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Abstract

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Mechanical Tension Controls Granulation Tissue Contractile Activity and Myofibroblast Differentiation

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We have examined the role of mechanical tension in myofibroblast differentiation using two *in vivo* rat models. In the first model, granulation tissue was subjected to an increase in mechanical tension by splinting a full-thickness wound with a plastic frame. Myofibroblast features, such as stress fiber formation, expression of ED-A fibronectin and α -smooth muscle actin (α -SMA) appeared earlier in splinted than in unsplinted wounds. Myofibroblast marker expression decreased in control wounds starting at 10 days after wounding as expected, but persisted in splinted wounds. In the second model, granuloma pouches were induced by subcutaneous croton oil injection; pouches were either left intact or released from tension by evacuation of the exudate at 14 days. The expression of myofibroblast markers was reduced after tension release in the following sequence: F-actin (2 days), α -SMA (3 days), and ED-A fibronectin (5 days); cell density was not affected. In both models, isometric contraction of tissue strips was measured after stimulation with smooth muscle agonists. Contractility correlated always with the level of α -SMA expression, being high when granulation tissue had been subjected to tension and low when it had been relaxed. Our results support the assumption that mechanical tension is crucial for myofibroblast modulation and for the maintenance of their contractile activity. (*Am J Pathol* 2001, 159:1009–1020)

Myofibroblasts are present in certain normal tissues, in healing wounds, and in fibrocontractive diseases.^{1–3} Wound contraction takes place when granulation tissue is populated with myofibroblasts⁴ that are characterized by the expression of α -smooth muscle actin (α -SMA),⁵ the actin isoform typical of vascular smooth muscle cells.⁶ Incorporation of α -SMA into microfilament bundles or stress fibers has been suggested to mediate granulation tissue contraction *in vivo*,³ and was shown to promote myofibroblast contraction *in vitro*.^{7–9} Stress fibers have been proposed to function as contractile organelles^{10,11}

and their presence has been correlated with the production of isometric tension.¹² The factors regulating the transition between fibroblastic and myofibroblastic phenotypes are not fully identified. Transforming growth factor- β 1 (TGF- β 1) is accepted to be the major cytokine involved in α -SMA expression and myofibroblast differentiation^{13,14} through the specific binding to TGF- β -receptor type II (TGF- β -RII).^{15,16} This TGF- β effect depends on the presence of the fibronectin (FN) splice variant ED-A FN in the extracellular matrix.¹⁷

Isometric tension has been shown to be important *in vitro* for the development of fibroblast contractile features such as stress fiber formation^{18–20} and α -SMA expression.²¹ Mechanical tension alters FN fiber assembly²² and fibroblast response to growth factors;^{23,24} it also prevents fibroblast apoptosis.¹⁹ An altered mechanical load of the matrix stimulates fibroblasts to adjust their contractile activity to achieve tensional homeostasis.²⁵ Moreover, isometric tension modulates signaling mechanisms regulating fibroblast contraction.²⁶ Most previous studies on the effect of mechanical tension on fibroblastic cells have been restricted to the *in vitro* use of collagen substrates with different mechanical properties.²⁷ In this work, we have used an *in vivo* approach using two different rat models in view of evaluating: the role of mechanical load in myofibroblast modulation (α -SMA expression in particular) and in granulation tissue contractility. In the first model, the contraction of full thickness wound granulation tissue was prevented by splinting with a plastic frame. In the second model, the wall of rat granuloma pouches⁴ was released from mechanical tension by evacuating the pouch exudate. In both models isometric contraction and expression of the myofibroblast markers F-actin, ED-A FN, α -SMA, TGF- β -RII, and TGF- β 1 were evaluated. Our results indicate that mechanical tension is crucial for differentiation and maintenance of the myofibroblast phenotype. In addition, we show that tissue contractility correlates with the expression of α -SMA.

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Materials and Methods

Preparation of Granulation and Granuloma Pouch Tissue

A total of 96 female Wistar rats (200 to 220 g) was used. After shaving the skin, full-thickness 25 × 25-mm wounds, including the cutaneous muscle, were made using surgical scissors in the middle of the dorsum on the first day of the experiments. Wounds were either allowed to heal spontaneously (unsplinted) or were subjected to mechanical tension (splinted) by fixing their edges on a 25 × 25-mm plastic frame using surgical thread (Supramid USP 2-0 DS 19; B. Braun-SSC AG, Emmenbrügge, Switzerland). Rats were sacrificed by CO₂ anesthesia and tissues were dissected daily for 2 to 12 days after wounding. In a second series of experiments that investigated the effect of tension release, wounds were splinted for 7 days, the plastic frame was then removed and animals were sacrificed at 1 and 2 days thereafter. Granulation tissue from 8-day and 9-day unsplinted and splinted wounds, served as controls. Granulation tissues were cleaned from the scab and transferred to ice-cold Krebs-Henseleit solution (118.7 mmol/L NaCl, 4.7 mmol/L KCl, 1.17 mmol/L KH₂PO₄, 2.41 mmol/L MgSO₄, 5.05 mmol/L CaCl₂, 11.1 mmol/L D-glucose, 25.0 mmol/L NaHCO₃), bubbling with 95% O₂ and 5% CO₂.

Granuloma pouches were induced in rats by injecting 15 ml of air into the dorsal subcutaneous tissue, followed by 1 ml of 1% croton oil in maize oil.^{28,29} This treatment produces the formation of an abscess-like structure whose wall is rich in myofibroblasts^{4,29,30} and has been used to study the effects of several smooth muscle contraction-inducing and -inhibiting agents on the isometric force development by a myofibroblastic tissue.^{29,30} Pouches were either left intact or released from internal tension by evacuating ~3 ml of the exudate after 14 days using an 18-gauge needle. Evacuation was repeated every 2 days to prevent new development of tension. To control whether the removal of exudate has other effects than tension release, we replaced it with an equal volume of physiological saline in an additional group. To study the role of TGF- β 1 in granuloma pouch wall development, we injected daily 14-day-old granuloma pouches with 1 ml of soluble TGF- β -R11 (final, 1 μ g/ml muTGF- β -sR; gift of Dr. V. Kotliansky, Biogen Inc., Cambridge, MA)^{31,32} for 7 days in another group. Tension-released granuloma pouches were dissected after 15, 16, 17, 18, and 21 days, intact pouches after 14 days and 21 days. Dissected pouches were transferred to ice-cold Krebs-Henseleit solution, bubbling with 95% O₂ and 5% CO₂, and thoroughly washed. Only tissue from the dorsal portion of granuloma pouches was used to standardize experiments. As control we used normal dermis and dorsal fascia covering the muscular layer. Fascia was exposed after full-thickness incision of skin and subcutaneous tissue and removed using fine forceps and scissors. Dermis was prepared by removing the epidermis and subcutaneous tissue with a scalpel blade. Fascia and normal dermis were further treated as described for granulation tissue and granuloma pouch.

Isometric Force Measurement

Dissected tissues were cut into strips of 5 × 10 mm in transversal orientation to the animal body axis. These were transferred into an organ bath containing 5 ml of Krebs-Henseleit solution bubbling with 95% O₂ and 5% CO₂ at 37°C and were mounted on the levers of an isometric force displacement transducer (FT03; Grass Instrument Co., West Warwick, RI). A resting tension of 1.5 g was applied in steps of 0.5 g with a 30-minute interval followed by 2 hours of equilibration. Tissue contraction was documented on an oscillograph writer (7WC16H; Grass Instrument Co.) and quantified by converting the analogue data with a MacLab 8/Chart digitization system (AD Instruments Pty. Ltd., Castle Hill, Australia). In a first series of experiments maximal tension development was determined after cumulative stimulation with various agonists of granulation tissue contraction in concentrations ranging from 10⁻¹⁰ mol/L to 10⁻⁴ mol/L. For all subsequent experiments agonists were applied at the following concentrations: angiotensin-II (AT-II; Bachem AG, Bubendorf, Switzerland), 10⁻⁵ mol/L; endothelin-1 (ET-1; Bachem), 10⁻⁷ mol/L; serotonin (Sigma Chemical Co., St. Louis, MO), 10⁻⁴ mol/L; histamine (Sigma Chemical Co.), 10⁻⁴ mol/L; KCl, 4 × 10⁻³ mol/L. Tension peaks were registered and mean values were calculated from four tissue strips per animal and three to five animals per experimental condition.

