Extracellular regulation of TGF-β activity in wound repair: growth factor latency as a sensor mechanism for injury

Georg Brunner, Robert Blakytny
Department of Cancer Research, Fachklinik Hornheide, Münster, Germany

Summary
The transforming growth factor-β (TGF-β) family of growth factors are major regulators of wound repair, scar formation, and fibrosis. One of the prominent features of TGF-β biology is the fact that this growth factor is secreted as a latent precursor, which may be directed to and stored at specific sites in the cellular microenvironment. Targeting and mobilization, and particularly extracellular activation of latent TGF-β control the biological action of this growth factor. This review will focus on mechanisms of extracellular TGF-β regulation relevant to and potentially operating in wound repair and scarring.

Keywords
TGF-β, TGF-β regulation, latent TGF-β activation, wound repair, scarring

TGF-β in wound repair and scarring
Wound healing is a multi-step process which is the result of complex interactions of cells present in the injured tissue with cellular and soluble blood constituents (1). These interactions are governed by the action of growth factors, chemokines, and hormones. Cellular responses as well as the action of soluble mediators are modulated by the extracellular matrix (ECM) present in the wound area. Wound repair proceeds through a sequence of different stages, each of them being fundamentally influenced by the TGF-β family members, TGF-β1, 2, 3, or activin (2-4).

Among the first responses to injury are activation of platelets and the initiation of blood coagulation, whereby platelets are a major source of TGF-β1 in the early stages of wound repair (5, 6). The release and activation of platelet TGF-β1 probably contributes to the initiation of early cellular responses, such as the chemotactic recruitment of inflammatory cells (7) and new blood vessels (8) into the wound area. In contrast, TGF-β1 delays re-epithelialization when added to organotypic cultures in vitro (9) or when overexpressed in the epidermis in vivo (10). This inhibitory effect of TGF-β signaling in keratinocytes has been confirmed in vivo by transgenic studies demonstrating accelerated re-epithelialization in mice deficient in TGF-β1 (11), in the signaling keratinocyte type II TGF-β receptor (12), or in the intracellular TGF-β signal transducer, Smad3 (13).

At later stages of normal wound repair, TGF-β1 is a major inducer of connective tissue production and myofibroblast activity. Consequently, TGF-β1 activity promotes wound contraction and enhances wound breaking strength (14). In addition, TGF-β1 stimulates scar formation and tissue remodeling and may also be involved in pathologic healing responses such as hypertrophic scarring and fibrosis (15, 16).

Impaired wound healing states (e.g. venous and diabetic ulcers or glucocorticoid-induced wound healing defects) are characterized by reduced expression of TGF-β1 and the type II...
TGF-β receptor (17-19). In these conditions, exogenously added TGF-β1 has the ability, when administered topically or systemically, to reverse growth factor deficits and promote healing by stimulating wound contraction and breaking strength (2, 20, 21).

In summary, TGF-β1 appears to have two major functions in wound repair: At early stages, TGF-β1 recruits inflammatory cells into the wound area, apparently at the expense of a delay in re-epithelialization. At later stages, TGF-β1 acts on fibroblasts to promote connective tissue production, wound contraction, and scar formation, thereby accelerating normal as well as impaired wound healing. Interestingly, TGF-β1’s stimulation of scar formation appears to require cooperation with TGF-β2 to be fully efficient (22).

While the role of TGF-β1 in wound repair has been well characterized, potential isoform-specific functions of other TGF-β family members are less well defined. As mentioned above, TGF-β2 appears to synergize with TGF-β1, at least regarding the stimulation of connective tissue production and scar formation. TGF-β3, however, counteracts TGF-β1 activity in these processes and reduces the influx of inflammatory cells into the wound area as well as connective tissue deposition and scarring (22). Activin A and B expression is strongly induced during wound repair in the skin (23), and activin overexpression in the epidermis enhances wound repair, causing dermal fibrosis and epidermal hyperthickening (24). Conversely, overexpression of the activin antagonist, follistatin, in the epidermis causes a severe delay in wound repair (25). This defect appears to be mainly due to a reduced formation of granulation tissue, resulting in diminished wound breaking strength. These data document the important role of activin in promoting various stages of wound repair.

