American Journal of Pathology, Vol. 154, No. 1, January 1999

Technical Advance

Active Transforming Growth Factor-β in Wound Repair

Determination Using a New Assay

Liju Yang, Cindy X. Qiu, Anna Ludlow, Mark W. J. Ferguson, and Georg Brunner

From the Division of Cells, Immunology and Development, School of Biological Sciences, University of Manchester, Manchester, United Kingdom

Transforming growth factor (TGF)-β regulates wound repair and scarring in an isoform-specific fashion. TGF-β is produced in a latent form, and its activation is a critical regulatory step controlling the bioactivity of this growth factor. To date, it has been impossible to determine latent TGF-β activation *in vivo* due to a lack of quantitative assays. We describe here a semiquantitative modification of the plasminogen activator inhibitor-1/luciferase bioassay (PAI/L assay) for TGF-β, which we used to determine active and latent TGF-β isoforms in frozen sections of rat wound tissue. We found that significant amounts of latent TGF-β were rapidly activated upon wounding (38% of the total TGF-β at 1 hour after wounding). A second peak of active TGF-β (17% of total) occurred at 5 days after wounding. The predominant isoforms were TGF-β1 and -2 with only minor amounts of TGF-β3 present. This is the first TGF-β bioassay allowing semiquantitative determination of active and latent isoforms present *in vivo*, and our results document the significance and temporal regulation of latent TGF-β isoform activation in wound repair. *(Am J Pathol 1999, 154:105–111)*

The transforming growth factor (TGF)-β family of growth factors are potent regulators of cell growth and differentiation (reviewed in Refs. 1–3). TGF-βs are expressed throughout embryogenesis[4] and are detected at high levels in areas undergoing rapid morphogenesis (reviewed in Ref. 2), suggesting an important role in development, tissue repair, and regeneration. TGF-βs exist in a number of structurally related but functionally distinct 25-kd homodimeric isoforms. In mammals, three isoforms, TGF-β1, -2, and -3, have been identified. Each isoform is synthesized as a large latent precursor that is unable to trigger signaling via high-affinity TGF-β receptors.[5,6] Therefore, activation of latent TGF-β isoforms appears to be a critical control point in regulating their biological activity.

In vitro, latent TGF-βs can be activated by a variety of treatments, such as incubation at extreme pH, high temperature, or with proteases (reviewed in Refs. 7 and 8) or by interaction with thrombospondin.[9] Evidence from *in vitro* models suggests that limited proteolysis by plasmin may be involved in the physiological activation of latent TGF-β.[10–14] However, the mechanisms of latent TGF-β activation *in vivo* are poorly understood.

A large body of evidence suggests that TGF-β has profound effects on wound repair (reviewed in Refs. 15 and 16). Wound healing is a highly regulated process involving inflammation, cell proliferation and migration, angiogenesis, and extracellular matrix (ECM) production (reviewed in Ref. 17). TGF-βs regulate most of these events, including the chemotaxis of inflammatory cells,[18] angiogenesis,[19] ECM deposition, and granulation tissue formation,[19,20] thereby promoting healing and contributing to scar formation.[21,22] TGF-β1 has also been implicated in abnormal wound healing such as hypertrophic scar and keloid formation[23,24] as well as in fibrotic diseases such as glomerulonephritis, liver cirrhosis, and pulmonary cirrhosis.[25–27] The TGF-β isoforms have distinct biological activities in regulating wound repair. Thus, manipulation of the ratio of TGF-β isoforms in adult rat wounds, particularly reducing the level of TGF-β1 and TGF-β2 relative to TGF-β3, reduces scarring.[28–30] By contrast, exogenous addition of TGF-β1 to fetal rabbit or

L. Yang was supported by an ORS award and a Frederick Craven Moore Research Studentship of the University of Manchester at the time of this work. This work was also supported by Johnson and Johnson Medical, Ltd.

Accepted for publication September 24, 1998.

