BMC Dermatology

BioMed Central

Research article

Phenotypic differences between dermal fibroblasts from different body sites determine their responses to tension and TGFβ1

Constantin C Chipev and Marcia Simon*

Address: Living Skin Bank, University Hospital, Dept. Oral Biology and Pathology, Dept. of Dermatology, HSC, SUNY at Stony Brook, NY 11794-9702, USA

E-mail: Constantin C Chipev - cchipev@notes.cc.sunysb.edu; Marcia Simon* - marsimon@notes.cc.sunysb.edu
*Corresponding author

Published: 21 November 2002

BMC Dermatology 2002, **2**:13

Received: 30 August 2002
Accepted: 21 November 2002

This article is available from: http://www.biomedcentral.com/1471-5945/2/13

Abstract

Background: Wounds in the nonglabrous skin of keloid-prone individuals tend to cause large disordered accumulations of collagen which extend beyond the original margins of the wound. In addition to abnormalities in keloid fibroblasts, comparison of dermal fibroblasts derived from nonwounded glabrous or nonglabrous skin revealed differences that may account for the observed location of keloids.

Methods: Fibroblast apoptosis and the cellular content of α-smooth-muscle actin, TGFβ1 receptorII and ED-A fibronectin were estimated by FACS analysis. The effects of TGFβ1 and serum were examined.

Results: In monolayer cultures non-glabrous fibroblasts were slower growing, had higher granularity and accumulated more α-smooth-muscle actin than fibroblasts from glabrous tissues. Keloid fibroblasts had the highest level of α-smooth-muscle actin in parallel with their expression level of ED-A fibronectin. TGFβ1 positively regulated α-smooth-muscle actin expression in all fibroblast cultures, although its effects on apoptosis in fibroblasts from glabrous and non-glabrous tissues were found to differ. The presence of collagen I in the ECM resulted in reduction of α-smooth-muscle actin. A considerable percentage of the apoptotic fibroblasts in attached gels were α-smooth-muscle actin positive. The extent of apoptosis correlated positively with increased cell and matrix relaxation. TGFβ1 was unable to overcome this apoptotic effect of matrix relaxation.

Conclusion: The presence of myofibroblasts and the apoptosis level can be regulated by both TGFβ1 and by the extracellular matrix. However, reduction of tension in the matrix is the critical determinant. This predicts that the tension in the wound bed determines the type of scar at different body sites.

Background

Normal wound healing requires fibroblast proliferation and migration into the wound bed followed by tightly regulated matrix deposition and contraction. Aberrations in these processes can lead to excessive collagen accumulation as found in keloids. These scars extend beyond the original wound margins and are excluded from glabrous surfaces (palms, soles). When grown in vitro keloid fibroblast cultures contain a high percentage of α-smooth muscle actin (α-SMA) expressing cells – myofibroblasts.

In spite of numerous studies the etiology of keloid formation remains obscure [1–11]. However, as TGFβ1 regulates the expression and deposition of collagenous extracellular matrix (ECM) [12–14] it is expected that keloids develop due to aberrant responses to this cytokine. While in vitro analyses of TGFβ1 levels in keloid and normal fibroblasts have yielded variable results [15–20], higher levels of TGFβ1 receptors and Smad3 activation were recently reported in keloid fibroblasts [21]. Thus, procedures that lower TGFβ1 expression may help prevent keloid development [15,16,18,22].

TGFβ1 also supports the differentiation of fibroblasts into myofibroblasts, which are a major constituent of the granulation tissue [23,24]. The process depends upon the deformability of the ECM [17,25] and is mediated by the ED-A splice variant of fibronectin [26,27]. ED-A fibronectin is expressed in the initial stages of wound healing and along with collagen I is positively regulated by TGFβ1 [26].