**TGF-β latency**

TGF-β1, 2, and 3 are secreted as latent protein complexes (Fig. 1; 26, 27). It is believed that the extracellular activation of these latent forms represents a crucial step in the regulation of...
TGF-β activity. Following proteolytic processing of pro-TGF-β by furin-like enzymes, the mature growth factor dimer remains associated with the dimeric TGF-β propeptide (the latency-associated peptide, LAP), forming the small latent TGF-β complex. This association maintains growth factor latency and inhibits TGF-β activity (28).

The LAP present in the small latent complex is usually disulfide-bonded to a fibrillin-like latent TGF-β binding protein (LTBP-1, 3, or 4) to form the large latent TGF-β complex (Fig. 1; 29). These binding proteins target latent TGF-β, depending on the LTBP isoform present in the complex, to specific binding sites in the ECM (27) and enable subsequent mobilization of latent growth factor from these extracellular reservoirs by limited proteolytic processing (Fig. 1; 30).

LAP is the target of various latent TGF-β activators, such as proteases, integrins, and thrombospondin-1 (TSP-1) (26). Latent TGF-β serves as a sensor transmitting a variety of extracellular signals, mostly generated by the disturbance of tissue homeostasis, into cellular responses (27). Thus, extracellular TGF-β activity is regulated at the level of bioavailability (release of latent TGF-β from ECM reservoirs) and latency (release of active TGF-β from the latent complex).

Although it is well established that growth factors of the TGF-β family are major regulators of various stages of wound repair and scarring, little is known regarding the levels of TGF-β activity in the wound area and the levels of latent TGF-β bound to ECM. While expression of all three TGF-β isoforms has been demonstrated in wound tissue in vivo, the potentially isoform-specific mechanisms of extracellular growth factor deposition, mobilization, and activation of the latent TGF-β complex during wound healing are largely unknown. Analysis of incisional wound repair in an animal model suggests that major latent TGF-β activation occurs at two peak time points, immediately following wounding and upon wound closure by re-epithelialization (Fig. 2; 31). Between and after these two peaks, TGF-β activity remains significantly increased.
above the levels in normal skin or adjacent tissue, suggesting continuous latent TGF-β activation up to 14 days post-wounding. Temporal regulation of TGF-β activity is most likely accompanied by spatial regulation of latent TGF-β activation within the wound area, probably involving different cell types, isoforms of TGF-β and LTBP, LTBP binding sites in the ECM, and mechanisms of latent TGF-β mobilization, activation, and turnover. These events will be the subject of the following sections.

**Platelet latent TGF-β activation**

One of the first responses to injury is the adherence and activation of platelets, which is accompanied by the release of large amounts of latent TGF-β1 into the wound area (6). Platelets, when stimulated with thrombin in vitro, activate part of the TGF-β that they release (32, 33), but TGF-β release and activation appear to be independent events. Thus, the early peak of latent TGF-β activation occurring in vivo (< 1 hour following incisional wounding; Fig. 2) can most likely be ascribed to platelet activation in the wound area.

The platelet α granule protein, TSP-1, binds and activates latent TGF-β1 and 2 in vitro, possibly by disrupting the non-covalent interactions between LAP and TGF-β (34, 35). TGF-β and LAP remain associated with TSP-1 forming a ternary, biologically active TSP-TGF-β complex (35). TSP-1 null and TGF-β1 null mice have similar phenotypes, which led to the hypothesis that TSP-1 is one of the major latent TGF-β activators in vivo (36). Wound healing in TSP-1 null mice is characterized by delayed angiogenesis and macrophage infiltration which correlates with decreased TGF-β activity in the wound area (37).

However, the molecular mechanism of activation remains somewhat controversial, since, in another study using a purified system, TSP-1 was reported to be unable to activate latent TGF-β1 in vitro (38). This could possibly be explained by differences in TSP-1 purification protocols or TGF-β assays used in these studies and/or might also indicate the involvement of additional co-factors in activation.

