapeutic agents should for now remain dedicated to TGF-β as past studies have already proven potential in reducing scarring, while the other options reviewed here remain clouded in uncertainty. Research has further investigated the effects of interactions between cell adhesion molecules such as integrins and cadherins and the various TGF-β isoforms in an effort to explore targeted therapeutic agents (Eslami, et al. 2009; Agarwal, 2014). Additionally, research has explored TGF-β as a potential target for anti-scarring gene therapy (Liu, et al. 2004). These and other efforts show promise in the development of successful clinical means of controlling scarring and perhaps even fibrotic disease.

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