Progression of granulation tissue to neodermis requires a decrease in cellularity through apoptosis of endothelial cells, fibroblasts and myofibroblasts [28,29]. Keloid fibroblasts demonstrate aberrant apoptotic behavior [30,31] although studies have given variable results [5,9,11,30–35]. Our initial data for serum-starvation-induced apoptosis in monolayer cultures of dermal fibroblasts demonstrated delayed apoptosis of keloid fibroblasts and a negative correlation with α-SMA expression [36]. Similar correlations were observed in rat lung fibroblasts, where TGFβ1 increased α-SMA content while acting as an antiapoptotic agent [37,38]. It should be noted however, that many of the myofibroblasts were still able to undergo apoptosis consistent with in vivo data on palatal wound healing [39]. Thus, TGFβ1 can promote both α-SMA expression in the initial stages of wound healing and apoptosis in later stages of wound healing. The latter effect may be related to the apoptosis induced by relaxation of the ECM or of the collagen gel [40,41].

In order to follow both the differentiation of fibroblasts into myofibroblasts and their apoptosis in relation to the tension in the surrounding ECM, we utilized two-color FACS analyses. Experiments were carried out with human dermal fibroblasts of different origins – keloid, nonaffected palmar sites of the corresponding patients, normal fibroblasts from palms, heels or nonpalmoplantar sites. α-SMA content and apoptosis were evaluated as a function of serum, TGFβ1 and collagen type I. The observed phenotypic differences between fibroblasts grown from glabrous vs. nonglabrous tissue suggest a reason for the exclusion of keloids from specific body sites of keloid prone individuals.

Methods

Cell growth, media, collagen coating and embedding in rafts

Skin samples from palmoplantar (PP) and non-PP loci were obtained under IRB approval from African-American keloid prone and normal donors [36]. Biopsies were rinsed with PBS, minced into small fragments and epidermis separated from dermis using a 4°C overnight incubation with Dispase II (2.5 mg/ml in DMEM). The epidermis was then removed with forceps and the dermal pieces were incubated with Collagenase D (3 mg/ml in *collagenase buffer* (see below)) for 15–30 min at 37°C. Any cells and pieces were collected by centrifugation and resuspended in trypsin/EDTA (0.5 mg/ml trypsin and 0.25 mM EDTA in PBS) for an additional 15–30 min at 37°C with occasional shaking. Fetal bovine serum 10% was added at the end to neutralize the trypsin. Cells were harvested by centrifugation (800 rpm in a clinical centrifuge) and were grown and serially passaged as previously described [36]. For experiments, cells from passages four to six were used with an initial plating density of 10–20 cells/mm^2. When added, ascorbic acid and TGFβ1 (R&D Systems) were used at 50μg/ml and 10 ng/ml, respectively. Fetal bovine serum(Hyclone) 10% was used as supplement to the media (low glucose DMEM (GIBCO, BRL)) when indicated.

Collagen type I coated plates were prepared by incubating tissue culture plates with 0.1 mg rat tail collagen per ml Phosphate Buffered Saline (PBS) for 3 hrs at room temperature, followed by a 30 min, 37°C incubation with 0.5%BSA (bovine serum albumin) in PBS with 0.5 mM Ethylene DiamineTetraacetic acid (EDTA) – PBS/EDTA, and final PBS washes.

Collagen gels (1.5 mg/ml) were prepared with fibroblasts harvested at confluence and resuspended to 10^6 cells/ml collagen solution (see also [36]). One ml of this cell suspension was added per well of a 6 well plate (Nunc). Gel relaxation was achieved by full detachment with a spatula. Gels with keloid fibroblasts tend to shrink, but remain attached to the center of the well. To harvest cells the gels were first incubated with 100 μl trypsin/EDTA (0.5 mg/ml trypsin;.0.25 mM EDTA) for 10 min at 37°C, and then with 400 μl collagenase D (5 mg/ml; Boehringer) in *collagenase buffer* (20 mM Hepes pH 7, 0.13 mM NaCl, 10 mM Ca Acetate) for 30 min at 37°C. The reaction was stopped by addition of fetal bovine serum to 10%. Cells were collected by centrifugation at 2000 rpm and the resultant pellet was washed with PBS.

