Upregulation of TGF-β1 Expression May Be Necessary but Is Not Sufficient for Excessive Scarring

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Transforming growth factor beta 1 (TGF-β1) upregulation has been implicated in hypertrophic scars and keloids, but it is unclear if it is the cause or an effect of excessive scar formation. In this study, we overexpressed TGF-β1 in fibroblasts and characterized its role. Normal human dermal fibroblasts were genetically modified to overexpress TGF-β1 as the wild-type latent molecule or as a mutant constitutively active molecule. TGF-β1 secretion was measured, as were the effects of TGF-β1 upregulation on cell proliferation, expression of smooth muscle cell alpha actin (SMC α-actin) and ability to contract collagen lattices. Fibroblasts were implanted intradermally into athymic mice and tissue formation was analyzed over time by histology and immunostaining. Gene-modified fibroblasts secreted ~20 times the TGF-β1 released by control cells, but only cells expressing mutant TGF-β1 secreted it in the active form. Fibroblasts expressing the active TGF-β1 gene had increased levels of SMC α-actin and enhanced ability to contract a collagen lattice. After intradermal injection into athymic mice, only fibroblasts expressing active TGF-β1 formed "keloid-like" nodules containing collagen, which persisted longer than implants of the other cell types. We conclude that upregulation of TGF-β1 by fibroblasts may be necessary, but is not sufficient for excessive scarring. Needed are other signals to activate TGF-β1 and prolong cell persistence.

Journal of Investigative Dermatology (2006) **126,** 1168–1176. doi:10.1038/sj.jid.5700200; published online 23 February 2006

INTRODUCTION

Fibrosis, the abnormal and excessive deposition of fibrous tissue, occurs in many diseases including liver cirrhosis, pulmonary fibrosis, scleroderma, and wound healing of the skin after trauma or surgery (O'kane and Ferguson, 1997). Fibrosis in the skin can lead to the formation of abnormal scars, hypertrophic scars or keloids, which can cause significant problems in tissue growth, function, and movement. The problem is particularly acute following large deep second- and third-degree burns.

Keloids and hypertrophic scars are both benign proliferations of the dermis. A keloid is a dermal lesion that spreads beyond the margins of the original wound, continues to grow over time, does not regress spontaneously, and commonly recurs following treatment. By contrast, a hypertrophic scar is a raised scar that remains within the boundaries of the original lesion, often regresses spontaneously several months after the initial injury and rarely recurs after treatment. Clinically, treatment is challenging and despite a wide array of options (corticoids, radiation, lasers, surgery, and interferon), recurrence of keloids after treatment is frequent, varying from 45 to 100% (Niessen *et al.*, 1999).

There is little understanding of the molecular mechanisms leading to the formation of keloids or hypertrophic scars, nor is there an adequate animal model for their study, as they occur only in humans. A leading theory proposes that upregulation of transforming growth factor beta 1 (TGF-β1) expression drives excessive cell proliferation and matrix synthesis (Border and Noble, 1994). TGF-β1 and its family members β2 and β3 are one of the only few cytokines known to stimulate collagen synthesis, and several studies have shown that it is upregulated in hypertrophic scars and keloids (Ghahary *et al.*, 1993; Younai *et al.*, 1994; Tredget *et al.*, 1998; Lee *et al.*, 1999; Wang *et al.*, 2000; Hanasono *et al.*, 2003). It is also well known that TGF-β1 is an autocrine factor produced by fibroblasts that is capable of upregulating its own synthesis (Van Obberghen-Schilling *et al.*, 1988; Tomasek *et al.*, 2002).

However, synthesis and activation of TGF-β1 and its family members β2 and β3 is a complex process. TGF-β1 is synthesized in a latent form as part of a large precursor molecule. TGF-β1 is proteolytically cleaved from the precursor as it is secreted, but remains noncovalently bound to the latency-associated peptide. Much of the TGF-β1–latency-associated peptide complex is stored in the extracellular

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Abbreviations: PBS, phosphate buffer solution; SMC α-actin, smooth muscle cell alpha actin; TGF-β1, transforming growth factor beta 1

Received 9 May 2005; revised 8 December 2005; accepted 13 December 2005; published online 23 February 2006

matrix because of the fact that latency-associated peptide is covalently bound to another protein, the latent TGF-β1-binding protein. Most importantly, TGF-β1 in this complex is inactive. Once released from this complex, TGF-β1 is active and can bind/signal its cellular receptor. TGF-β1 is liberated by extremes of pH and heat *in vitro* as well as by proteases such as furin and plasmin (Gleizes *et al.*, 1997; Khalil, 1999). Other proteins, such as thrombospondin, have been implicated in the release of TGF-β1 *in vivo*, although other mechanisms may be operable (Murphy-Ullrich and Poczatek, 2000).

Although upregulation of TGF-β1 expression has been implicated in the formation of hypertrophic scars and keloids, it is unclear if it is the cause or merely an effect of the formation of these scars. Moreover, activation of TGF-β1 is probably a critical regulatory step in this process. In this study, we used genetic modification to directly test the hypothesis that TGF-β1 upregulation contributes to fibrosis in the skin. We genetically modified normal human dermal fibroblasts to overexpress TGF-β1 in one of two forms; the wild-type latent molecule or a mutant form that is constitutively active. The modified cells secreted high levels of TGF-β1, but only cells transfected with the mutant gene secreted it in the active form. These cells also had increased levels of smooth muscle cell (SMC) α-actin and an enhanced ability to contract a collagen lattice. When implanted intradermally into athymic mice, only cells expressing the active mutant TGF-β1 formed nodules and these structures persisted longer than implants of control cells or cells overexpressing latent TGF-β1. The role of TGF-β1 in hypertrophic scars and keloids are discussed in light of these results.