Immunohistochemistry, Confocal Laser-Scanning Microscopy, and Quantification of F-Actin by Fluorescence Image Analysis

Strips from granuloma pouch and wound granulation tissues were fixed in 4% neutral-buffered formalin and embedded in paraffin. Sections of 4 μ m were stained with hematoxylin and eosin or with Masson's trichrome. For immunohistochemistry deparaffinized sections were immersed in methanol containing 0.5% H₂O₂ for 10 minutes. Sections were incubated with primary antibodies either overnight at 4°C (anti-TGF- β -R11, rbAb; Santa Cruz Biotechnology Inc., Heidelberg, Germany) or 60 minutes at room temperature (anti-TGF- β 1, rbAb; Santa Cruz), followed by 30 minutes of incubation with a secondary biotinylated goat anti-rabbit antibody (DAKO, Copenhagen, Denmark). Presence of the specific protein was evaluated by means of the streptavidin-biotin complex peroxidase method (DAKO) and peroxidase activity was visualized with 3-amino-9-ethylcarbazole (Sigma). Slides were counterstained with hematoxylin and mounted in Eukit. Pictures were acquired using a Zeiss Axiophot microscope equipped with Plan-Neofluar ×20/0.50 objective (Carl Zeiss Inc., Oberkochen, Germany) and with a digital color camera (Coolview; Photonic Science, London, UK).

For confocal laser-scanning microscopy tissue strips were snap-frozen in precooled liquid isopentane and embedded in OTCTM resin (Miles Scientific, Naperville, IL). Cryosections of 4 μ m were stained for α -SMA (anti- α SM-1, IgG2a mAb),⁶ F-actin (phalloidin-Alexa 488; Mo-

lecular Probes, Eugene, OR), ED-A FN (IST-9, IgG1 mAb; gift from Dr. L. Zardi, National Institute for Cancer Research, Laboratory of Cell Biology, Genoa, Italy),^{33,34} desmin (D33, IgG1 mAb; DAKO), smooth muscle myosin heavy chain (SMMHC, rb polyclonal Ab³⁵ or BT-562, rb polyclonal Ab; Biomedical Technologies, Stoughton, MA), and non-muscle myosin heavy chain (NMMHC, rb polyclonal Ab)³⁵ and DNA [4,6-diamidino-2-phenylindole (DAPI)]. As secondary antibodies goat anti-mouse IgG2a tetramethylrhodamine B isothiocyanate-conjugated, goat anti-mouse IgG1 fluorescein isothiocyanate-conjugated, and goat anti-rabbit tetramethylrhodamine B isothiocyanate-conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA) were used. All slides were mounted in buffered polyvinyl alcohol. Images were taken by means of confocal laser-scanning microscopy (LSM 410, Zeiss) using a Plan-Neofluar $\times 10/0.50$ objective or a $\times 63/1.4$ Plan-Neofluar oil immersion objective with a digitally extended focus depth of 1 μm , reconstructed from five optical sections at 0.2- μm z-resolution. All digital images were processed for printing using Adobe Photoshop and printed with a digital Fujifilm Pictography 4000 printer (Fujifilm, Tokyo, Japan).

To quantify F-actin by fluorescence image analysis 4- μm sections of granulation tissue strips were fixed and triple-stained for F-actin (red), desmin (green), and cell nuclei (blue) as described above. For a given region of interest digital images with a resolution of 515 \times 512 pixels were taken at red-, green-, and UV-light excitation, respectively, using a Plan-Neofluar $\times 20/0.50$ objective (Zeiss), color-camera (Coolview, Photonic Science Ltd.), and image grabber software (ImageAccess V2.04K; Imagic Bildverarbeitung AG, Glattbrugg, Switzerland). Constant gain values and integration times were used for the digital camera to obtain similar exposure times for all specimens. To determine the level of F-actin, images were then automatically processed by KS400 software (Zeiss). First, the three 24-bit Red \cdot Green \cdot Blue-images per region were converted to 8-bit gray value images with pixel intensity values ranging from 0 (black) to 255 (white). Desmin staining, specific for vascular smooth-muscle cells, was automatically selected by a thresholding process and corresponding pixel values were set to 255. To exclude vessels from F-actin quantification, desmin-positive areas were then subtracted from F-actin and DAPI images. Second, the background of the desmin-corrected F-actin image was calculated from a manually chosen F-actin-negative area and subtracted from the mean pixel intensity of the whole image, thereby quantifying the level of F-actin expression in the region of interest. Finally, the F-actin expression level was related to the number of cells in the region, represented by the number cell nuclei calculated from the desmin-corrected DAPI image. Tissue was collected from three animals per experimental condition, five sections were prepared per animal, and five regions of interest were analyzed per section.

Western Blot Analysis

Dissected tissues were immediately frozen in liquid nitrogen, crushed, and dissolved in sample buffer: 10 mmol/L

EGTA (Sigma), 1 mmol/L *N* α -tosyl-L-arginine methyl ester hydrochloride (TAME; Fluka, Buchs, Switzerland), 1 mmol/L phenylmethyl sulfonyl fluoride (Fluka), 4% aprotinin (Bayer AG, Zurich, Switzerland), 0.5 mmol/L benzamide (Sigma), and 1 mmol/L di-isopropylfluorophosphate (Fluka). Samples were sonicated (Branson Sonic Power Company, Danbury, CT), boiled for 3 minutes, and protein concentration was determined according to Bradford as described previously.³⁶ Equal amounts of total protein (5 to 20 μg) were loaded to 10 or 7% sodium dodecyl sulfate minigels (Bio-Rad Laboratories AG, Glattbrugg, Switzerland), separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany). Membranes were then probed with the primary antibodies: anti- α -SMA, (α SM-1), β -actin (β 74, rbAb),³⁷ total-actin (β 74+AL-20), ED-A FN (IST-9), desmin (DAKO), SMMHC, NMMHC,³⁵ and TGF- β -RII (Santa Cruz). Secondary goat anti-mouse and anti-rabbit antibodies, conjugated with horseradish-peroxidase (Jackson ImmunoResearch Laboratories) were used and signals were detected by ECL chemiluminescence (Amersham, Rahn AG, Zürich, Switzerland). Subsequently, membranes were stripped of bound antibodies and reprobbed for vimentin (mAb, clone V9; DAKO). Bands were digitized with a scanner (Arcus II; Agfa, Köln, Germany) and the ratio between all band densities of one blot was calculated by commercial computer software (ImageQuant V3.3I Molecular Dynamics, Sunnyvale, CA). Relative protein expression was normalized to the respective values for vimentin. Samples from three different animals per experimental condition were tested and protein/vimentin mean ratio was calculated from three scanned Western blots per animal sample.

Statistical Analysis

Quantitative results are presented as mean values \pm SD. Mean values were tested for the likelihood to belong to the same numeric population by means of a two-tailed heteroscedastic Student's *t*-test. Differences were considered to be statistically significant at values of $P \leq 0.01$. In graphs, differences with statistical significance are indicated by filled symbols. In column charts P values ≤ 0.01 are indicated by an asterisk (*) and $P \leq 0.005$ by a double asterisk (**). Positive linear correlation between isometric tissue contraction and expression of myofibroblast markers, quantified by Western blot analysis was statistically tested by calculating the square of the Pearson correlation product (r^2 value).