^{3}H-Thymidine incorporation

Fibroblasts at 70% confluence were incubated for 4 hrs in serum-free DMEM containing 4 μCi/ml methyl- ^{3}H-thymidine (87 Ci/mmol) (NEN, Boston, MA). The cells were

harvested by trypsinization, sonicated, and incubated for 1 hr in 10% tri-chloro-acetic acid (TCA) on ice. They were then applied to a glass filter (Whatman GF/C glass microfibre) and washed in a filter assembly (Sigma) with 10%TCA and once with 95% ethanol, before being dried and counted in an LKB liquid scintillation counter. The cell number was determined by direct counting. The data were an average of three experiments.

Preparation and performance of the FACS analysis

For each FACS assay about one million cells were fixed by suspension in the non-formaldehyde tissue fixative Histo-Choice (Amresco, Ohio) for 20-minute at room temperature. For permeabilization cells were treated for 3 min at 4°C with 0.3% Triton in PBS. The ED-A antibody gave nearly identical results for permeabilized and nonpermeabilized cells.

Apoptosis levels were measured using the TUNEL assay (Boehringer – In Situ Cell Death Detection Kit, Fluorescein) according to manufacturer's recommendations on cells, which were resuspended in 50 μl reaction mix and incubated for 1 hr at 37°C with regular agitation. After two PBS washes cells were resuspended in PBS and incubated with the primary antibody.

The primary antibodies against human α-SMA (monoclonal Asm-1, Roche Molecular Biochemicals), TGFβ1 (pan specific, produced in rabbits, R&D systems), ED-A fibronectin (MAS 521, Accurate Chemicals, Westbury, NY); and TGFβ1 receptor II (affinity purified rabbit polyclonal, H-657, Santa Cruz Biotechnology Inc.) were diluted 1:100. The secondary antibodies – FITC and Phycoerythrin (PE) – conjugated IgG (Jackson ImmunoResearch Labs) were used at 1:100 dilutions. Incubations with antibodies (primary and secondary) were all carried out for one hour at room temperature with several PBS washes in between.

The samples were filtered through 35 μm cell strainer caps of Falcon polystyrene tubes before being applied to a Beckton Dickinson Fascan equipped with CellQuest software version 3.2. Controls included cells incubated only with the secondary antibody, cells incubated only with the primary antibodies, cells after the TUNEL reaction only, and cells without the TUNEL reaction. These controls were used to determine the settings for the FACS analyses. The background fluorescence had values around 20–30 in arbitrary fluorescence units for all of the controls. Cells with fluorescence above this value were considered immunopositive and/or apoptotic.

The results of the FACS analyses are presented using a combined measure of both percentage immunopositive/apoptotic cells (with fluorescence above the background) and their GeoMean fluorescence: [% immuno-positive or apoptotic cells]* [GeoMean-background fluorescence]. (For more details, please, see additional file 5: Appendix to Methods.doc).

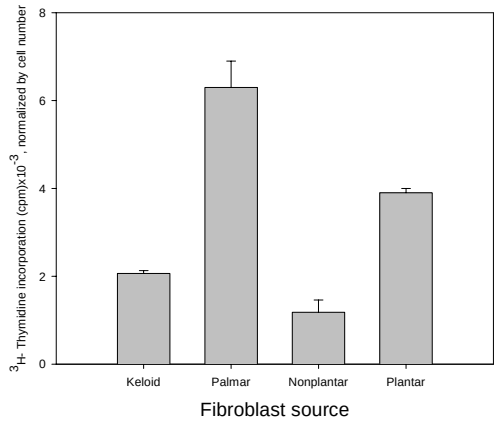

Figure 1
^{3}H-thymidine incorporation in keloid and normal nonpalmoplantar fibroblasts compared to palmoplantar fibroblasts.

The data presented were obtained in three independent experiments with fibroblasts from two keloid prone individuals (labeled as keloid D and keloid T), as well as with fibroblasts from one non-affected individual. Similar results (not presented here) were found with fibroblasts from other keloid donors and nonaffected individuals.