Address reprint requests to Dr. Georg Brunner, School of Biological Sciences, University of Manchester, 3.239 Stopford Bldg., Oxford Road, Manchester M13 9PT, UK. E-mail: georg.brunner@man.ac.uk.

mouse wounds, which normally heal without a scar, induces scar formation.[31,32]

As the activation of the latent form of TGF-β is a critical step in the regulation of the bioactivity of this growth factor, modulation of latent TGF-β activation *in vivo* might be a promising approach to modulate TGF-β action, eg, in wound repair and scarring. However, although potent effects of TGF-β isoforms have been clearly demonstrated, it has so far not been possible, due to the lack of a quantitative assay for active TGF-β, to monitor latent TGF-β activation *in vivo*. Here, we report, for the first time, a semiquantitative assay for the measurement of active TGF-β isoforms present *in vivo* in frozen tissue sections. The assay is based on the ability of TGF-β to induce plasminogen activator inhibitor-1 (PAI-1) expression and is a modification of a bioassay using a truncated PAI-1 promoter/luciferase construct (PAI/L assay).[33] Using this assay, we demonstrate that significant amounts of all three latent TGF-β isoforms are activated shortly after wounding as well as at later stages of wound repair.

Materials and Methods

Reagents

Recombinant human TGF-β1 and neutralizing chicken or goat antibodies to TGF-β1 (AB-101-NA), -2 (AB-112-NA), or -3 (AB-224-NA) were purchased from R&D Systems (Abingdon, Oxon, UK). Irrelevant chicken IgY, irrelevant goat IgG, aprotinin, geneticin (G418), and methyl cellulose were supplied from Sigma (Dorset, UK). Pyrogen-poor bovine serum albumin (BSA) was from Pierce (Rockford, IL). Luciferin and cell lysis buffer were purchased from Analytical Luminescence Laboratory (Ann Arbor, MI), and Microlite 96-well plates were supplied by Dynatech Laboratories (Chantilly, VA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), L-glutamine, penicillin, and streptomycin were purchased from Gibco BRL (Paisley, UK).

Dermal Wound-Healing Model

Adult male Sprague-Dawley rats (Charles River UK, Kent, UK) weighing 225 to 250 g were anesthetized by inhalation of halothane, nitrous oxide, and oxygen. The backs of the rats were shaved, and four full-thickness linear incisions of 1 cm length, including the panniculus carnosus, were made on the back of each animal using a scalpel as described by Shah et al.[29] The wounds were left unsutured to heal by secondary intention. The animals were allowed to recover, housed separately, and fed *ad libitum* until the wounds were harvested. The animals were killed by chloroform overdose at 1 hour, 6 hours, or 1, 2, 3, 4, 5, 7, 10, or 14 days after wounding. The wounds were harvested and processed as described below. All animal experiments were carried out under appropriate licenses according to UK Home Office regulations.

Preparation of Cryosections

The wound area was swabbed with 70% ethanol. The wounds were carefully dissected from surrounding tissue and subsequently embedded in 8% sterile methyl cellulose and snap-frozen in liquid nitrogen. Four wounds from each animal were embedded in one block of methyl cellulose. Four tissue samples of equivalent size representing surrounding tissue adjacent to the wound were also excised and processed as described above. Normal skin from the back of nonwounded rats was used as a control. The embedded tissue samples were stored at −80°C for a maximum of 3 months. Thick cryosections (20 μm) were cut and placed on sterilized 13-mm round coverslips and analyzed in duplicate. The coverslips, each carrying four cryosections of the wound, were placed into a 24-well cell culture plate and temporarily stored at −20°C until transferred onto mink lung epithelial cells (MLECs).