Results

Increased Mechanical Tension Enhances the Contractility of Wound Granulation Tissue

It has been shown that fibroblasts subjected to mechanical tension develop a contractile phenotype *in vitro*.^{20,21} To test whether mechanical tension induces similar effects on fibroblasts *in vivo*, we have first increased the

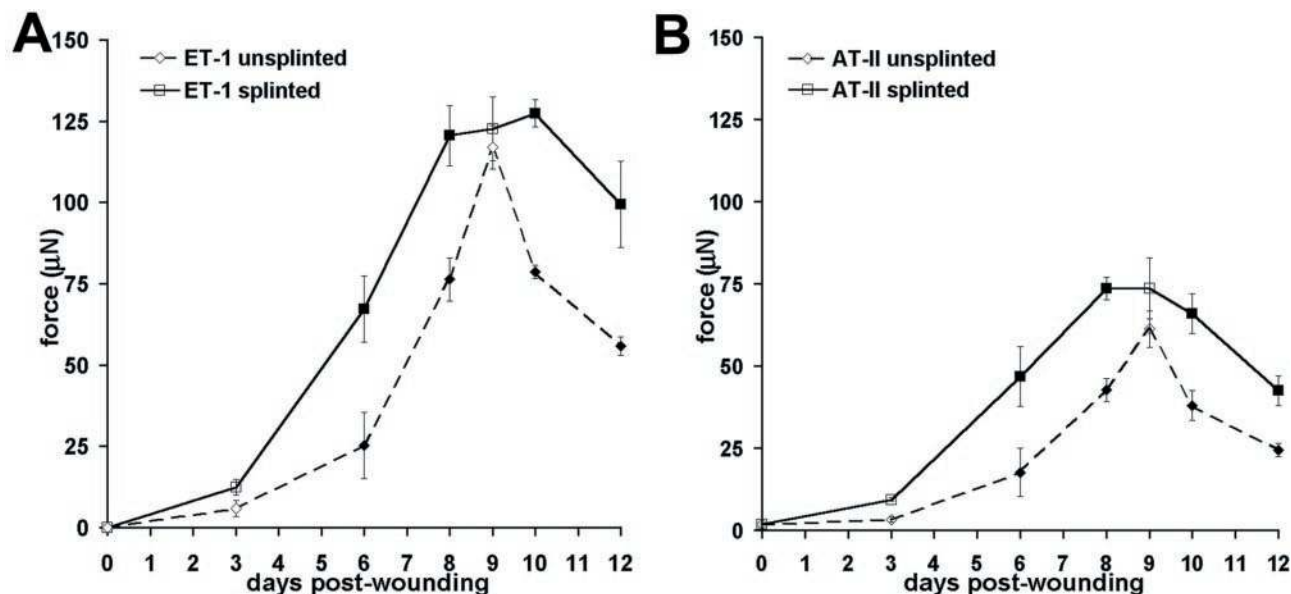


Figure 1. Isometric contraction of wound granulation tissue. Maximum contraction of tissue strips taken from 3- to 12-day-old wound granulation tissue and normal dermal tissue (0 days) was quantified after stimulation with ET-1 (**A**) and AT-II (**B**). Tissue from splinted wounds (**continuous line**) exhibits significantly higher contractility already at early stages of development (6 days) and maintains high levels of contractility up to 10 days after wounding compared to unsplinted granulation tissue (**dashed line**). Normal dermal tissue shows no significant contractility (0 days). Bars indicate SD of mean values. **Filled squares** indicate $P \leq 0.01$ for comparison between unsplinted and splinted tissue of the same age.

mechanical tension of rat granulation tissue by splinting wounds with plastic frames and compared the contractility of tissues from splinted and unsplinted wounds (Figure 1). Isometric contraction was stimulated with various smooth muscle agonists in the following order of efficiency: ET-1 (10^{-7} mol/L), AT-II (10^{-5} mol/L), and serotonin (10^{-4} mol/L). Wound tissue contracted only weakly after stimulation with histamine (10^{-4} mol/L) and potassium (4×10^{-3} mol/L). Control tissues (0 days) did not show any significant response after stimulation with all agonists (not shown). The contraction of unsplinted and splinted granulation tissue increased from 3-day-old to 9-day-old tissue after stimulation with ET-1 (Figure 1A); moreover, splinted tissues contracted more strongly at 6 days (266%) and 8 days (185%) compared to unsplinted tissues. Surprisingly, splinted and unsplinted tissues exhibited comparable contraction after 9 days. At 10 days the contractile activity of unsplinted tissues decreased (67% compared to 9 days), whereas the contractile activity of splinted tissues was unchanged compared to 9 days. At 12 days unsplinted tissues showed a further decrease in contractility (71% compared to 10 days); splinted wounds also decreased their contractility compared to 10 days (78%) but maintained a higher contractility (168%) compared to unsplinted tissues. Results obtained after stimulation with AT-II (Figure 1B) and serotonin (not shown) were similar to those obtained after ET-1 stimulation.

Mechanical Tension Results in an Early Expression of Myofibroblast Markers in Wound Granulation Tissue

To assess whether mechanical tension stimulates myofibroblast differentiation *in vivo*, we compared the expres-

sion of myofibroblast markers such as F-actin, ED-A FN, α -SMA, TGF- β -RII, TGF- β 1, desmin, SMMHC, and NMMHC in the different situations. F-actin was not present in fibroblasts of normal dermis (not shown), started to be visible in 3-day-old unsplinted wounds (Figure 2Aa) and gradually increased (Figure 2, Ab to Ad). Splinting induced noticeable accumulation of F-actin already at 1 day (not shown) and importantly enhanced F-actin levels throughout the following days (Figure 2, Ae to Ah). Moreover, splinting improved the alignment of F-actin along the lines of mechanical tension, ie, parallel to the wound surface. F-actin bundles were devoid of α -SMA up until day 6 in unsplinted and until day 4 in splinted tissue, as evaluated by double staining (not shown). Quantification of Alexa 488-phalloidin fluorescence intensity confirmed higher levels of F-actin in splinted compared to unsplinted granulation tissues at any wound age (Figure 2B, between 376 and 226% increase). No significant changes of marker distribution were observed in the different wound locations. Quantification of granulation tissue cellularity by means of DAPI staining demonstrated a linear increase in cell number from 3- to 12-day-old granulation tissue (120%) and no difference between unsplinted and splinted tissue. ED-A FN was detected in low levels in 3-day-old unsplinted granulation tissue (Figure 2Aa), expression increased between day 6 and day 9 (Figure 2, Ab and Ac), and was reduced in 12-day-old granulation tissue (Figure 2Ad). Splinting induced ED-A FN expression starting from day 1 (not shown) and enhanced its expression compared to unsplinted granulation tissue at all stages of wound healing (Figure 2, Ae to Ah). No differences in vascularity were observed in splinted *versus* unsplinted wounds.