Results and Discussion

Growth rates and morphologic characteristics of palmoplantar and non-palmoplantar fibroblasts

The proliferation of preconfluent fibroblasts grown on tissue culture plastic was measured as a function of ^{3}H-thymidine incorporation into TCA-precipitates. As shown in Fig. 1, fibroblasts, derived either from the palmar skin of keloid-forming donors or the plantar skin of nonaffected donors, incorporated more ^{3}H-thymidine than did those isolated from keloid or normal nonglabrous trunk skin. This apparent difference in proliferation rate is consistent with the microscopic observation (data not shown) that palmoplantar (PP) fibroblasts reached confluence 2–3 days earlier than did the non-palmoplantar (non-PP) cultures, when plated at the same density. Earlier work from other laboratories had indicated that normal fibroblasts grew more quickly [42], similarly [6,43] or more slowly [16,34] than did keloid fibroblasts. Such variations may reflect differences in culture conditions at the time of assay. For example, in confluent cultures the growth rate of PP fibroblasts slows to less than that of non-PP fibroblasts (results not shown).

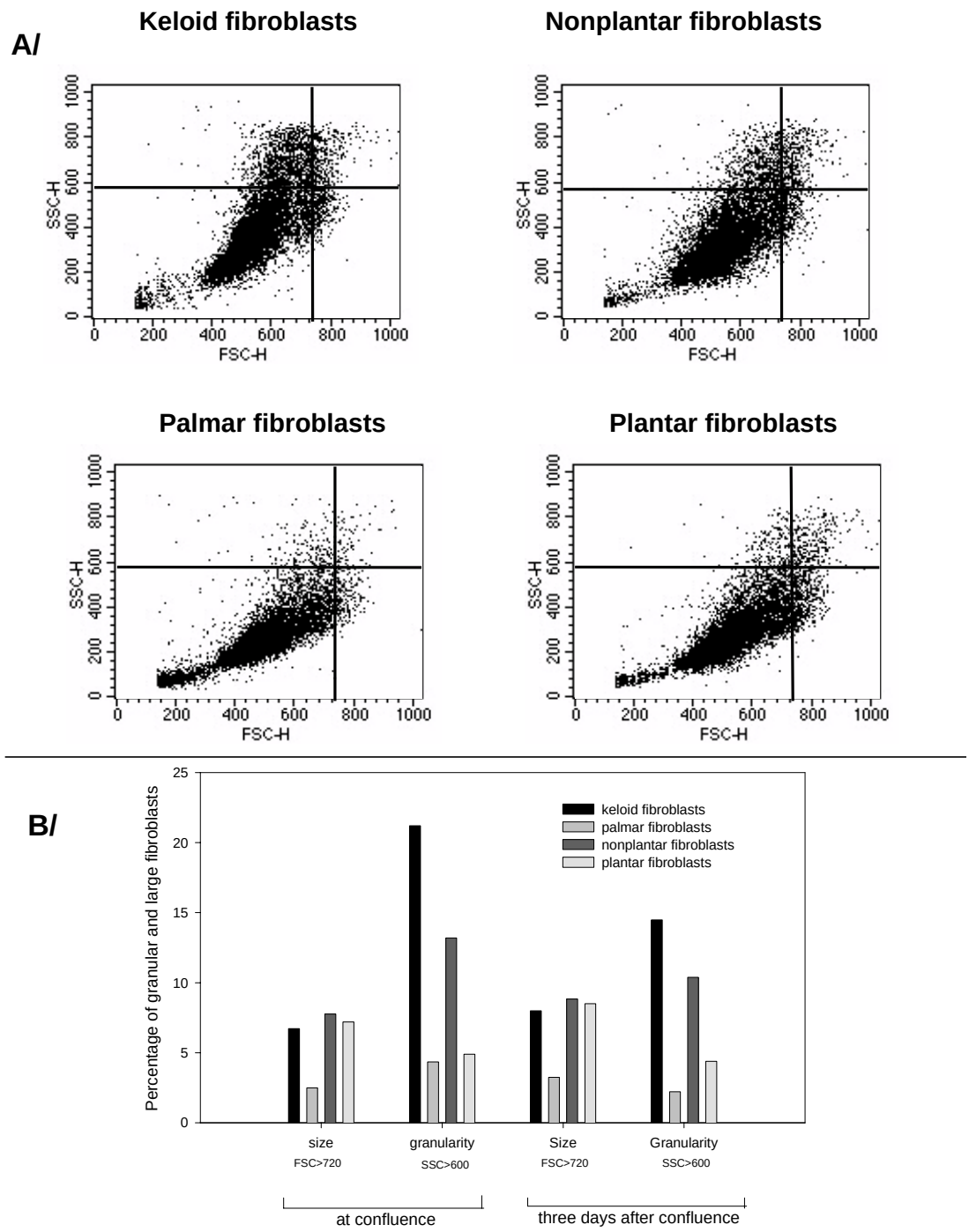

Figure 2
Size and granularity in fibroblast cultures from different origin. A/: Forward (FSC-H) and side scattering (SSC-H) data from FACS analysis of cultures grown with serum for three days after confluence. **B/**: Difference in size (the total percentage of cells with forward scattering in the Upper and Lower Right quadrants) and granularity (the total percentage of cells with side scattering in the Upper Left and Right quadrants). The FSC and SSC intervals are arbitrarily chosen.

As shown in Fig. 2A and 2B, PP cultures can also be distinguished by a paucity of fibroblasts with high granularity (side scattering), a characteristic often associated with the elaboration of RER (rough endoplasmic reticulum) [44]. In both confluent and post-confluent cultures, the populations of cells with the highest granularity were found in the non-PP isolates (keloid and nonplantar). The differences in proliferation rate and granularity predict and are consistent with differences in protein expression [[36], below]. Palmar, but not plantar, isolates were also found to have smaller cell size (judged by forward scattering, Fig. 2A,2B and light microscopy (data not shown)).