It is surprising that, although both TSP-1 and latent TGF-β1 are co-expressed in platelet α granules and are released upon degranulation, TSP-1 is not required for latent TGF-β activation in this system in vitro and in vivo (32, 39). Upon activation, TSP-1-deficient platelets produce normal levels of extracellular active TGF-β1 in vitro and in vivo, and peptides competing with the TSP-LAP interaction do not interfere with platelet latent TGF-β activation. The mechanism of activation appears to be proteolytic, since platelets express a furin-like enzyme, which is released upon activation and is involved in the extracellular activation of latent TGF-β (32).

**Pericellular reservoirs of latent TGF-β**

Platelets activate only a small fraction of the latent TGF-β that they release (32). The majority of the released large latent TGF-β complex is probably either directed to extracellular binding sites in the wound area or is flushed out of the tissue into the circulation. It has been reported that approximately one third of the released TGF-β represents small latent complex which remains associated with the platelets in an RGD-dependent manner (40). Thus, platelets may provide a long-term source for TGF-β activity by generating a solid-phase reservoir of latent growth factor in the wound area accessible to various cell types and mechanisms of activation.

The LTBP directs the large latent TGF-β complex to specific binding sites in the ECM (Fig. 1). Latent TGF-β is incorporated into ECM via transglutaminase-catalyzed cross-linking of TBP’s N-terminus (41). This interaction most likely involves one of the 8-Cys domains of the protein and possibly fibronectin as the binding site in the ECM (42). Alternatively, LTBP may also associate with microfibrils through interaction of its C-terminus with fibrillin (43). Thus, LTBP anchors the large latent TGF-β complex between two structural connective tissue elements, microfibrils and extracellular matrix fibers. Latent TGF-β released during wound repair by keratinocytes, fibroblasts, macrophages, neutrophils, or vascular cells could be deposited into such ECM-associated growth factor reservoirs. In fact, the essential role of LTBP in latent TGF-β activation (44) might be related to its function in targeting the latent complex into ECM and controlling its release from these sites. Platelets, however, release a shortened form of LTBP-1, whose ability to interact with ECM has not yet been determined.

Efficient latent TGF-β activation requires appropriate localization of the latent growth factor complex in the ECM (27). Furthermore, activation is most likely preceded by the mobilization of latent TGF-β from these extracellular reservoirs, e.g. by enzymatic cleavage of the protease-sensitive regions in LTBP (Fig. 1). Wound repair is characterized by the induction of a panel of ECM-degrading proteases including plasmin, thrombin, mast cell chymase, leucocyte elastase, MMP-2, MMP-3, MMP-9, MT1-MMP, some of which have been shown to be involved in latent TGF-β release from ECM and/or in its activation (45-50). This suggests that ECM-bound latent TGF-β may be mobilized and activated during wound repair by multiple, possibly redundant proteolytic mechanisms.

**Mechanisms of latent TGF-β activation**

Wound repair is accomplished by a number of different cell types cooperating in a spatio-temporal strictly controlled manner. It is therefore likely that regulation of TGF-β action in
wound repair involves multiple mechanisms of activation resulting in distinct biological activities, probably depending on the TGF-β isof orm, the stage of wound repair, and the location within the wound area. This is reflected by the occurrence of two distinct peaks of TGF-β-like activity, as described above, during the repair process of incisional skin wounds in an animal model (31; Fig. 2). The early peak of activation immediately following wounding can most likely be ascribed to the activation of platelet latent TGF-β1 involving a furin-like enzyme (32; see above).

Activated TGF-β1 then helps to recruit inflammatory cells to the wound site which also secrete and activate latent growth factor, possibly contributing to the elevated TGF-β activity levels seen during the time period between the two main peaks (Fig. 2). For instance, lipopolysaccharide-stimulated macrophages activate endogenous latent TGF-β1. This is accomplished via a proteolytic mechanism requiring the enzymatic function of the plasminogen activation cascade (i.e. cell surface plasmin generation by receptor-bound urokinase) and of tissue type II transglutaminase as well as latent TGF-β binding to ECM and to the cellular cation-independent mannose 6-phosphate / insulin-like growth factor type II receptor (M6P/IGFII-R) (51). A pentameric complex of the components involved (M6P/IGFII-R, latent TGF-β, plasminogen, urokinase receptor, urokinase plasminogen activator) has been shown to be necessary and sufficient to activate latent TGF-β in vitro (52).