α -SMA was not expressed in fibroblasts of normal dermis (not shown) and of 3-day-old granulation tissue

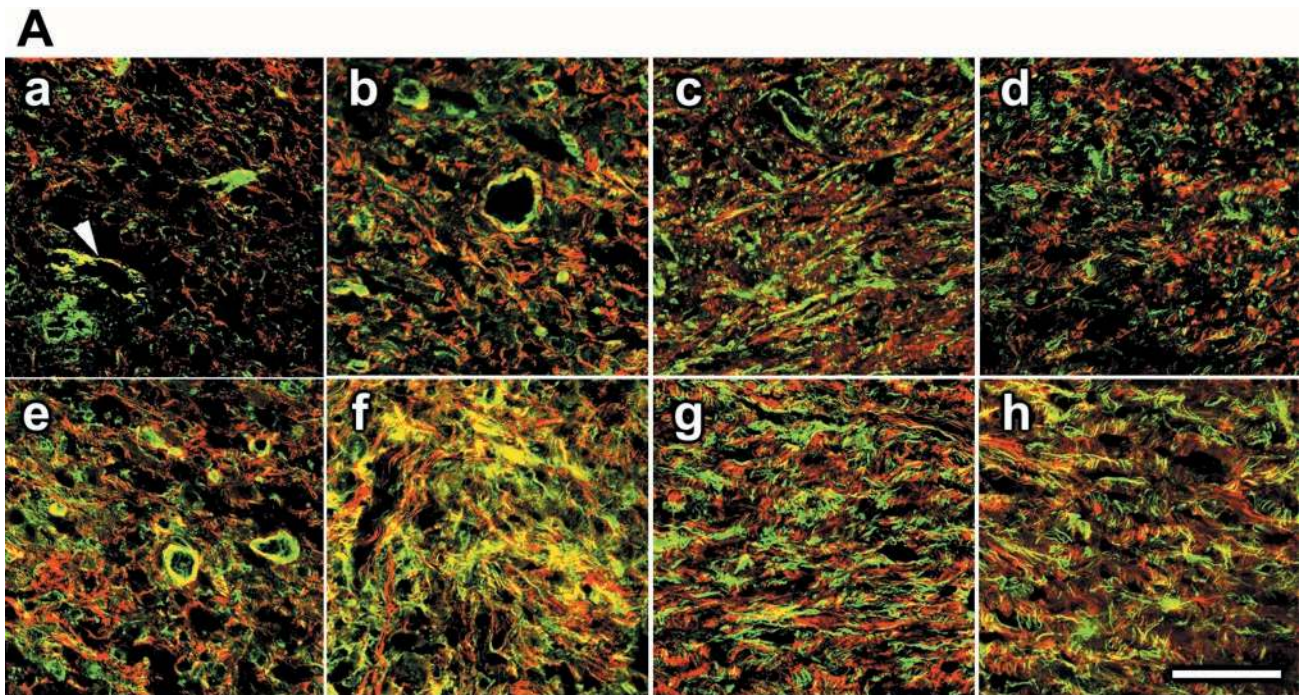


Figure 2. Mechanical tension enhances expression of F-actin and ED-A FN in wound granulation tissue. **A:** Sections of 3-day-old (**Aa, Ae**), 6-day-old (**Ab, Af**), 9-day-old (**Ac, Ag**), and 12-day-old (**Ad, Ah**) granulation tissue were double-stained for F-actin (green) and ED-A FN (red) and examined by confocal laser-scanning microscopy. Tissues from unsplinted wounds (**Aa–Ad**) are compared to those from splinted wounds (**Ae–Ah**). **B:** F-actin expression in unsplinted (dashed line) and splinted granulation tissue (continuous line) from 3 days to 12 days assessed by image analysis of immunostained tissue sections (normal dermis = 0 days). In 3-day-old unsplinted granulation tissue (**Aa**) fibroblastic cells exhibit low levels of *de novo*-appearing F-actin and ED-A FN whereas fibroblasts in 3-day splinted tissue (**Ae**) already show important expression and co-localization of the two proteins (yellow); co-localization is also seen in vascular smooth muscle cells (**Aa**, arrowhead). Expression of both proteins gradually increases and with partial co-localization at 6 days to 12 days after wounding. At any wound age fibroblastic cells of splinted wound granulation tissue exhibit higher expression of ED-A FN and F-actin compared to control (**B**). Scale bar, 50 μ m.

(Figure 3, A and E), and was not detected in fibroblasts of 6-day-old unsplinted granulation tissue (Figure 3B). In unsplinted wounds, it was first detected after 7 days (not shown), was strongly expressed after 9 days (Figure 3C), and expression was clearly reduced after 12 days (Figure 3D). In contrast, splinting induced α -SMA expression by granulation tissue fibroblasts already at 4 to 6 days after wounding (Figure 3F); the level of α -SMA expression increased in splinted tissue to a maximum at 9 days (Figure 3; E to G) and remained at an apparently similar level at 12 days (Figure 3H). Three-day-old to 12-day-old splinted wounds exhibited increased expression of both TGF- β 1 and TGF- β -RII; however, no clear differences were observed between splinted and unsplinted wound tissue by means of immunohistochemistry (not shown). Desmin and SMMHC were not expressed by granulation

tissue fibroblasts, and expression of NMMHC did not change in any condition as tested by means of immunofluorescence and Western blotting (not shown).

Analysis of myofibroblast marker expression in wound granulation tissue by Western blotting (Figure 4) essentially confirmed immunolocalization results. In unsplinted tissue ED-A FN expression was continuously increased from 3 days to 9 days (630%) and exhibited a reduction by 160% between 9 days and 12 days (Figure 4, A and B). Splinting enhanced the amount of ED-A FN (Figure 4A) with the largest difference at 3 days (Figure 4B, 347%). Expression of α -SMA moderately increased from 3-day to 6-day unsplinted tissue (195%), steeply increased from 6 days to 9 days (400%), and was reduced by 145% between 9 days and 12 days (Figure 4, A and C). Splinted tissue exhibited increased α -SMA expres-

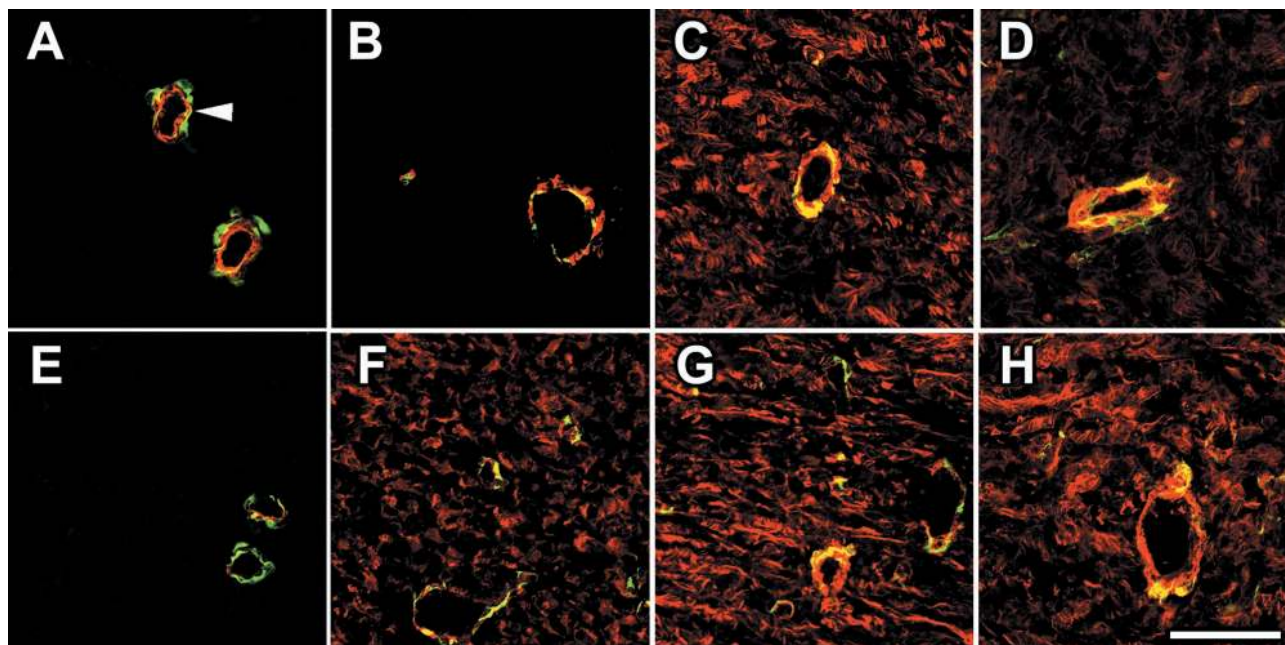


Figure 3. Wound splinting promotes early expression of α -SMA in granulation tissue. Sections of 3-day-old (A, E), 6-day-old (B, F), 9-day-old (C, G), and 12-day-old (D, H) granulation tissue were double-stained for α -SMA (red) and desmin (green). Protein expression in tissue from unsplinted wounds (A–D) is compared to that in splinted wound tissue (E–H). α -SMA is constitutively expressed by smooth muscle cells, co-localizing with desmin in vessels (A, arrowheads). Fibroblastic cells in unsplinted wound tissue express α -SMA at 9 days after wounding (C) and exhibit decreased expression levels after 12 days (D). Mechanical tension results in expression of α -SMA already at 6 days (E) that gradually increases up to 12 days (H). Scale bar, 50 μ m.