TGFβI receptor and ED-A fibronectin in palmoplantar and non-palmoplantar fibroblasts

Earlier work demonstrated that palmoplantar fibroblasts express less α-SMA than do keloid or to a lesser extent normal non-palmoplantar fibroblasts [36]. As TGFβ1 is a positive regulator of α-SMA, these observations could reflect the relative levels of active TGFβ1 or the levels of those proteins responsible for the transduction of its signal. We therefore measured the expression of α-SMA together with of the expression of those proteins participating in the initial signal transduction event: TGFβ1, its type II receptor (TGFβ1-RII), and ED-A fibronectin. No consistent differences were detected in the levels of intracellular or secreted, latent or active TGFβ1 (data not shown). However, as shown in Figs. 3 (for FACS analysis examples, please, see additional file 1: Additional data #1.ppt), the expression of both TGFβ1-RII and ED-A fibronectin was lower in PP cultures than in non-PP cultures. In contrast to previous reports [21], the level of TGFβ1-RII in keloid fibroblast cultures was similar to that in plantar cultures or even lower than that of normal nonplantar fibroblast cultures – Fig. 3. α-SMA content correlated most closely with the levels of ED-A fibronectin (Fig. 3). For keloid fibroblasts, the size and granularity of the α-SMA expressing cells was similar to those expressing ED-A fibronectin rather than TGFβ1-RII (not shown, please, see additional file 2: Additional data #2.ppt). Cells with the highest granularity expressed TGFβ1-RII, while cells with medium granularity had high levels of α-SMA and ED-A fibronectin (not shown, please, see additional file 2: Additional data #2.ppt). Together with our previous results demonstrating higher levels of thrombospondin-1 in keloid fibroblasts [36], the data suggest that ***keloid fibroblasts (and to a lesser extent normal non-PP fibroblasts) appear primed to more effectively transmit the TGFβ1 signal and thereby to express the myofibroblast phenotype.***