To measure the volume of the tissue sections, serial cryosections from the same specimen were stained with hematoxylin and eosin (H&E). Color images of H&E-stained wound sections were captured using a color video camera (3-CCD JVC KY-F55) connected to an Olympus Vanox AHBS3 microscope. The image-capturing process employed a Spynt Capture Board (Synoptics, Cambridge, UK) in a Dan 486 66-Mhz computer running PC Image (Foster Findlay, Newcastle, UK) in Windows 3.11. The area of the wound was measured using the stylus of a digitizing tablet (Summgraphics, summesketch III). The volume of the wound was calculated from the area of the wound and the thickness of the section. The average volume of four wound sections was 0.1 to 0.2 mm^3.

Cell Culture

MLECs transfected with the PAI/L construct were a generous gift from Dr. Daniel B. Rifkin (New York University Medical Center, New York, NY). MLECs were cultured in DMEM supplemented with 5% FCS, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and geneticin (250 μg/ml). Cells were used between passage 8 and 20.

Determination of Active/Latent TGF-β in the PAI/L Assay

For the PAI/L assay, a truncated TGF-β-inducible PAI-1 promoter was fused to a firefly luciferase reporter gene and subsequently transfected into MLECs generating a highly sensitive TGF-β-responsive cell line.[33] The PAI/L assay is specific for active TGF-β1, -2, and -3, and the detection limit is approximately 5 to 10 pg/ml. This assay has been used previously for the quantification of active TGF-β in conditioned medium, blood samples, or other liquid samples.[33–35]

The original PAI/L assay for the quantification of TGF-β in liquid samples was modified to measure active/latent TGF-β levels present at the wound site *in vivo*. MLECs

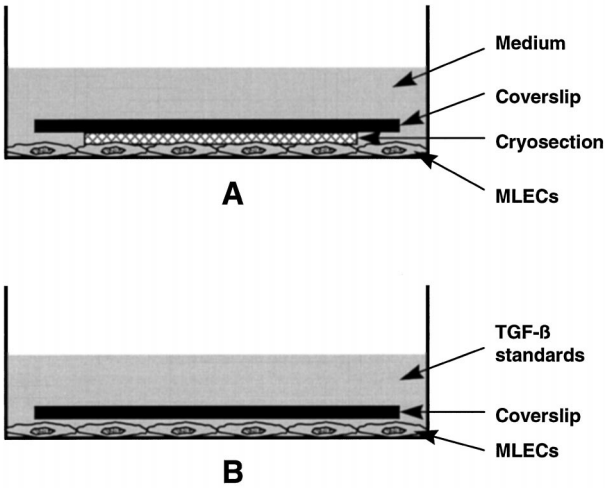

Figure 1. PAI/L assay of cryosections. MLECs were plated for 4 hours into 24-well plates (3×10^5/ml, 500 μl/well) and incubated overnight with coverslips carrying four cryosections each. The coverslips were placed upside down onto the cells (**A**). To generate a TGF-β standard curve, MLECs were incubated with serial dilutions of recombinant human TGF-β1 and coverslips coated with methyl cellulose (**B**). Methyl cellulose used for tissue embedding did not significantly affect the PAI/L assay.

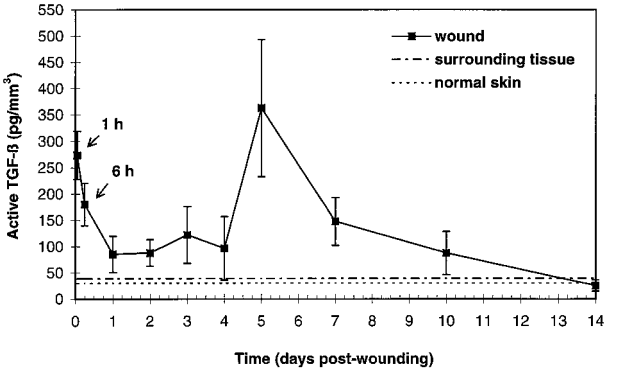

Figure 2. Time course of active TGF-β generation in incisional wound repair. Wound tissue, surrounding tissue, and normal skin were embedded in methyl cellulose. Four cryosections (20 μm) representing four wounds of one animal were prepared on duplicate coverslips and incubated overnight with MLECs. Twenty animals were used for this time course (two for each time point). MLECs were lysed and luciferase activity was determined and converted to TGF-β concentration (pg/mm³) using a recombinant human TGF-β1 standard curve. The data represent mean values ± SEM of eight determinations in duplicate.