RESULTS

Fibroblasts transfected with the mutant gene produce high levels of active TGF-β1

Diploid human fibroblasts were genetically modified with a recombinant retrovirus encoding either wild-type (latent) TGF-β1, which is secreted as a latent molecule and requires activation, or a mutant form of TGF-β1 (cysteines 223 and 225 were converted to serines), which is secreted as an active molecule and does not require activation (Figure S1). To determine how much TGF-β1 was secreted, control and gene-modified fibroblasts were grown to confluence and aliquots of the medium harvested at different time points, and levels of TGF-β1 measured by ELISA (Figure 1). To distinguish between TGF-β1 secreted in the latent form versus the active form, samples were either activated by acidification or not activated before ELISA. In samples that were activated, cells expressing the wild-type latent form of TGF-β1 accumulated 18 times more TGF-β1 than control unmodified cells after 48 hours, and cells expressing the mutant active form of TGF-β1 accumulated 21 times the amount (Figure 1a). The rates of total TGF-β1 production (latent plus active) were 2.6 ng/10^6 cells/48 h for control cells (1.1 ng/ml at 24 hours), 48.5 ng/10^6 cells/48 h for latent cells (17.9 ng/ml at 24 hours), and 56.7 ng/10^6 cells/48 h for mutant active cells (8.3 ng/ml at 24 hours). These ELISA data of activated samples measure the level of TGF-β1 in the latent as well as the active form. To determine how much TGF-β1 was secreted in the active form, the same samples were assayed without activation before ELISA (Figure 1b). Cells expressing latent TGF-β1 accumulated the same amount of active TGF-β1 as control cells (0.22 ng/10^6 cells/48 h) (0.084 ng/ml at 24 hours), but the rate of active TGF-β1 production by cells expressing the mutant gene was 300 times higher (67.2 ng/10^6 cells/48 h) (4.4 ng/ml at 24 hours).

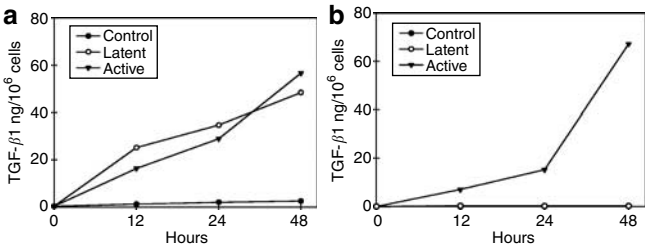

Figure 1. Fibroblasts expressing the mutant gene secrete high levels of active TGF-β1. Control unmodified cells and gene-modified cells expressing either latent TGF-β1 or mutant active TGF-β1 were grown to confluence and aliquots of the medium harvested at various times and measured for levels of TGF-β1 by ELISA. The ELISA cannot measure TGF-β1 in the latent form. (**a**) Samples were acidified to release latent TGF-β1 before ELISA or (**b**) samples were not acidified before ELISA.

Proliferation is decreased in fibroblasts expressing active TGF-β1

To determine whether TGF-β1 expression altered fibroblast proliferation, control and modified cells were plated into six-well plates, trypsinized and counted every day (Figure 2). For the first 48 hours, the proliferation of all cells was comparable. After 2 days, control and cells expressing latent TGF-β1 proliferated at comparable rates and reached confluence in 5 days. In contrast, cells expressing active TGF-β1 proliferated slower, and required an additional 48 hours to reach confluence (Figure 2a). The doubling time for control cells and cells expressing latent TGF-β1 was ~20–24 hours versus ~48 hours for cells expressing active TGF-β1. A similar result was obtained when performing the growth curve with WST-1 (Figure 2b). After reaching confluence, cells were followed for up to 21 days to evaluate cell morphology (Figure 2c). In the first few days, the cell morphology for all groups was comparable. By the end of 21 days, the slower growing cells expressing active TGF-β1 had about half as many cells (60×10^4 cells/well) as control cells or cells expressing latent TGF-β1 (111×10^4). One week after reaching confluence, cells expressing active TGF-β1 started to form aggregates and clusters that grew larger with time, whereas control cells and cells expressing latent TGF-β1 did not.

Levels of SMC α-actin are increased in fibroblasts expressing active TGF-β1

To determine whether levels of SMC α-actin were altered, control and modified fibroblasts were grown to confluence and incubated in medium containing 1% serum for 48 hours. Cells were lysed with a buffer containing 1% SDS, and equal amounts of total protein were fractionated by SDS-PAGE, transferred to nitrocellulose membranes and probed by