Alternatively, for activated alveolar macrophages, cell surface localization of latent TGF-β has been proposed to occur via TGF-β complex formation with TSP-1 followed by binding of the biologically active complex to the TSP receptor, CD36 (53).

At later stages of wound healing, latent TGF-β activation might occur during angiogenesis in the granulation tissue, involving a similar mechanism of proteolytic activation (Fig. 2). Co-cultures of capillary endothelial cells and pericytes or smooth muscle cells, in contrast to mono-cultures of the respective cell types, activate latent TGF-β1 (54, 55). Similar to activated macrophages, activation in co-cultures also requires plasmin generation, transglutaminase activity, and LTBP-mediated latent TGF-β binding to ECM as well as cell surface binding to M6P/IGFII-R (26).

Plasminogen activation is a characteristic feature of a variety of cell types activated during wound repair, including macrophages, endothelial cells, migrating keratinocytes, and fibroblasts. Apart from possibly being involved in latent TGF-β activation by macrophages and vascular cells, the fundamental and essential function of cell-associated plasmin generation in wound repair is the dissolution of the fibrin clot (56). It has been proposed that, during this process, the pool of platelet small latent TGF-β1 complex associated with the fibrin clot (see above) is activated by plasmin (40). Thus, the role of plasmin in the sequence of events leading to the generation of active TGF-β may be manifold. Besides activating small latent TGF-β complex directly by cleaving LAP (see above), it might release large latent TGF-β complex from ECM by cleaving the LTBP’s protease-sensitive regions. In addition, plasmin activates pro-MMP-9, which has been suggested to lead to the activation of latent TGF-β in interleukin-13-induced tissue fibrosis (57).

The late peak of latent TGF-β activation coincides with wound closure suggesting involvement of epithelial-mesenchymal interactions (Fig. 2). The function of the TGF-β-like activity at this stage of wound healing might be to induce cell differentiation of epithelial cells and/or fibroblasts and to regulate wound contraction and scar formation. This is supported by the findings that co-cultures of keratinocytes and fibroblasts, in contrast to mono-cultures of each cell type, activate or induce several isoforms of the TGF-β family (TGF-β1, 2, 3 and activin A) leading to the expression of a myofibroblastic phenotype (58, and our unpublished data). Epithelial cells can activate latent TGF-β1 and 3 via interactions of LAP with αvβ6 integrin (59, 60). This mechanism might be operating at the time of wound closure and could contribute to the second peak of TGF-β activity, since, at this stage of wound healing, αvβ6 integrin expression is strongly induced in basal and suprabasal keratinocytes (61, 62).

Transgenic approaches targeting latent TGF-β activators

Most of the mechanisms of latent TGF-β activation described here have been identified and characterized in cell models in vitro, and their biological significance in vivo, particularly in wound repair, is largely unknown. Several of the genes encoding proteins involved in these mechanisms have been knocked out and/or overexpressed in mice, and wound healing has been studied in three of these animals (deficient in plasminogen and TSP-1, respectively, or overexpressing β6 integrin) (63-67). However, evaluation of the physiological role of latent TGF-β activators using gene knockout approaches is not straightforward, since deficiency in TGF-β1 or Smad3-mediated signaling does not result in a striking wounding defect. Instead, it accelerates re-epithelialization and produces features such as decreased epithelial thickness, local inflammatory responses, granulation tissue formation, and scarring (11, 13).

In contrast to TGF-β1 null mice, mice deficient in plasminogen suffer from defective re-epithelialization caused by severely delayed fibrinolysis (63). Wound healing problems related to this might conceal subtle phenotypic features caused by a potential lack of TGF-β activation at certain stages of healing. The wound healing defect in these mice is corrected by fibrinogen gene deletion (68). Thus, whereas plasmin-catalyzed fibrinolysis is required for efficient repair of skin wounds, the above gene knockout studies did not reveal an essential role for plasmin in generating active TGF-β in this process.
Based on gene knockout studies, TSP-1 has been proposed to be one of the major activators of latent TGF-β in vivo (36). The role of TSP-1 in wound repair, however, remains somewhat controversial: Transgenic TSP-1 overexpression in the epidermis results in a delay in wound repair, which appears to be caused by diminished granulation tissue formation and angiogenesis and by inhibition of fibroblast migration (65). These features are incompatible with an increase in latent TGF-β activation. Intriguingly, antisense targeting of endogenous TSP-1 in wounds, which is mainly expressed in macrophages, also caused a delay in wound repair, probably due to a decreased rate of re-epithelialization and dermal re-organization (64). In this case, the latter feature could indeed be explained by reduced levels of active TGF-β. This is consistent with the findings that skin wounds of TSP-1-deficient mice, at early stages of healing, contain decreased TGF-β activity and wound healing is delayed (66). Furthermore, the repair process in these mice exhibits other features consistent with diminished latent TGF-β activation, such as an impaired inflammatory response and less densely packed collagen fibers. Taken together, these findings suggest that endogenous macrophage TSP-1 is involved in latent TGF-β activation at defined stages of wound repair.