were plated into 24-well cell culture dishes (3×10^5/ml, 500 μl/well) in complete DMEM and incubated for 4 hours at 37°C. Then, serum-containing medium was replaced with 500 μl of DMEM containing 0.1% pyrogen-poor BSA, aprotinin (1 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) (DMEM/BSA medium). The coverslips carrying cryosections were placed upside down onto the MLECs in the 24-well plates (Figure 1A). To generate standard curves for TGF-β activity, serial dilutions of recombinant human TGF-β1 (0 to 250 pg/ml) in DMEM/BSA medium were added to the MLECs together with methylcellulose-coated coverslips (Figure 1B). Samples and TGF-β standards were incubated with the MLECs overnight at 37°C. MLECs were then washed with PBS and lysed with 200 μl of luciferase cell lysis buffer for 20 minutes at room temperature. Forty-five microliters of the lysates were transferred in triplicate to a Microlite 96-well plate. Luciferase activity in the lysates was determined using an MLX Microtiter Plate Luminometer (Dynex Technologies, Chantilly, VA).

The ratios of active TGF-β isoforms in the wound tissue samples were determined by addition of isoform-specific neutralizing antibodies to TGF-β1, -2, or -3 to the PAI/L assay. The cryosections were preincubated with anti-TGF-β1, -2, or -3 antibodies (1 μg/ml, 5 μg/ml, and 5 μg/ml) for 1 hour at room temperature and then transferred onto the MLECs together with the antibody solution to measure residual TGF-β activity. Nonimmune chicken IgY and goat IgG were used as controls.

To measure total TGF-β (active plus latent) in the wound tissue samples, the coverslips carrying the cryosections were submerged in 500 μl of DMEM/BSA medium in a sterile glass embryo dish covered with a glass lid (BDH, Merck, UK) and incubated for 20 minutes at 80°C to heat activate latent TGF-β. After heat activation, the coverslips were transferred, together with the medium, onto MLECs for TGF-β quantification in the PAI/L assay. Alternatively, to determine the amount of TGF-β solubilized into the medium during heat activation and remaining bound to the cryosections, respectively, coverslips and medium were analyzed separately.

To determine whether TGF-β present in the cryosections can be eluted into the medium, wound sections were incubated with DMEM/BSA for 10 hours at 37°C. The medium was collected and assayed for active or total TGF-β.

Results

Latent TGF-β Activation during Incisional Wound Repair

To characterize latent TGF-β activation in wound repair, we analyzed tissue samples at various time points after wounding for active TGF-β in the PAI/L assay. Figure 2 shows the levels of active TGF-β in cryosections of normal skin, wound tissue, and surrounding tissue. The average amounts of active TGF-β in normal skin and tissue surrounding the wounds were found to be 30 pg/mm³ and 39 pg/mm³, respectively. As the detection limit of the assay is approximately 25 to 50 pg/mm³, these levels are almost negligible. Immediately after wounding, the tissue level of active TGF-β increased up to ninefold (at 1 hour after wounding). Active TGF-β levels declined within 24 hours but remained significantly higher than those in normal skin or surrounding tissue during the following 3 days after wounding (Figure 2). A second major peak of active TGF-β was detected at 5 days after wounding. By 14 days after wounding, the level of active TGF-β had returned to that of normal skin (Figure 2). Although there was some variation in the absolute levels of active TGF-β between experiments, the kinetics and time points of the two peaks of growth factor activity were highly reproducible.