sion levels at 6 days (Figure 4C, 420%) and showed similar α -SMA expression at 9 days compared to unsplinted granulation tissue. Splinting maintained high α -SMA expression levels at 12 days in contrast to the decreased expression in unsplinted wound tissue (Figure 4C). TGF- β -RII expression increased from 0 to 12 days after wounding with no significant difference between splinted and unsplinted granulation tissue (Figure 4, A and D). To relate changes in myofibroblast marker expression to enhanced tissue contractility during the evolution of unsplinted and splinted wound granulation tissue, we calculated correlation coefficients. Highest correlation was observed between the temporal course of ET-1-stimulated tissue contraction (Figure 1A) and expression of α -SMA ($r^2 = 0.98$). Lower correlation was calculated for TGF- β -RII ($r^2 = 0.92$), ED-A FN ($r^2 = 0.90$), and F-actin ($r^2 = 0.84$), no correlation for β -actin ($r^2 = 0.69$), NMMHC ($r^2 = 0.61$), desmin ($r^2 = 0.57$), and vimentin ($r^2 = 0.55$).

Loss of Mechanical Tension Decreases Tissue Contractility and Expression of Myofibroblast Markers

Because increased mechanical tension seems to induce myofibroblast differentiation and increase granulation tissue contractility, we sought to investigate whether reduction of tension may have the opposite effect. First, wounds were splinted for 7 days, then released from tension for 1 or 2 days and compared to granulation of the same age but still splinted. One-day-released gran-

ulation tissue contracted similarly compared to 8-day splinted tissue ($96 \pm 7\%$). However, tension release for 2 days significantly reduced contraction compared to 9-day splinted tissue to $63 \pm 3\%$ after stimulation with AT-II and to $63 \pm 2\%$ after stimulation with ET-1. Contraction of 2-day-released granulation tissue was also lower ($71 \pm 4\%$) when compared to 9-day-unsplinted granulation tissue. Characterization of released tissue by immunostaining and Western blotting revealed no changes after 1 day, but a decrease of F-actin and α -SMA after 2 days. Expression levels of ED-A FN, TGF- β -RII, TGF- β 1, NMMHC, desmin, and vimentin were not significantly changed 2 days after release (not shown).

To investigate further the chronological relationship between tissue contraction and myofibroblast modulation after release of mechanical tension, we used the granuloma pouch model.²⁹ Tension was released in granuloma pouch tissue at 14 days by evacuating the exudate and isometric contraction was measured 1 to 7 days after release (Figure 5). Contraction of 21-day control pouch tissue was maximally stimulated by ET-1 (10^{-7} mol/L) and by the following agonists in percentage of ET-1-response: AT-II (10^{-5} mol/L), 60%; serotonin (10^{-4} mol/L), 48%; potassium (4×10^{-3} mol/L), 45%; and histamine (10^{-4} mol/L), 6%. Results are presented for ET-1 (Figure 5A) and AT-II (Figure 5B). Tissue contractility was higher in intact 21-day-old compared to 14-day-old pouches (Figure 5, dashed lines), confirming previous observations.^{30,38} Release of mechanical tension at 14 days decreased pouch tissue contraction beginning 2 days after release, exhibited a minimum at 3 days after release