The mechanism of platelet latent TGF-β activation has also been analyzed by gene knockout approaches, using mice deficient in plasminogen and TSP-1, respectively, as well as in M6P/IGFII-R null mice (32). Generation of active TGF-β1 by platelets from these mice was normal, indicating that these proteins are not involved in TGF-β regulation at early stages of wound repair.

Intriguingly, transgenic β6 integrin overexpression in the epidermis leads to the development of chronic wounds which contain increased amounts of TGF-β1 protein (67). This not only represents a potentially very useful animal model for impaired wound healing, but also suggests that αvβ6-integrin mediated epithelial latent TGF-β activation is involved in the pathology of chronic skin wounds.

Modulation of TGF-β activity by proteoglycans

Active TGF-β binds to proteoglycans on the cell surface and in the ECM. These interactions serve two main functions, regulation of TGF-β bioavailability by growth factor sequestration into ECM and modulation of TGF-β signaling by acting as a cell surface co-receptor (type III TGF-β receptor). In particular, the cell surface proteoglycan, betaglycan, presents active TGF-β2 to the signaling type II TGF-β receptor, forming a trimeric complex (69). Betaglycan is expressed on a variety of cells, including fibroblasts, macrophages, and smooth muscle cells, probably rendering these cells more responsive towards TGF-β during wound repair. Intriguingly, when expressed in renal epithelial cells, betaglycan inhibits TGF-β signaling, which appears to be due to alterations in the glycosaminoglycan modifications (70). This indicates that the effects of betaglycan on TGF-β signaling are cell-type specific.

The type III receptor proteoglycan, endoglin (CD105), which is expressed on endothelial cells, macrophages, keratinocytes and fibroblasts, indirectly binds several TGF-β family members including TGF-β1, -β3 and activin, but not TGF-β2. In contrast to betaglycan, endoglin inhibits TGF-β responsiveness of cells by associating with type I and II receptors (71), not allowing growth factor – receptor interactions to occur.

These positive and negative proteoglycan regulators with specificities for different TGF-β isoform are expressed on the surface of cells involved in wound repair, allowing a complex fine tuning of TGF-β responses during the healing process.

Sequestration of TGF-β1, 2, or 3 into ECM may occur via binding to the core proteins of the small proteoglycans with leucine-rich repeats (SLRPs), decorin, biglycan, or fibromodulin (72). Whether these interactions prevent or possibly even promote TGF-β receptor binding and influence biological activity is still under debate. All three SLRPs are expressed in skin, and decorin and biglycan are, in contrast to fibromodulin, up-regulated during adult wound healing, possibly as part of a negative feedback regulation of TGF-β action (73). Intriguingly, in the same study fibromodulin expression was found to be induced during scarless repair in the fetus, suggesting a role for this proteoglycan in counteracting TGF-β activity during scar formation.

Termination of TGF-β action

Upon wounding, large quantities of latent TGF-β are released from a variety of cellular sources, and significant fractions of it are activated at various stages of repair. Since TGF-β action during wound healing and scarring is strictly controlled (see also Fig. 2), precise timing not only of latent growth factor activation but also of inactivation of active growth factor is important.