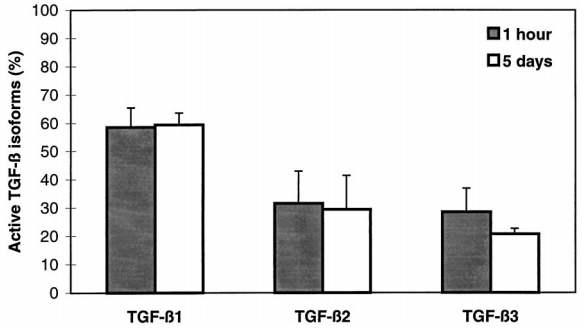

Figure 3. Isoforms of active TGF-β in wound tissue. Cryosections of wound tissue were assayed in the PAI/L assay as described in the legend of Figure 2 except that isoform-specific antibodies to TGF-β1, -2, or -3 were included in the assay. The data represent mean values ± SEM of four separate experiments in duplicate. Four animals were used for each time point.

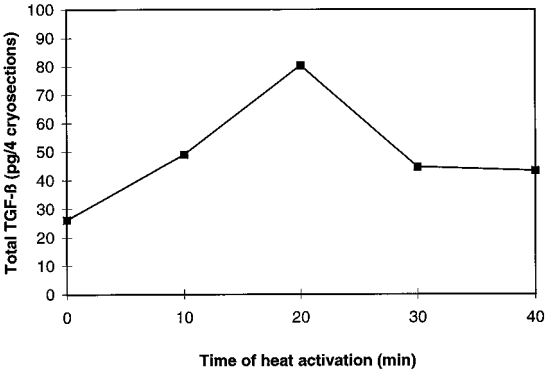

Figure 4. Heat activation of latent TGF-β in cryosections. Coverslips with cryosections of 5-day wounds were placed into 500 μl of DMEM/BSA medium and incubated at 80°C in a dry oven. Total TGF-β was determined using the PAI/L assay. Similar results were obtained in three separate experiments.

Ratios of TGF-β Isoforms in Wound Repair

The ratios of active TGF-β isoforms in the wound area were determined by assaying TGF-β activity in cryosections of wound tissue samples taken at the two peak time points, 1 hour and 5 days after wounding, in the presence of neutralizing antibodies to TGF-β1, -2, or -3. At both time points, TGF-β1 was the predominant isoform, representing approximately 60% of the total active TGF-β. TGF-β2 accounted for 32% and 29% of TGF-β activity at 1 hour and 5 days after wounding, and TGF-β3 for 28% and 21%, respectively (Figure 3). The combined inhibitory effect of all three isoform-specific antibodies exceeded 100%. This is probably due to cross-reactivity of the anti-TGF-β3 antibody used with TGF-β2 (50% cross-reactivity at the concentrations used, data not shown). Thus, the amount of TGF-β3 was significantly overestimated in these experiments. These observations indicate that the active TGF-β present in the wound area is predominantly TGF-β1 and -2.

Total TGF-β in Wound Tissue

Total TGF-β in cryosections taken from wound samples at the time points 1 hour and 5 days after wounding was measured after heat activation.[36] We found that the optimal time period for heat activation of latent TGF-β in cryosections was 20 minutes (Figure 4). Longer time periods caused partial inactivation of TGF-β. Active TGF-β represented 35% of the total TGF-β at 1 hour and 17% at 5 days after wounding, respectively (Figure 5). When cryosections and medium were analyzed separately after heat activation, all of the TGF-β activity was found in the medium and no remaining TGF-β was detected in the heated sections (data not shown). This indicates that all of the TGF-β detectable in the cryosections had been released into the medium during heat activation.

Elution of TGF-β from Cryosections

As TGF-β was readily released into the medium during heat activation, we determined how much of the active/total TGF-β could be eluted into the fluid phase before heat activation. Cryosections taken from 1-hour and 5-day wounds were incubated with DMEM/BSA medium for 10 hours at 37°C, and the eluate was assayed for both active and total TGF-β. As shown in Figure 5, the levels of active TGF-β in the eluate were similar to those in cryosections, indicating that virtually all active TGF-β detectable in the cryosections was released into the medium during incubation. In contrast, total TGF-β levels in the medium were lower than those in cryosections, indicating that a significant portion of total TGF-β remained associated with the cryosections and could, therefore, be detected only after heat activation.