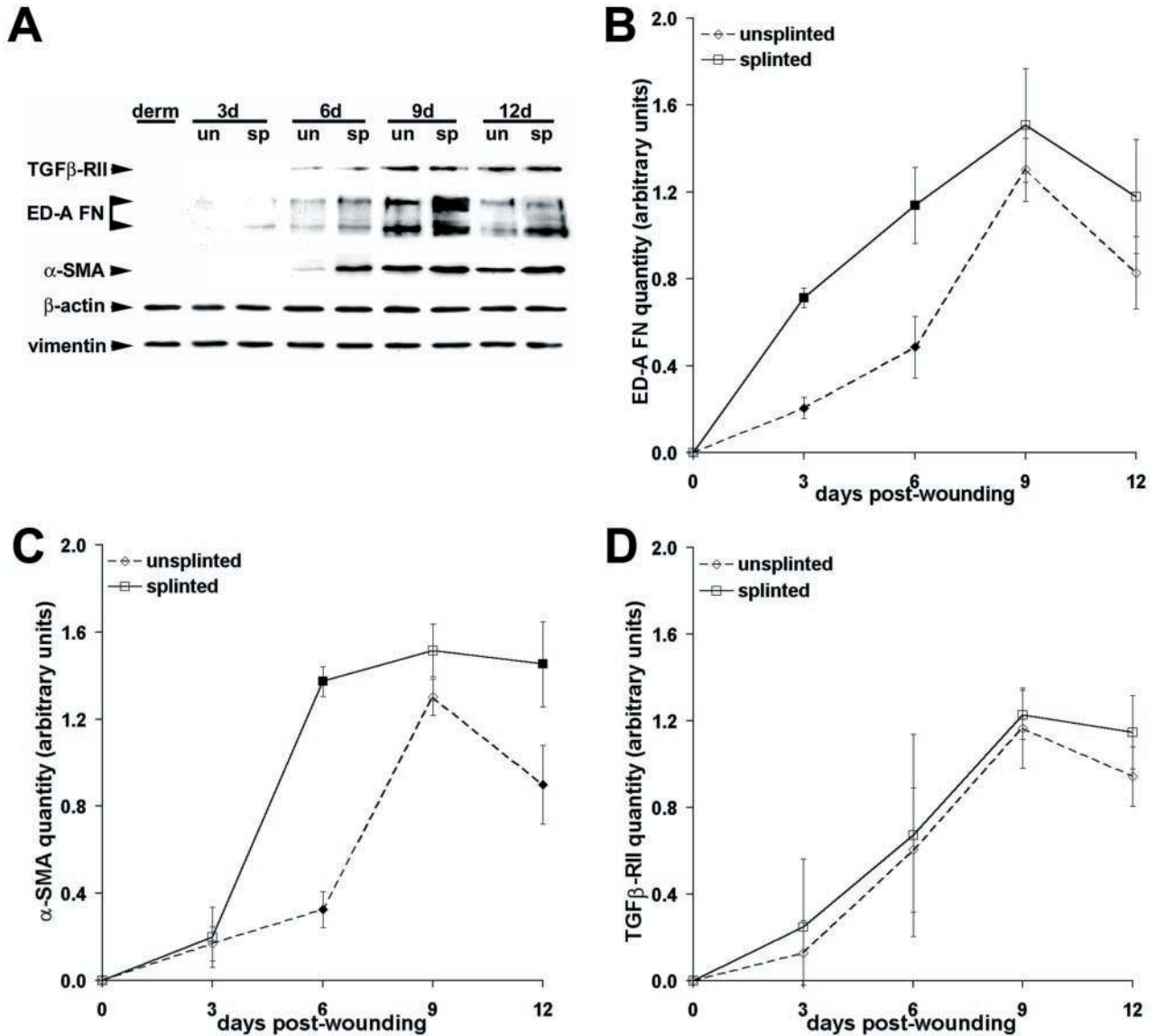


Figure 4. Quantification of myofibroblast markers in granulation tissue by means of Western blot. **A:** Expression of β -actin, vimentin, and the myofibroblast markers TGF β -RII, ED-A FN, and α -SMA was investigated. Extracts of 3- to 12-day-old granulation tissue from splinted wounds (sp) are compared to those of unspliced wounds (un) and of normal dermis (derm). Expression of myofibroblast markers gradually increases in evolving granulation tissues up to 9 days after wounding. No change in expression of β -actin and vimentin is observed. **B-D:** Protein bands were scanned, quantified by image analysis, and normalized to vimentin. Expression of ED-A FN is significantly higher in 3 day and 6 day splinted tissue compared to the respective control (**B**), as observed for α -SMA expression at 6 days and 12 days (**C**). Expression of TGF β -RII is similar in unspliced and splinted wound granulation tissue at any age (**D**). **Filled squares** indicate $P \leq 0.01$ for comparison between unspliced and splinted tissue of the same age.

(14d+3dR), and kept this level until 7 days. Compared to intact pouches of the same age (21 days), tension release resulted in a significantly lower tissue contraction (38% for ET-1 and 46% for AT-II). As a control, we substituted the evacuated exudate with an identical volume of physiological saline for 7 days (Figure 5, 14+7dS). Saline-injected pouch tissue contraction did not differ from intact pouches of the same age (21 days).

Alexa 568-phalloidin (Figure 6, insets) revealed an intense F-actin staining in 14-day-old granuloma pouch fibroblasts, that was clearly decreased already 2 days after release (Figure 6B); this decrease was more important at 7 days after release (Figure 6C, inset). ED-A FN and α -SMA were co-localized and their expression in-

creased in 21-day-old compared to 14-day-old intact granuloma pouch tissue (Figure 6, A and D). Three days after tension release α -SMA expression was reduced (Figure 6B); 7 days after tension release, ED-A FN expression was also reduced and α -SMA was hardly detectable (Figure 6C). TGF- β 1, previously shown to be expressed in granuloma pouch tissue,^{39,40} and TGF- β -RII were both predominantly expressed in the external collagen-rich layer of the pouch tissue. TGF- β 1 and TGF- β -RII expression patterns in 7-day-released pouches did not differ significantly from 1- to 5-day-released tissue or 21-day intact pouch tissue, but the thickness of the collagen-rich layer decreased progressively during the 7-day period of tension release to ~70% compared to 21-day intact pouches (not shown).

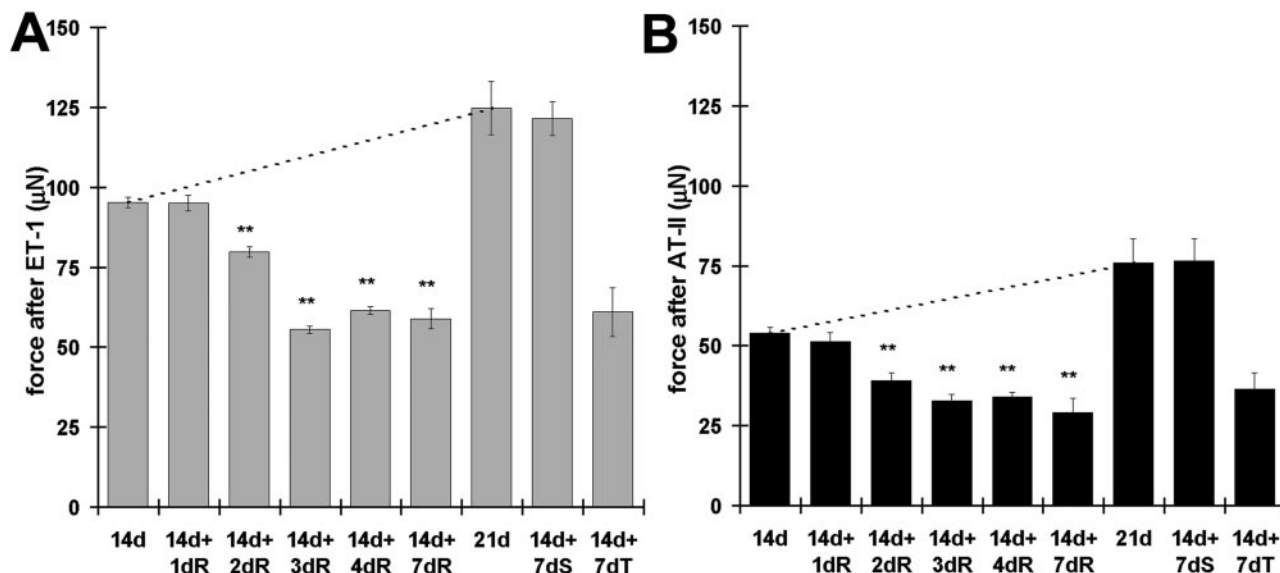


Figure 5. Loss of mechanical tension decreases granuloma pouch tissue contraction. Maximum contraction of tissue strips from granuloma pouches was quantified after stimulation with ET-1 (**A**) and AT-II (**B**). Pouches were either left intact for 14 days and 21 days or were released from tension by removing exudate after 14 days for 1 to 7 days. A significant increase in tissue contraction is observed between 14- and 21-day-old intact pouches (**dashed lines**). Loss of tension decreases granuloma pouch tissue contractility time-dependently and starts 2 days after release (14d+2dR). Note the dramatic difference in tissue contractility between 21-day intact pouches (21 day) and 21-day-old pouches, which were released for 7 days (14d+7dR). Exchange of exudate by physiological saline shows no effect (14d+7dS), whereas injection of TGF- β -R11 significantly decreases pouch tissue contraction (14d+7dT). **, $P \leq 0.005$ compared to tissue contraction mean values of 14-day intact pouches.

As determined by Western blotting, α -SMA was significantly reduced 2 to 3 days after release, compared to 14-day-old intact pouch tissue, whereas decrease of ED-A FN expression started 4 days after release (Figure 7). TGF- β -R11 expression was only moderately diminished throughout the 7-day period after release and no change was observed for β -actin and vimentin expression (Figure 7A). Compared to 21-day-old intact pouch tissue, 7-day tension release reduced α -SMA expression to 51%, ED-A FN to 47%, and TGF- β -R11 to 81% (Figure 7B). In contrast to wound granulation tissue fibroblasts, which did not express desmin and SMMHC, low expression of both proteins was observed in fibroblastic cells of 14-day and 21-day intact granuloma pouches. Tension release did not change desmin and NMMHC expression and only moderately diminished expression of SMMHC 7 days after release as evaluated by Western blotting and immunofluorescence (not shown). When removed exudate was replaced by physiological saline, no changes in protein expression were detected (Figure 7A, 14d+7dS).

Injection of soluble TGF- β -R11 into the granuloma pouch for 7 days decreased tissue contraction to 49% after stimulation with ET-1 (Figure 5A, 14d+7dT) and to 48% after AT-II stimulation (Figure 5B, 14d+7dT) compared to 21-day-old control pouch tissue. It reduced the expression levels of TGF- β , TGF- β -R11, ED-A FN, F-actin, and α -SMA significantly compared to 21-day-old control pouch tissue as demonstrated by Western blotting (Figure 7A) and immunofluorescence (not shown).