Besides receptor-mediated uptake into cells present in or migrating into the wound area, active TGF-β binds to a number of proteins and proteoglycans possibly involved in the inactivation and/or clearance of excess growth factor. One of the most important TGF-β binding proteins with a clearance function is α2-macroglobulin. It binds all three TGF-β isoforms, in particular TGF-β1 and 2, and thereby neutralizes their biological activity (74). α2-Macroglobulin can be converted, possibly by neutrophil oxidants or proteases expressed during wound repair, from the “slow” to the “fast” form (75). This modification renders α2-macroglobulin competent for binding to the α2-macroglobulin receptor (the low density lipoprotein receptor-related protein, LRP) expressed on macrophages, fibroblasts,
and liver hepatocytes. Thus, by binding the growth factor, α2-macroglobulin might mediate the clearance of excess active TGF-β, via uptake into cells within the wound area or in the liver. The potential physiological significance of this mechanism is supported by the finding that α2-macroglobulin-deficient mice exhibit increased levels of active TGF-β, possibly contributing to the increased resistance to endotoxin challenge and to the higher lethality of acute pancreatitis observed in these animals (76, 77).

Excess TGF-β activated during wound repair may also be inactivated via sequestration into the ECM, e.g. by binding to SLRPs expressed in the skin (see above). The binding constant for this interaction ranges between 1 – 20 nM (72). TGF-β-SLRP complexes could then be cleared from the wound area by proteolytic dissociation from the ECM followed by uptake in the liver or by endothelial and kidney tubular epithelial cells (78). Active TGF-β has also been found in urine, part of it being complexed to decorin, indicating clearance via kidney excretion of TGF-β-decorin complexes (78). The concentration of these complexes dramatically increases in diabetic nephropathy, suggesting an anti-fibrotic role of decorin in this pathologic condition.

Active TGF-β also binds to the ECM proteins, fibronectin and vitronectin. The binding constant for the interaction with vitronectin ranges between 0.3 – 7.3 nM depending on the vitronectin isoform (79), while TGF-β binding to fibronectin is almost irreversible (80). Fibronectin and vitronectin both occur in soluble form, e.g. in the plasma, and are endocytosed by a number of cell types. Thus, similar to the potential function of SLRPs, excess TGF-β in the wound area might be inactivated by complex formation with these proteins resulting in sequestration into ECM and/or endocytosis of soluble, circulating protein complexes. Whether active TGF-β sequestration into the granulation tissue, mediated by fibronectin or vitronectin, may also regulate the bioavailability and biological activity of this growth factor in wound repair, remains to be investigated.

Conclusions

Paracrine and autocrine signaling events initiated by the TGF-β family members, TGF-β1, 2, 3, and activin, regulate early as well as late wound healing stages. Immediate responses to injury, such as inflammation, are most likely initiated by the activation of platelet latent TGF-β1, catalyzed by a furin-like platelet protease. This enzyme as well as potential additional molecular determinants involved in the activation process remain to be identified. Characterization of this platelet-mediated mechanism of latent TGF-β activation will potentially allow for the modulation of the inflammatory response at early stages of wound repair.

Medium- and long-term responses to injury, e.g. granulation tissue formation, re-epithelialization, angiogenesis, and scar formation, are most likely mediated by large latent TGF-β complex. This growth factor precursor may be deposited into ECM reservoirs, e.g. anchored between microfibrils and ECM fibres, and probably functions as a sensor for injury (27): Latent TGF-β complex, proteolytically mobilized from ECM, is accessible to a number of proteolytic and non-proteolytic mechanisms of activation, potentially operating at sequential or overlapping stages of wound repair. Whether some of these mechanisms will turn out to be TGF-β isofrom-specific remains to be determined.

It is not yet clear which of the mechanisms of active TGF-β generation identified in vitro are in fact operating in wound repair in vivo. Results from transgenic studies have provided evidence for potentially important roles of TSP-1 and β6 integrin in latent TGF-β activation at defined stages of healing. Involvement of mechanisms unidentified so far cannot be excluded. Attempts are currently being made to identify novel latent TGF-β activators, e.g. using a genetic screen (81). Fine tuning of TGF-β signaling is achieved by presentation and/or sequestration of active growth factor by type III receptor proteoglycans and SLRPs, respectively.

TGF-β is an important regulator of normal and impaired wound healing, and its activity is controlled at the level of latent precursor activation. The mechanisms of activation, therefore, represent promising therapeutic targets for the treatment of wound healing disorders and fibrosis.

References