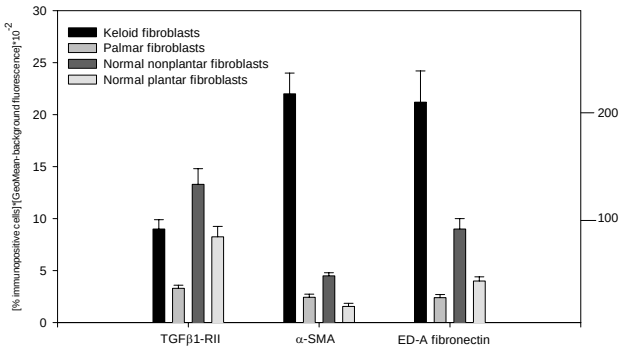

Figure 3
TGFβI Receptor II, ED-A fibronectin and α-Smooth Muscle Actin (α-SMA) in fibroblasts from different body sites. The product of [Percentage immunopositive cells]*[GeoMean of the fluorescence distribution – background] is used to estimate the antigen content. The scale to the right is for the ED-A fibronectin only. (For examples of actual FACS scans, please, see additional file 1: Additional data #1.ppt).

Myofibroblast formation and apoptosis

Resolution of normal dermal wound healing requires a decrease in scar cellularity so as to prevent excessive collagen accumulation and wound contraction. The reduction in cellularity occurs through the process of apoptosis in fibroblasts [28] and possibly myofibroblasts ([29], and see below). To determine the extent to which the myofibroblast phenotype is expressed and the extent to which myofibroblasts undergo apoptosis, α-SMA content and apoptosis were measured simultaneously using two-color FACS analyses. Shown in Fig. 4 are the four classes of fibroblasts which could be distinguished: α-SMA$^-$, apoptosis$^-$ (lower left quadrant LL, "naïve" fibroblasts); α-SMA$^+$, apoptosis$^-$ (upper left quadrant UL, myofibroblasts); α-SMA$^-$, apoptosis$^+$ (lower right quadrant LR, apoptotic fibroblasts) and α-SMA$^+$, apoptosis$^+$ (upper right quadrant UR, apoptotic myofibroblasts). The analyses by FACS of fibroblasts grown directly on tissue culture plates (monolayer cultures) or on collagen I revealed several differences between PP and non-PP cultures in response to confluence, serum, TGFβ1 and embedding within collagen gels (Figs. 5,6,7).

"Monolayer" cultures

Comparisons of confluent cultures with three-day postconfluent cultures revealed an increase in α-SMA levels independent of media serum content – Fig 5A (for examples of the actual FACS analyses, please, see additional file 3: Additional data #3.ppt). The postconfluent PP cultures had considerably less α-SMA than either the keloid or the normal non-PP control (Fig. 5A). These results are consistent with data reported for palatal fibroblasts [43], but contrast with observations on corneal fibroblasts [45]. In the

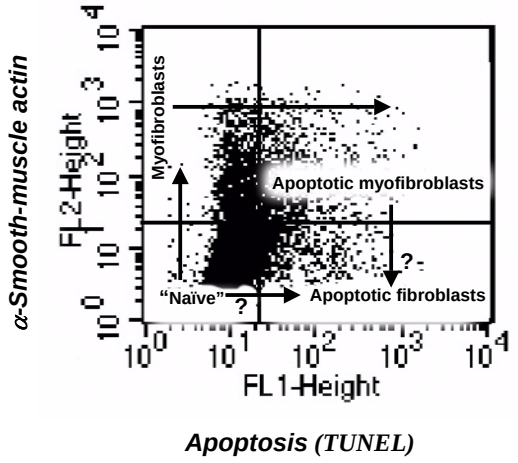

Figure 4
Schematic of the FACS analysis Two color FACS analysis of the levels of: apoptosis, measured by the TUNEL assay (using fluorescein-dUTP) – FL1; α-SMA – FL2, using a Phycoerythrin-conjugated secondary antibody. Quadrants are defined by background fluorescence of fluorescein and phycoerythrin. Lower Left (LL) quadrant – α-SMA-negative, non-differentiated, non-apoptotic ("Naïve") fibroblasts; Upper Left (UL) quadrant – non-apoptotic cells with higher α-SMA content – non-apoptotic myofibroblasts); Upper Right (UR) quadrant – apoptotic myofibroblasts; Lower Right (LR) quadrant – apoptotic fibroblasts who have either lost the myofibroblast phenotype or have not developed it at all. Apoptotic fibroblasts eventually detach from the plate. Question marks point to putative transitions.

absence of exogenous TGFβ1, keloid fibroblasts accumulated the most α-SMA – Fig. 5A.