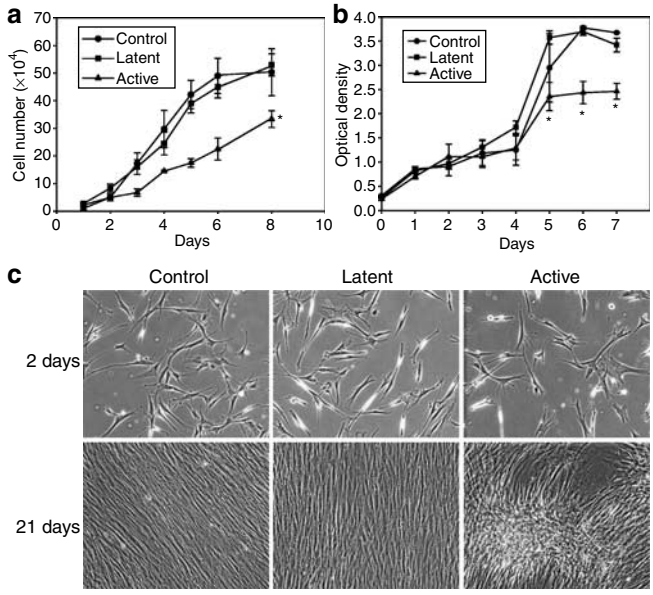

Figure 2. Fibroblasts expressing active TGF-β1 proliferate slower and form cell clusters. (**a**) Control cells and gene-modified cells expressing either latent TGF-β1 or active TGF-β1 (3×10^3 cell/cm^2) were plated and at various time points, the cells were trypsinized and counted. Cells were also plated in 96-well dishes, incubated each day with the WST-1 reagent to assess viability and cell number. (**b**) Absorbance was read in an ELISA plate reader. Error bars represent standard deviation for three replicates (*P<0,001). (**c**) Photographs of cells using phase contrast microscopy at day 2 (preconfluent) and day 21 (postconfluent) shows the formation of cell clusters by those cells expressing active TGF-β1 (Bar = 100 μm).

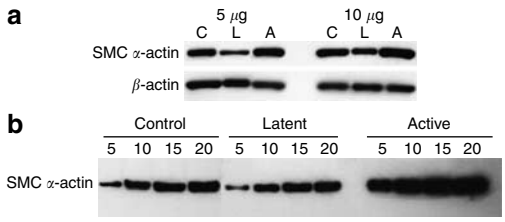

Figure 3. Levels of SMC α-actin are increased in fibroblasts expressing active TGF-β1. (**a**) Cell extracts (5 or 10 μg of protein) from control cells (C), and gene-modified cells expressing either latent TGF-β1 (L), or active TGF-β1 (A) were separated by SDS-PAGE, transferred to a membrane and levels of SMA α-actin measured by immunostaining. The membrane was stripped and reprobed for levels of β-actin by immunostaining to ensure equal loading. (**b**) Relative levels of SMA α-actin of the same cells were measured in the same way by loading 5, 10, 15, and 20 μg extract protein and immunostaining. Image analysis of the signal from the different concentrations of extracts showed that SMA α-actin levels were increased 2–3-fold in cells expressing active TGF-β1.

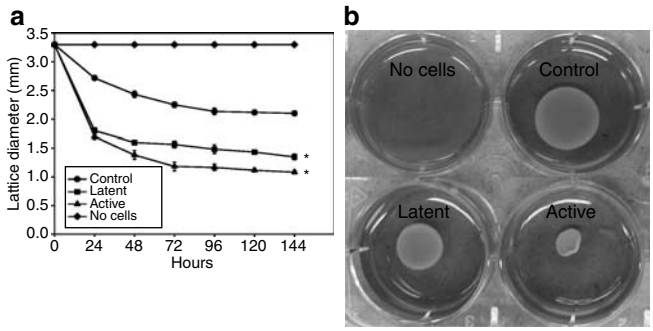

Figure 4. Time course of contraction of fibroblast-populated collagen lattices. (**a**) Control cells and gene-modified cells expressing either latent TGF-β1 or active TGF-β1 were cast in floating collagen gels and the rate of contraction determined by measuring lattice diameter. Error bars represent the standard deviation of six replicates (*P<0.001). Results were significant at each time point throughout the curves. (**b**) Photograph of the lattices after 144 hours of contraction.

Western blotting. Compared to controls and cells expressing latent TGF-β1, cells expressing active TGF-β1 had increased levels of SMC α-actin (Figure 3a). The same membrane was stripped and probed for β-actin, showing that differences in SMC α-actin levels were not due to differences in loading, as the levels of β-actin were comparable. To estimate the increase in SMC α-actin levels, varying amounts of total protein (5, 10, 15, and 20 μg) were loaded and probed by Western blotting. These data show that SMC α-actin levels were 2–3-fold higher in cells expressing active TGF-β1 versus control cells and cells expressing latent TGF-β1 (Figure 3b).

Contraction of collagen lattices is enhanced by gene-modified fibroblasts

To evaluate the ability of fibroblasts to contract collagen lattices, control and modified cells were cast into floating collagen lattices and monitored contraction over time (Figure 4). Collagen lattices were rapidly contracted by transfected cells from a starting diameter of 3.3 cm to less than 2 cm in the first 24 hours. Lattices with cells expressing latent TGF-β1 and active TGF-β1 contracted from 3.3 to 1.8 cm and 3.3 to 1.7 cm, respectively; whereas lattices with control cells contracted from 3.3 to 2.7 cm in the first 24 hours. After 144 hours, contraction was greatest for cells expressing active TGF-β1 (32% of its initial diameter) compared to cells expressing latent TGF-β1 (58%) and control cells (63%).

Cells expressing active TGF-β1 form nodules and persist longer after implantation

To evaluate their growth and tissue organization *in vivo*, control and gene-modified fibroblasts were implanted by intradermal injection into athymic mice. Injection sites were excised and pictures taken at each time point (Figure S2). At 3 days after injection, cells expressing latent or active TGF-β1 formed macroscopic nodules that were visible on the inner side of the skin. Nodule formation by control cells was not obvious. At 7, 10, and 14 days after injection, nodules were only obvious for those cells expressing active TGF-β1 and they had an enhanced macroscopic vascularization, mainly at 7 days. Compared to their size at day 7, nodules formed by cells expressing active TGF-β1 were somewhat smaller and appeared to be contracted at days 10 and 14. At day 28 and beyond, macroscopic nodules were not apparent for any of the cell groups. These results were found in all animals tested (eight animals/time point) with minimal variations in nodule size and time of formation and disappearance.