Discussion

TGF-β is a potent regulator of cell function in a variety of physiological and pathological conditions. TGF-β action is controlled via the extracellular activation of latent forms. Analysis of TGF-β action *in vivo* has not been possible to date because of the lack of methods to quantify TGF-β present in tissues. Here, we describe a modification of the PAI/L bioassay to measure active/latent TGF-β isoforms present in tissue cryosections. We used

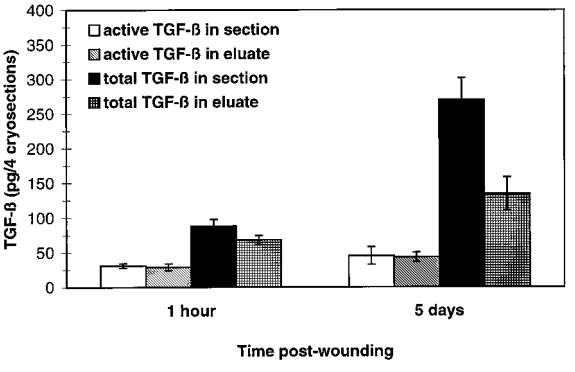

Figure 5. Elution of active/total TGF-β from cryosections. Active and total TGF-β in cryosections were measured as described in the legends to Figures 2 and 4. Alternatively, cryosections were incubated with 500 μl of DMEM/BSA for 10 hours at 37°C, and active/total TGF-β in the eluates was determined using the PAI/L assay. Four animals were used for each time point. The data are expressed as mean values ± SEM of three separate experiments in duplicate.

this assay to analyze TGF-β expression and activation in wound repair.

Although some variation in the absolute values for active TGF-β present in wound tissue was observed between experiments, the time course of latent TGF-β activation during wound repair was highly reproducible. TGF-βs are biologically active at picomolar concentrations,[18,37] and the PAI/L bioassay has a detection limit of approximately 5 to 10 pg/ml.[33] To detect significant TGF-β activity in cryosections of wound tissue, it was necessary to assay four wound sections on one coverslip. As the total volume of four wound sections amounts to 0.1 to 0.2 mm^3, the detection limit for TGF-β in wound tissue using this assay is 25 to 50 pg/mm^3.

Latent TGF-β is produced by a number of cell types involved in wound repair, including platelets, macrophages, fibroblasts, endothelial cells, and keratinocytes.[15,38–41] We found that significant amounts (up to 274 pg/mm^3) of latent TGF-β were activated immediately after wounding. As platelets rapidly accumulate at the wound site,[38] platelet-derived TGF-β1 might be one of the major sources of active TGF-β generated at early time points after wounding. The origin of the TGF-β2 and -3 detected at early stages of wound repair is presently unknown. The rapid decline in TGF-β activity observed during day 1 after wounding may be due to TGF-β uptake by cells, diffusion of platelet-derived TGF-β out of the wound area, and/or degradation by proteases.

During wound healing, several cell types, including macrophages, fibroblasts, keratinocytes, and endothelial cells, migrate into the wound area, and all of them may produce and activate TGF-βs (reviewed in Ref. 15), possibly contributing to the second peak of TGF-β activity occurring during wound repair (at 5 days after wounding). Alternatively and additionally, the second peak of TGF-β activity might be due to the release of pre-existing active or latent TGF-β from pools of soluble or extracellular matrix TGF-β-binding proteins.[42–49] For example, latent TGF-βs can be incorporated into extracellular matrix via latent TGF-β-binding protein-1 (LTBP-1) followed by release and activation catalyzed by proteases such as plasmin and thrombin.[50] In addition, it has been suggested that small latent TGF-β1 complex released from platelets is retained in the fibrin clot through binding to the platelet surface receptor, glycoprotein IIb/IIIa, and that it can be released into the wound area in an active form when the fibrin clot is dissolved by plasmin.[51] These mechanisms of active TGF-β generation may also contribute to the second peak observed at day 5 after wounding.