Discussion

It is accepted that mechanical tension plays an important role in the modulation of cultured fibroblasts into myofi-

broblasts by favoring the formation of stress fibers and the expression of α -SMA.²⁷ Here, we demonstrate that mechanical tension also influences myofibroblast differentiation *in vivo*. Fibroblasts populating the granulation tissue of splinted wounds exhibit earlier formation of stress fibers, expression of ED-A FN and of α -SMA compared to nonsplinted wounds. Inversely, release of tension in wound granulation tissue and in granuloma pouch leads to a sequential loss of stress fibers, α -SMA, and ED-A FN. Moreover, our study indicates a correlation between the level of α -SMA expression and contractility of the tissues obtained during the different experimental situations. The acquisition of contractile activity by granulation tissue on stimulation with smooth muscle agonists is a well-accepted phenomenon and has been suggested to represent a useful indication of the retractile potential of granulation tissue.^{29,30,41} Acquisition of an increased contractile activity by wound splinting⁴² has been correlated to the induction of microfilament bundles in fibroblasts.¹⁸ Our results corroborate these observations and establish a correlation between changes in tissue contractility and cytoskeletal protein expression and/or organization after mechanical load modulation.

In both splinted and unsplit wounds, the granulation tissue strip contractile activity evolves according to three phases, as depicted schematically in Figure 8. During the first phase (red) the contractility of granulation tissue increases slowly, whereas during the second phase (blue), the increase is steep; the third phase (green) corresponds to a decrease of contractile activity. In the first phase there is an increase of F-actin and of ED-A FN expression that is clearly more important in splinted wounds. The onset of high contractile activity correlates always with the *de novo* expression of α -SMA,

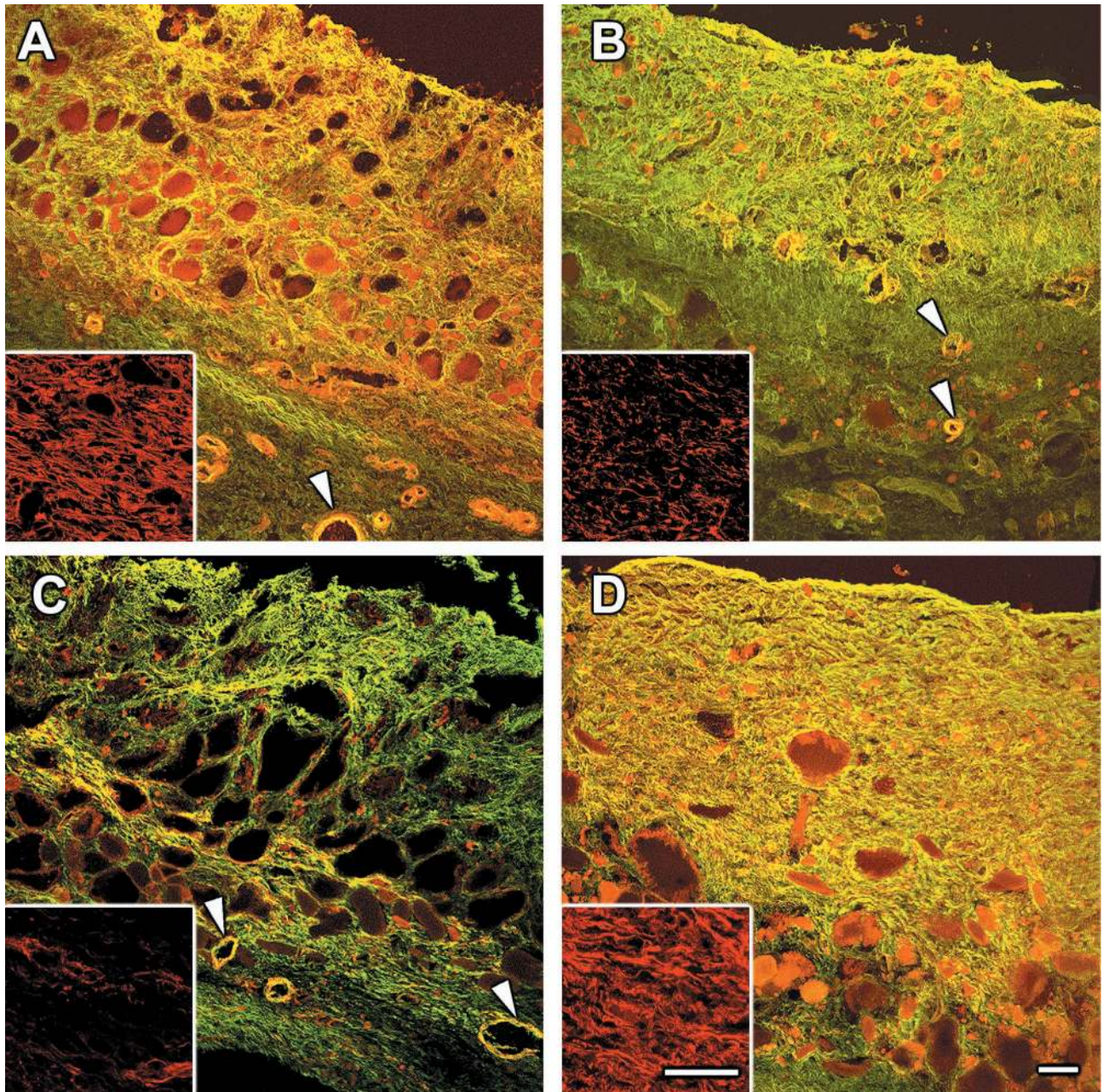


Figure 6. Loss of mechanical tension reduces myofibroblast differentiation in granulation pouch tissue. Tissue sections of intact (**A, D**) and tension-released granulation pouches (**B, C**) of different ages were double-stained for α -SMA (red) and ED-A FN (green) or stained for F-actin (**insets**, red) and examined by confocal laser-scanning microscopy. α -SMA and ED-A FN show important expression by fibroblastic cells and co-localization (yellow) in tissue from 14-day (**A**) and 21-day (**D**) intact granulation pouches, as well as in vascular smooth muscle cells (**arrowheads**). Loss of tension in 14-day pouches results in decreased levels of F-actin and α -SMA expression 3 days after release (**B**) followed by decreased expression of ED-A FN 7 days after release (**C**). Note that expression of both proteins in small vessels is not affected by pouch relaxation. Scale bars, 50 μ m.

assembled into stress fibers. It is noteworthy that the first phase is shorter and the second phase is longer in splinted compared to unsplinted wounds and corresponds to the maximal level of α -SMA expression. Interestingly, the maximal level of contractile capacity is similar in both situations. Finally, a decrease in contractile activity takes place more slowly in splinted than in unsplinted wounds. The observed correlation between α -SMA expression and granulation tissue contractility is in agreement with the previously reported correlation between α -SMA expression and contraction of fibroblast-

populated anchored collagen gels.^{8,9} More recently we have shown that 3T3 fibroblasts exhibit a significantly higher contractile activity after transient and/or stable transfection with α -SMA cDNA compared to fibroblasts transfected with β -actin, γ -actin, or even α -cardiac actin cDNA in the absence of any change of myosin heavy chain expression.⁷ All these data are compatible with the assumption that α -SMA expression alone plays a role in the enhancement of fibroblast contractility *in vitro*. Our results allow to hypothesize that also *in vivo* α -SMA expression plays a crucial role in the development of gran-