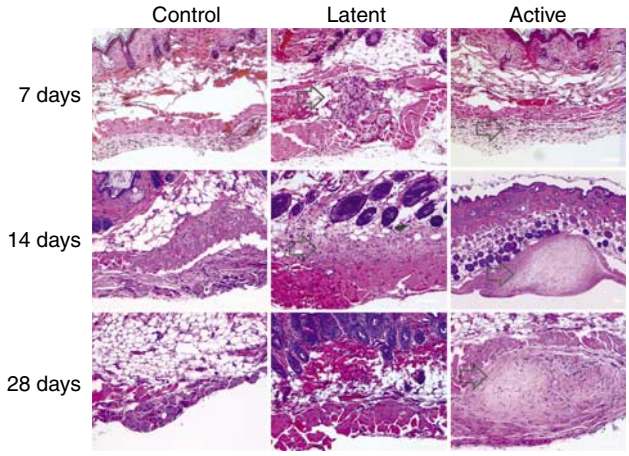

Figure 5. Fibroblasts expressing active TGF-β1 form keloid-like tissue. Tissue from injection sites were harvested at different times, fixed, paraffin-embedded, sectioned (5 μm) and stained with hematoxilin & eosin. Little organization of injected cells was evident for all groups at day 7. By day 14, only fibroblasts expressing active TGF-β1 had formed nodular structures with ''keloid-like'' appearance. Note the high density of cells at the periphery compared to the center and the nodule appears to be encapsulated by mouse connective tissue. Nodules formed by fibroblasts expressing active TGF-β1 were evident at day 28, whereas the location of control fibroblasts or fibroblasts expressing latent TGF-β1 were not obvious. Arrows mark sites of obvious implants (Bar = 100 μm).

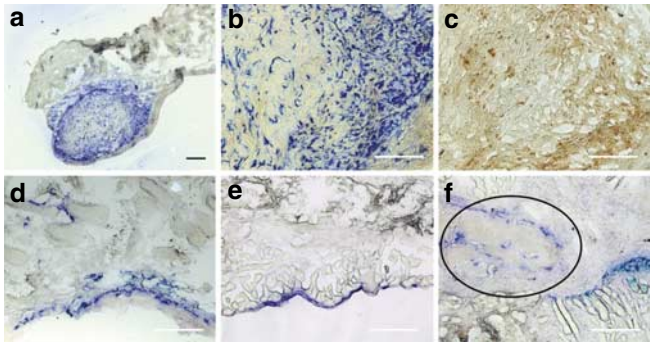

Figure 6. Human fibroblasts and collagen type I are present in nodules formed by cells expressing active TGF-β1. Cryosections (8 μm) of injection sites frozen in OCT were immunostained (**a, b, d, e, f**) for vimentin to reveal human fibroblasts (**c**) or for collagen type I to highlight connective tissue and collagen synthesis. (**a, b**) Nodules at day 14 formed by injected fibroblasts expressing active TGF-β1 immunostained for human vimentin show the variation in density of human cells in the nodule as well as the presence of surrounding cells of the host. (**c**) Immunostaining of a serial section shows that the nodule contains collagen type I. Immunostaining for vimentin of injection sites at day 28 of (**d**) control fibroblasts, (**e**) fibroblasts expressing latent TGF-β1, (**f**) and fibroblasts expressing active TGF-β1 show that human cells are present for all groups at day 28. Control fibroblasts and fibroblasts expressing latent TGF-β1 are integrated into the structures of the lower dermis and panniculus carnosus, whereas fibroblasts expressing active TGF-β1 appear to be surrounding the remains of a nodule (inside circle) (Bars: **a, d, e** and **f** = 200 μm; **b** and **c** = 100 μm).

To examine their organization, the sites of injection were evaluated by histology (hematoxilin & eosin) (Figure 5). At days 3 and 7, the injection sites for modified cells as well as control cells could be identified even though the control cells did not form a visible nodule. At these early time points, the injection sites of all groups had areas of high cell density that were approximately the same size, except for cells expressing active TGF-β1 that were bigger, and with little tissue organization. Control cells and cells expressing latent TGF-β1 tended to be more spread out from the injection site, whereas cells expressing active TGF-β1 were collected in a more sphere-like formation. Compared to control cells, blood vessels seemed more abundant for cells expressing active TGF-β1, followed by cells expressing latent TGF-β1. From day 10 onward, it was difficult to conclusively identify the control cells and those expressing latent TGF-β1, whereas cells expressing active TGF-β1 formed distinct nodules, most obvious at day 14. At this point, cell density of the nodules was lower than sites at early times after injection and cell density was not homogenous. Nodules had the highest density of cells at the periphery and a decrease in cell density toward the center. The entire nodule also appeared to be encapsulated by mouse connective tissue. Blood vessels were located predominately around the periphery of the nodule. Nodules formed by cells expressing active TGF-β1 persisted for as long as day 28 but decreased significantly in size.

To definitively identify which cells were human fibroblasts, cryosections were immunostained with a monoclonal antibody that recognizes human vimentin and does not crossreact with murine vimentin (Figure 6). Immunostaining confirmed, that at all time points for all groups where a nodule was macroscopically visible, it contained human fibroblasts. Immunostaining also revealed human fibroblasts, even if no nodule was visible. Control cells and cells expressing latent TGF-β1 were detected 28 days after injection. These cells were fewer in number and were more integrated into the mouse tissue than cells expressing active TGF-β1. Active TGF-β1-expressing cells were generally confined to a nodule. Immunostaining also revealed that the cells in the center and the periphery of the nodules were human cells and clearly showed the boundary between human cells and the mouse cells encapsulating the nodule. When immunostained for collagen type 1, it was clear the entire nodule contained abundant collagen, and the injected human cells were producing collagen.