The ratios of active *versus* total TGF-β observed in dermal wound healing *in vivo* (35% at 1 hour and 17% at 5 days after wounding) are relatively high compared with those reported for latent TGF-β activation in cell culture systems (1% to 8%).[10,14,52] Assuming that the determination of total TGF-β is more efficient than that of active TGF-β, due to the heat activation step probably releasing the majority of TGF-β from the cryosection, the ratios of active *versus* total TGF-β are probably underestimated in our assays. Our data therefore suggest that latent TGF-β activation in wound repair *in vivo* is significantly more efficient than that in cell culture models *in vitro*. This might be explained by suboptimal activation conditions in *in vitro* models.

The pool of active TGF-β measured in wound tissue using the PAI/L assay appears to be in the fluid phase as it can be eluted from cryosections. Although the majority of latent TGF-β was also eluted into the medium, a significant portion of it was retained in the cryosections, suggesting that latent TGF-β may be cross-linked to extracellular matrix proteins, eg, via LTBPs. We found that mature TGF-β could be released from this pool by heat activation. Thus, as the PAI/L assay might detect TGF-β only in the fluid phase, we currently do not know the efficiency for the measurements of active or latent TGF-β in cryosections using this assay. Therefore, we probably underestimated the absolute TGF-β amounts present, and the assay can be considered only semiquantitative. However, this is a common problem of TGF-β measurements, as all cell culture models used to study latent TGF-β activation determine TGF-β only in the fluid phase.[10,12,14,52,53]

As TGF-β1, -2, and -3 have been shown to have distinct functions in wound healing and scarring,[28–30] we determined the ratios of active TGF-β isoforms at early (at 1 hour after wounding) and late stages (at 5 days after wounding) of wound repair. TGF-β1 and -2 were identified in wound tissue at both time points with TGF-β1 being the predominant isoform. Although a significant fraction (28% at 1 hour and 20% at 5 days after wounding) of TGF-β activity was neutralized by anti-TGF-β3 antibodies, a considerable proportion of this inhibition was probably due to cross-reactivity of the antibodies with TGF-β2 (estimated as 16% and 15% at 1 hour and 5 days after wounding, respectively). Thus, the proportion of active TGF-β3 in wound tissue appears to be relatively small (approximately 12% and 5% at 1 hour and 5 days after wounding, respectively). These results are consistent with previous reports indicating that the predominant TGF-β isoforms in adult wound tissue and wound fluid are TGF-β1 and -2.[28,29,54]

As latent TGF-β activation is considered a key point in the regulation of TGF-β action, our recent studies have focused on the modulation of latent TGF-β activation *in vivo* to improve the quality of wound repair and scarring.[55,56] Our findings suggest that inhibition of the activation of latent TGF-β1 and/or -2 results in a reduction in scarring. However, due to the lack of a quantitative assay for active TGF-β present *in vivo*, this hypothesis could not be tested. The assay described here will allow the monitoring of latent TGF-β activation *in vivo* and will be employed to further investigate the mechanisms by which latent TGF-β isoform activation modulates wound repair and scarring.

Apart from wound repair, TGF-βs play important roles in other pathological conditions, such as fibrosis and cancer,[57,58] and active TGF-β present in tissues might be an important prognostic and therapeutic marker in these disease states.[59] Therefore, measurements of active TGF-β isoforms in fibrotic, precancerous, or cancer tissue by the modified PAI/L assay described here may

contribute to the development of improved strategies for prognosis, therapy, and/or postoperative treatment in these pathological conditions.

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