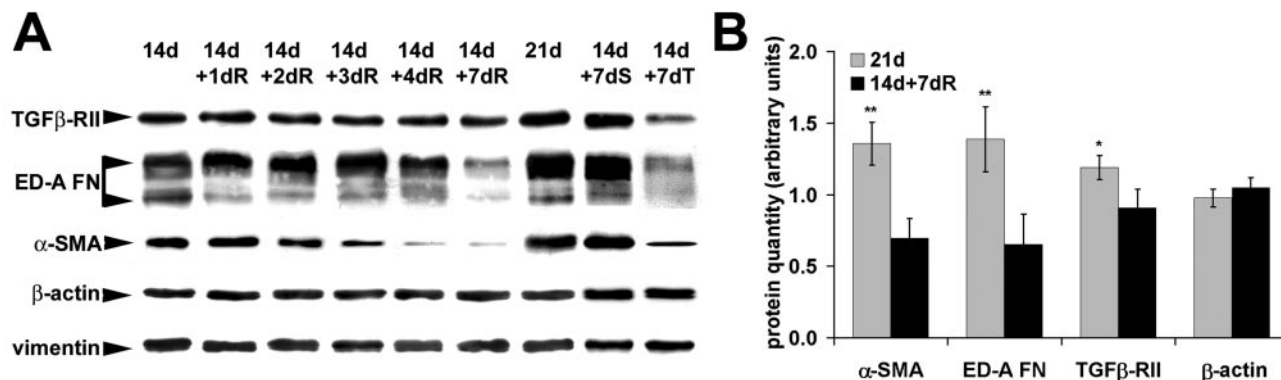


Figure 7. Quantification of myofibroblast markers in granuloma pouch tissue by means of Western blot. **A:** Protein expression in intact granuloma pouches (14d, 21d) was compared to 14-day-old pouches, which were subsequently released from mechanical tension for 1 to 7 days. Expression of α -SMA gradually decreases starting 2 days after release (14d+2dR), whereas decrease in ED-A FN expression occurs later (14d+7dR) after release. TGF- β -RII expression shows a moderate decrease after 7 days and no change is observed for β -actin and vimentin expression. Exchange of exudate by saline has no effect (14d+7dS), whereas injection of TGF- β -sRII significantly decreases expression of all myofibroblast markers (14d+7dT). **B:** Protein bands obtained from 21-day intact (21d) and 7-day-tension-released (14d+7dR) pouch tissue were scanned, their density was quantified by image analysis, and obtained values were normalized to vimentin. Tissue from 7-day-released pouches exhibits significantly reduced levels of α -SMA and ED-A FN expression, moderately reduced TGF- β -RII expression and no change in β -actin compared to 21-day intact pouches. *, $P \leq 0.01$; **, $P \leq 0.005$.

ulation tissue contractile capacity. In all situations, increased tension results in a greater expression of α -SMA and an increased force production, whereas decreased tension has the opposite effect.^{2,21,43,44} Although *in vivo* and *in vitro* data indicate that α -SMA expression is stimulated by mechanical stress, it remains unclear how fibroblasts translate the physical signal into protein expression. In cells such as cardiomyocytes, endothelial and smooth muscle cells, mechanical stress was shown to stimulate the production and secretion of growth factors that mediate stress-induced cell responses.⁴⁵ In par-

ticular, expression of TGF- β 1, the major inducer of α -SMA expression^{13,14} and contractility in myofibroblasts,^{8,21} is up-regulated in mechanically stressed mesangial cells.⁴⁶ Under our conditions the expression of TGF- β 1 and of its receptor TGF- β -RII was gradually increased, but similarly in splinted tissue and unsplinted tissues. Nevertheless, blocking TGF- β 1 by an excess of soluble receptor without changing the mechanical load reduced α -SMA expression and tissue contraction in our experimental conditions, implying a role of TGF- β 1. Enhanced mechanical stress increases growth factor sensitivity^{23,24,47} and loss of mechanical tension reduces the growth factor response of cultured fibroblasts.^{48,49} It remains to be examined whether TGF- β 1 activation changes in our experimental situations.

Changes in mechanical stress may directly affect the level of extracellular matrix proteins.⁵⁰⁻⁵² Recently, it was shown that during wound healing ED-A FN expression^{53,54} precedes the appearance of α -SMA-positive myofibroblasts and is essential to mediate TGF- β 1-induced α -SMA expression.¹⁷ Splinting induces ED-A FN expression in granulation tissue, suggesting a possible regulatory function of this protein during stress-induced myofibroblast differentiation. Regulation may occur at the level of FN expression,⁵⁵ alternative splicing,^{56,57} fibril assembly,²² or accessibility of functional residues in the ED-A domain of FN⁵⁸ as it has been demonstrated for plasma FN.⁵⁹ Integrins of focal adhesions are potential receptors and transducers of these extracellular signals.⁶⁰

When myofibroblasts were released from mechanical stress by removing the wound splint or by evacuating the granuloma pouch, disappearance of α -SMA preceded the decrease of ED-A FN and TGF- β 1 levels. Thus, ED-A FN and TGF- β 1 are crucial for the induction of α -SMA expression but are not sufficient to maintain myofibroblast differentiation in the absence of a mechanical stimulus. Taking into account the early formation of F-actin during wound healing and the rapid loss of actin stress fibers after load release, we suggest that the physical integrity of stress fibers⁶¹ provides an important prereq-

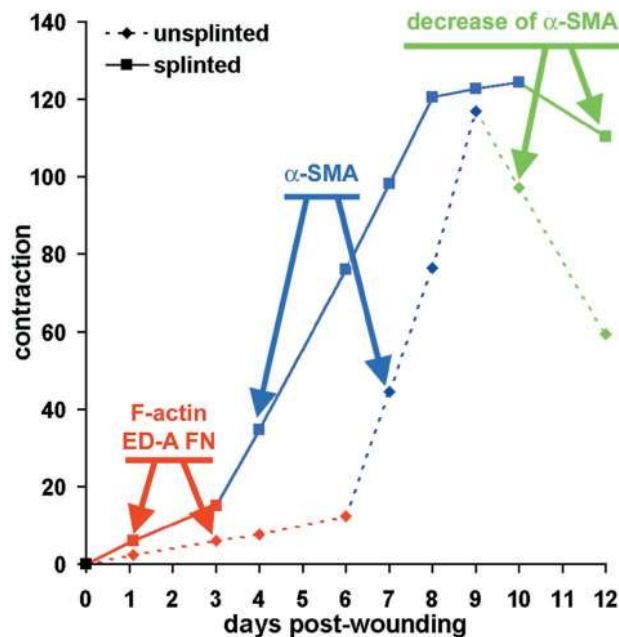


Figure 8. Evolution of granulation tissue contractility. The chronological evaluation of granulation tissue contractile activity distinguished three phases characterized by: 1) slow increase (red), 2) steep increase (blue), and 3) decrease (green). These phases correlate with the onset of F-actin and ED-A FN expression (phase 1), *de novo* expression of α -SMA (phase 2), and decrease of α -SMA expression (phase 3) as indicated by the arrows. The first two phases clearly start earlier and the third phase is delayed in splinted (continuous line) compared to unsplinted wounds (dotted line).

uisite for α -SMA expression and myofibroblast contraction. Mechanical stress induces actin polymerization and stress fiber formation when applied externally⁶² or by increasing intracellular tension.^{10,63} The ratio between G-actin/F-actin was shown to directly influence β -actin synthesis.^{64,65} However, the role of the G-actin/F-actin ratio for the expression of other actin isoforms, such as α -SMA, has not been evaluated. It is conceivable that mechanical tension induces actin expression without discriminating among isoforms whereas factors such as TGF- β and ED-A FN exert a specific stimulation of α -SMA.¹⁷ A similar phenomenon has been shown for the stress-dependent expression of α -skeletal actin by cardiomyocytes in the presence of AT-II.⁴⁵

In conclusion our results show that mechanical tension is a prerequisite for the development and maintenance of myofibroblast differentiation and hence of granulation tissue contraction. Given the reciprocal relationship between fibroblast contractility and the mechanical state of the matrix, the modulation of extracellular and intracellular tension may help to influence wound healing and development of fibrocontractive diseases.

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