DISCUSSION

Keloids and hypertrophic scars are manifestations of an abnormal process of tissue repair after trauma to the skin. Options for treatment are limited by our lack of understanding of the molecular and cellular mechanisms governing the formation of these scars. Moreover, as these lesions are unique to humans, animal models for the development of new therapeutic interventions are severely limited. Current models, based on the implantation of human scar tissue into athymic animals are cumbersome, limited by the availability and variability of human tissue and are not widely used, nor have they been used to pinpoint the molecular and cellular events underlying these scars (Estrem *et al.*, 1987; Kischer *et al.*, 1989; Waki *et al.*, 1991; Hillmer and MacLeod, 2002).

TGF-β1 induces collagen synthesis and has been shown to be upregulated in scars and so a leading hypothesis is that chronic overexpression of TGF-β1 or one of its fellow family members β2 and β3 drives the formation of keloids and hypertrophic scars. Yet, each TGF-β is secreted as a latent molecule that requires activation and no study has established a cause and effect relationship between TGF-β overexpression and the formation of keloids or hypertrophic scars. To investigate the role of TGF-β1 and its activation in fibrosis of the skin, we genetically modified diploid human skin fibroblasts to overexpress either wild-type TGF-β1, which is secreted in a latent form, or mutant TGF-β1, which is secreted in a constitutively active form. The mutations at positions 223 and 225 (cysteines to serines) are located in the latency-associated peptide portion of the gene and do not alter the mature TGF-β1 polypeptide sequence; however, they do disrupt latency-associated peptide folding and prevent the formation of the latent complex with TGF-β1 (Brunner *et al.*, 1989). Modified fibroblasts expressing the wild-type gene secreted 18-fold more TGF-β1 than control cells, but it was in the latent form and levels of active TGF-β1 released by these cells were comparable to controls. In contrast, modified fibroblasts expressing the mutant gene secreted 21-fold more TGF-β1 than control cells and it was in the active form. These data show that human fibroblasts are able to synthesize high levels of wild-type TGF-β1, but they are unable to activate it. Moreover, these data show that levels of secreted TGF-β1 (latent or active) are not limited by the levels of latent TGF-β-binding protein gene expression (Myazono *et al.*, 1991; Saharinen *et al.*, 1996). Others have used a gene transfer approach to overexpress TGF-β1 in the skin via transgenic animals or direct gene transfer (Sellheyer *et al.*, 1993; Benn *et al.*, 1996).

Fibroblasts expressing active TGF-β1 proliferated slower than either control cells or fibroblasts overexpressing latent TGF-β1. Prior studies have shown that TGF-β1's action on fibroblast proliferation is complex. Proliferation of mink lung fibroblasts is stimulated by low concentrations of TGF-β1 (5–10 ng), and inhibited by higher concentrations (Ghahary *et al.*, 2002), whereas cell proliferation of post confluent cultures of human dermal fibroblasts is stimulated by TGF-β1 (Clark *et al.*, 1997). In most proliferation studies, cells are exposed to a bolus of active TGF-β1, whereas in our study, cells are continually exposed to TGF-β1 (latent or active) during the entire time they are cultured. In fact, when cells were grown beyond confluence, only those cells expressing active TGF-β1 formed distinct cell clusters or aggregates. Based on observations from a prior study where fibroblasts treated with TGF-β formed stratified cell layers interposed by abundant extracellular matrix, our cell clusters could be sites of excessive extracellular matrix deposition that stimulated the local contraction of cells into aggregates (Clark *et al.*, 1997). This may be a dose-dependent phenomenon and so it would be interesting to select for gene-modified cells expressing different levels of TGF-β and determine its influence on cell proliferation.

TGF-β1 is thought to induce the differentiation of fibroblasts into myofibroblasts that reorganize extracellular matrix and exert tensile forces on the provisional matrix of healing wounds (Rudolph *et al.*, 1992; Tomasek *et al.*, 2002). Compared to control cells and cells expressing latent TGF-β1, fibroblasts expressing active TGF-β1 had a 2–3-fold increase in the expression of SMC α-actin, a marker of myofibroblasts that is upregulated by TGF-β1 (Desmoulière *et al.*, 1993; Serini and Gabbiani, 1996; Tomasek *et al.*, 2002). Moreover, contraction of collagen lattices was greatest for cells expressing active TGF-β1 versus control cells. Interestingly, contraction was also enhanced, but not as much, by cells expressing latent TGF-β1, even though they did not express higher levels of SMC α-actin. This is a consistent observation and may be a limitation of the collagen gel contraction assay possibly due to local effects (eg, pH) or the presence of other factors in the serum or collagen preparation that may activate TGF-β1.

When injected intradermally into athymic mice, the tissue organization of fibroblasts expressing active TGF-β1 was distinctly different than control cells or cells expressing latent TGF-β1. These cells formed macroscopically evident nodules that were well vascularized, appeared to contract with time and persisted longer than controls or cells expressing latent TGF-β1. At early times, cell density of control and both types of modified cells was high and the cells had little if any organization. Over time, cell density decreased for all groups and cells expressing active TGF-β1 formed distinct nodules encapsulated by mouse connective tissue. Within a nodule, the density of human fibroblasts was greatest around the periphery with few cells in the center that contained abundant collagen. These features show similarities to the histology of keloids and hypertrophic scars, although these lesions are often confused and there is significant overlap in their histology. Keloids are characterized by the presence of whorls of thick, hyalinized collagen bundles or keloidal collagen with mucinous ground substance and relatively few fibroblasts; hypertrophic scars are characterized by nodular structures with fine randomly organized collagen fibers (Ehrlich *et al.*, 1994; Lee *et al.*, 2004). However, unlike keloids that grow and hypertrophic scars that persist long term, nodules formed by fibroblasts expressing active TGF-β1 decreased in size over time.

These data demonstrate that upregulation of TGF-β1 by fibroblasts may be necessary but it is not sufficient for the formation of keloids or hypertrophic scars. This conclusion is supported by the observation that only those fibroblasts expressing active TGF-β1, but not fibroblasts overexpressing the wild-type latent TGF-β1 gene, formed keloid-like nodules. Thus, simple upregulation of the TGF-β1 gene, as seen in keloids or hypertrophic scars (Ghahary *et al.*, 1993; Younai *et al.*, 1994; Tredget *et al.*, 1998; Lee *et al.*, 1999; Wang *et al.*, 2000; Hanasono *et al.*, 2003), is not enough. Present in the scar environment must also be a signal that activates latent TGF-β1. Although latent TGF-β1 can be activated by heat, extremes of pH, chaotropic agents and detergents, the physiological relevance of these is unknown. More relevant to scars is the fact that latent TGF-β1 is activated by glycosidases, proteases such as plasmin and cathepsin D and interaction with the

adhesive protein thrombospondin-1 (Gleizes *et al.*, 1997; Khalil, 1999).

Even if TGF-β1 is activated, as was the case for our fibroblasts expressing mutant active TGF-β1, the keloid-like structures that these cells formed, decreased in size with time. Parallel to this decrease in nodule size was a significant loss of cells and a decrease in cell density at the injection site. These data suggest that another signal must also be present that either serves to maintain scar size, as is the case for hypertrophic scars, or to expand scar size, as is the case for keloids. We hypothesize that formation of keloids and hypertrophic scars is a multistep process. Signals that serve to maintain scar size might include apoptosis inhibition (Desmoulière *et al.*, 1995; Sayah *et al.*, 1999; Teofoli *et al.*, 1999; Luo *et al.*, 2001; Nakazono-Kusaba *et al.*, 2004). Signals that might stimulate the growth and expansion of a scar might include platelet-derived growth factor (PDGF), a factor well known for its ability to stimulate fibroblast proliferation (Deul, 1987; Messadi *et al.*, 1998), or other factors like basic fibroblast growth factor and connective tissue growth factor (Frazier *et al.*, 1996; Takehara, 2000). Moreover, several reports using a surgical model of scar implantation into athymic rats have suggested that TGF-β2 and not TGF-β1 has a causative role in the formation of proliferative scars (Polo *et al.*, 1999; Smith *et al.*, 1999; Wang *et al.*, 1999 and others have reported that a dysregulated response to glucocorticoids may contribute to the pathology (Russell *et al.*, 1995).

Recombinant retroviruses can be used for the stepwise genetic modification of human fibroblasts to express multiple factors and the tissue organization of these cells can be evaluated by intradermal injection as was done in this paper. In this new approach to an animal model for studying the steps to excessive scarring, we may be able to define tissue structures that are intermediates to either a keloid or a hypertrophic scar caused by the dysregulation of specific growth factors and define the role of specific genes and signaling pathways. The increased understanding of the molecular and cellular events leading to the formation of keloids and hypertrophic scars gained by this approach as well as other approaches such as the biochemical characterization of fibroblasts from keloids/hypertrophic scars, the implantation of human proliferative scars, the sustained *in vivo* delivery of factors that promote fibrosis and the use of transgenic animals may lead to new therapeutic options.

MATERIALS AND METHODS

Culture of human cells

Human dermal fibroblasts were derived from neonatal foreskins, obtained at the Women & Infants Hospital of Rhode Island, Providence, RI (approved by the Institutional Review Board and in adherence to the Declaration of Helsinki Principles). Foreskins were trimmed with scissors to remove excess fatty tissue, rinsed repeatedly with sterile phosphate buffer solution (PBS), and diced into small fragments. The fragments were allowed to adhere to the bottom of a tissue culture plate in a humidified 10% CO_2 atmosphere, at 37°C for 1 hour, and were covered with DMEM (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT) containing 100 U of penicillin and 100 μg of streptomycin (Gibco) per ml. Over a period of 14 days, fibroblasts migrated from the tissue fragments and formed a confluent layer on the culture plate. Fibroblasts were harvested with a 0.05% trypsin/EDTA (Gibco) solution and subcultured in DMEM containing 10% fetal bovine serum and penicillin/streptomycin.

Retroviral constructs and genetic modification

The retroviral vector, MFG-TGF-β1 was assembled in a two-step process to remove all 5′ and 3′ noncoding sequences of the wild-type human TGF-β1 cDNA. A fragment from the 5′ end of TGF-β1 (*Bgl*I to *Bam*HI) was isolated, combined with a double-stranded oligonucleotide (5′-CATGCCGCCCTCC-3′ and 5′-GGGCGG-3′) and ligated as a three-part ligation into purified MFG vector that had been previously cut with *Nco*I and *Bam*HI. This intermediate clone, containing the TGF-β1 5′ end, was cut with *Bam*HI, treated with calf intestinal phosphatase and gel purified. Into this *Bam*HI site was cloned a PCR product of the remaining 3′ end of the TGF-β1 cDNA. This PCR product, prepared using a forward primer (5′-GAAGTGG ATCCACGAGCCCAAGGGCT-3′) and a reverse primer (5′-CTG CTGGATCCAGATCTTCAGCTGCACTTGCAGGAGCG-3′) was digested with *Bam*HI and gel purified. A similar strategy was used to construct an MFG retrovirus encoding a mutant form of TGF-β1 that is secreted as an active molecule and that does not require activation (Brunner *et al.*, 1989). In this case, cysteines at positions 223 and 225 were converted to serines. The sequences of the final vectors encoding either wild-type (latent) TGF-β1 or mutant (active) TGF-β1 were verified by DNA sequencing. Vectors were transfected into a packaging cell line to generate a stable cell line producing recombinant retrovirus.

The passage 4 fibroblasts were genetically modified as previously described (Morgan *et al.*, 1995, Le Doux *et al.*, 2001). Briefly, virus-producing cells were grown to confluence in a 10 cm dish, the medium changed (10 ml/plate) and after 24 hours, the medium was removed, filtered using a 0.45 μm filter, and stored at −80°C. For transduction, 1.15×10^5 fibroblasts were plated in a 35 mm dish and incubated overnight. Stocks of recombinant retrovirus were thawed and brought to 80 μg/ml of chondroitin sulfate C from shark cartilage (Sigma, St Louis, MO) using a 100× stock. After incubation for 10 minutes at 37°C, the virus stock was then brought to 80 μg/ml of polybrene (hexadimethrine bromide, 1,5-dimethyl-1,5-diazaundecamenthylene polymethobromide) (Sigma) using a 100× stock and incubated for an additional 10 minutes at 37°C. The solution was centrifuged at 10,000 r.p.m. in a 1.5 ml microcentrifuge tube for 5 minutes at room temperature. The visible pellet was resuspended in cell culture medium to one-tenth of its original volume. The medium of the human fibroblasts was aspirated and the virus solution was added and incubated for 24 hours at 37°C. The medium was removed and the cells washed once with fresh culture medium, and then replaced with new medium. After another 24 hours, the cells were trypsinized and transferred to 10 cm dishes and grown to confluence.

Measurement of growth factors levels by ELISA

Control and genetically modified fibroblasts were grown to confluence in 10 cm dishes and incubated 48 hours in 1% serum medium. The medium was changed, aliquots harvested at different time points, and levels of TGF-β1 measured by ELISA (R&D Systems,

Minneapolis, MN). The antibodies used in the ELISA kit are only able to detect TGF-β1 in its active form, thus samples are routinely activated by acidification (HCl) before ELISA. To measure levels of latent and active TGF-β1, samples were activated according to the manufacturer's instructions. To measure levels of TGF-β1 that were secreted in the active form, the activation step was omitted. After harvesting the last time point, cells were treated with trypsin, counted and the values of TGF-β1 were normalized by the volume of medium in the dish at each time point divided by the final cell number.

Measurement of cell proliferation

Control and modified cells (3×10^3 cell/cm^2) were plated in six-well plates (triplicate) in medium containing 10% serum. Cells were trypsinized and counted in a hematocytometer daily, until reaching confluence. Cell viability and proliferation were also assessed with the WST-1 reagent (Roche Diagnostics Corp, Indianapolis, IN) according to the manufacturer's recommendations. Cells of each cell type were plated in a 96-well dish (1×10^3 per well) in triplicate with 100 μl of medium and incubated for 24 hours. Daily up to 7 days, 10 μl of the Wst-1 reagent was added to each well, incubated for 2 hours at 37°C and read with an ELISA plate reader at 450 nm. Cells were followed in culture for up to 3 weeks, and phase contrast microscopy (Olympus IX70 microscope with a Axio Cam MRc camera and MRGrab 1.0 Software) (Carl Zeiss Vision GmbH, Germany) was used to document changes in cell morphology over time.

Western blot analysis

Control and modified fibroblasts were grown to subconfluence in 60 mm dishes and incubated 48 hours in medium containing 1% serum. The medium was changed and after 48 hours, cells were lysed with a buffer containing 1% SDS (Sigma). Protein concentration was determined by bicinchoninic acid (BCA) assay (Sigma). Cell extracts were mixed with an equal volume of SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 2% bromophenol blue, and 5% β-mercaptoethanol) and boiled at 100°C for 5 minutes. Equal amounts of total protein (5–10 μg) were loaded onto 7% SDS-minigels and separated by PAGE. Separated proteins were transferred by electrophoresis to a Hybond ECL nitrocellulose membrane (Amersham Biosciences Corp, Piscataway, NJ) overnight at 4°C, in a buffer containing 25 mM Tris, 192 mM glycine, 20% methanol (vol/vol). Membranes were treated with blocking buffer (5% nonfat dry milk, 0.05% Tween 20 in PBS) at room temperature for 2 hours. Following a brief rinse, membranes were probed with a mouse monoclonal antibody to SMC α-actin (1:4,000 dilution in blocking buffer, clone 1A4, Sigma) for 2 hours at room temperature. This was followed by 3 washes of 5 minutes each in PBST (0.05% Tween 20 in PBS). Subsequently, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000 in blocking buffer; Chemicon, Temecula, CA) at room temperature for 1 hour. Following three washes in PBST, the immunoblots were then incubated with Supersignal West Pico chemiluminescence substrate (Pierce, Rockford, IL) for 5 minutes and exposed to X-OMAT Kodak film. The membranes probed for SMA α-actin were stripped with 2% SDS, 100 mM mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8 for 30 minutes at 70°C, and reprobed with a mouse monoclonal antibody to β-actin (1:5,000 in blocking buffer, clone AC-15, Sigma), followed by the same procedure. For quantitation, the films corresponding to each Western blot were photographed using the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290), and analyzed with the Image J Software (National Institute of Health) obtaining the sum of the pixel values of band areas. Densitometric analysis results were presented as fold increase.

Measurement of collagen lattice contraction by fibroblasts

Before preparing the gels, 5 ml of BSA (Sigma) solution (0.1% BSA in PBS, filter sterilized) was pipetted into each well of a six-well plate, incubated at 37°C for 1 hour. 1.4 ml of bovine dermal collagen Vitrogen (Cohesion, Palo Alto, CA) was mixed with 0.4 ml of 5 × DMEM in a 15 ml centrifuge tube cooled on ice. The pH was adjusted to a range of 7.2–7.5, using a sterile solution of 1 N NaOH. The control and modified cells were trypsinized from a confluent tissue culture plate and 0.2 ml of the cell suspension (containing 5×10^5 cells) was added to the collagen solution, gently mixed and poured into a well of the six-well plate. Collagen lattices were allowed to gel for 60 minutes in a 5% CO_2 atmosphere at 37°C. After 60 minutes, the collagen lattices were detached from the surface of the well by rimming the lattice with a sterile spatula and gently swirling the six-well plate. Four milliliters of serum-free medium was added to each well. Plates were incubated in a 10% CO_2 atmosphere at 37°C, and the medium changed every 2 days. To measure contraction, the tissue culture dishes were periodically placed on top of a transparent metric ruler on an opaque background, and the diameters of the lattices were recorded as the average values of the major and minor axes (Pins *et al.*, 2000). Experiments were repeated 6 times and the values presented with the standard deviation.

Cell implantation into athymic mice

NCr athymic outbread mice, 5–6-week-old males, were purchased from Taconic (Germantown, NY), and acclimated for 1 week before undergoing any procedure. All procedures were approved by the Brown University Animal Care Committee and carried out in accordance with the National Institute of Health *Guide For The Care And Use Of Laboratory Animals.*

Confluent fibroblasts (modified and unmodified) contained in a triple flask (500 cm^2) were trypsinized, counted and pelleted by centrifugation. The pellet was resuspended in DMEM and placed in a 28-gauge 1 ml disposable insulin syringe (Becton-Dickinson, Frankin Lakes, NJ). Intradermal injection of cells into the dorsal skin (0.1 ml of DMEM with 1×10^6 cells) was administered immediately following cell preparation. Mice were anesthetized using inhalatory isofluorane (Baxter, Deerfield, IL). Each mouse was injected with duplicates of control (unmodified cells), cells expressing latent TGF-β1, cells expressing active TGF-β1, and vehicle alone (DMEM). The skin was marked with a permanent marker to indicate the injection sites, which were separated by 1 cm of nontreated skin. A Tegaderm™ (3M, St Paul, MN) dressing was applied after injections to prevent the animal from disturbing the sites. Animals were killed by CO_2 asphyxiation at days 3, 7, 10, 14, 21, and 28. A total of four batches of animals were used, with two animals for each time point. All animals survived throughout the experiments.

One injection site was fixed in 10% formalin and embedded in paraffin for routine histology. The other injection site was immediately embedded in Tissue-tek OCT compound (Sakura

Finetek Inc., Torrence, CA) and snap frozen for immunostaining. There was no tissue response when vehicle without cells was injected.

Histology and immunostaining

Paraffin-embedded samples were sectioned (5 μm) and stained with hematoxylin and eosin. For immunostaining, frozen sections (8 μm) were fixed in cold acetone (−20°C) for 2 minutes, rinsed in PBS and incubated in a solution of four parts methanol to one part of 6% H_2O_2 (in PBS) for 20 minutes to block endogenous peroxidase activity. Nonspecific binding sites were blocked for 1 hour in 10% goat serum (Sigma) and 5% BSA in PBS. Sections were incubated with a mouse monoclonal anti-vimentin, clone V9 (DakoCytomation, Carpinteria, CA), diluted 1:50 in the blocking solution for 1 hour at room temperature in a humidified container. Sections were washed 3 times in PBS, incubated 30 minutes in blocking buffer and 30 minutes with a biotinylated HRP-conjugated secondary antibody goat anti-mouse (Chemicon) diluted 1:2,500 in blocking buffer. Sections were developed with 3, 3′, 5, 5′-tetramethylbenzidine for 10 minutes (Vector, Burlingame, CA). For collagen staining, serial sections were incubated with a mouse monoclonal anticollagen type I (Chemicon) diluted 1:20 in blocking buffer overnight at 4°C, incubated with the secondary antibody as above (1:,1000 in blocking buffer) for 4 hours, and developed with 3, 3′-diaminobenzidine (Vector). Negative controls were prepared using the same tissue and were stained with primary antibody alone, secondary antibody alone and substrate alone. Pictures were taken using an Olympus IX70 microscope with an Axio Cam MRc camera and MRGrab 1.0 Software (Carl Zeiss Vision GmbH, Germany).

Statistics

Statistical comparisons between sample groups were made using an analysis of variance with $P \leqslant 0.05$ indicating a significant difference between the groups.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported, in part, by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Education Ministry, Brazil) and the Shriners Hospital for Children. We thank Joon Paek and Xudong Cao for advice in the conduct of this study.

SUPPLEMENTARY MATERIAL

Figure S1. Complex pathway for synthesis, secretion, and activation of TGF-β1.

Figure S2. Implanted fibroblasts expressing active TGF-β1 form nodules that persist.

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