To determine whether TGFβ1 could eliminate differences in α-SMA levels between PP and non-PP fibroblasts, cultures were incubated at confluence for three days with serum-free or serum-containing media supplemented with 0 ng/ml or 10 ng/ml active TGFβ1, and α-SMA and apoptosis were evaluated (Fig. 5A,5B; for examples of actual FACS analyses, please, see also additional files: Additional data #3.ppt). All fibroblast cultures responded to TGFβ1 with an increased accumulation of α-SMA, although the α-SMA content of PP cultures again remained lower than either the keloid or the normal non-PP isolates. While the two keloid and palmar pairs differed in absolute α-SMA levels, the difference between paired keloid and palmar samples was statistically significant ($p = 0.05$) (not shown, please, see also additional file 4: Additional data #4.ppt). In both paired samples keloid fibroblasts contained more α-SMA than did palmar fibroblasts. Lower concentrations of TGFβ1 (2.5 ng/ml and 5 ng/ml) were also found (data not shown) to be equally effective in up-regulating α-SMA in accordance with other data [45,46].

Simultaneous evaluation of apoptosis was carried out. Serum withdrawal increased the percentage of apoptotic cells (Fig. 5B; for examples of actual FACS analyses, please, see additional files: Additional data #3.ppt) – a considerable number of which were α-SMA positive. The impact of serum withdrawal was found to be greater in PP cultures than in non-PP cultures (Fig. 5B). In addition, during serum starvation about 10% of PP fibroblasts detached and were found floating. FACS analysis demonstrated that these cells were all apoptotic (not shown, see also [36]). Although TGFβ1 acted anti-apoptotically in PP cultures (palmar and normal plantar fibroblasts) and prevented the shedding of cells into the media, its effect was dissimilar in non-PP cultures, where TGFβ1 had either no impact (keloid T and normal nonplantar fibroblasts) or was pro-apoptotic (keloid D cultures).

In the presence of serum, TGFβ1 acted anti-apoptotically only on the normal plantar cultures and was without observable effect on the palmar cultures (Fig. 5B). However, the addition of TGFβ1 to the non-PP cultures always resulted in increased apoptosis in the presence of serum: in keloid D small increases were observed and in keloid T and in normal nonplantar cultures large increases were observed.

Thus, in contrast to the ability of TGFβ1 to induce the expression of α-SMA in all fibroblast cultures, its effect on apoptosis was fibroblast-origin dependent.

Impact of collagen on levels of α-SMA and apoptosis in fibroblasts

Measurements were made of the α-SMA content and apoptotic status of fibroblasts embedded within attached or detached(relaxed) gels incubated for three days in the presence or absence of serum and TGFβ1 (Figs. 6 and 7). The data were compared with those obtained prior to embedding and with three day post confluent monolayer cultures (Fig. 5A,5B). In attached gels, α-SMA content modestly increased in PP fibroblasts (e.g. UL in 6a', Fig. 7A; for the FACS at confluence see examples in Additional data #3). TGFβ1 further induced the expression of α-SMA (e.g. UL+UR in Fig. 6B, Fig. 7A), although in no instance were the levels increased to those observed in the three day postconfluent cultures (Fig 5A). In contrast to PP fibroblasts the α-SMA content of keloid and normal non-PP fibroblasts did not increase post collagen embedding. Even TGFβ1 was modestly effective at increasing α-SMA levels in keloid and normal non-PP fibroblasts (Fig. 7A). The inability of keloid fibroblasts to respond with increased α-SMA to TGFβ1 may be a consequence of their ability to initiate gel contraction (only the central portion

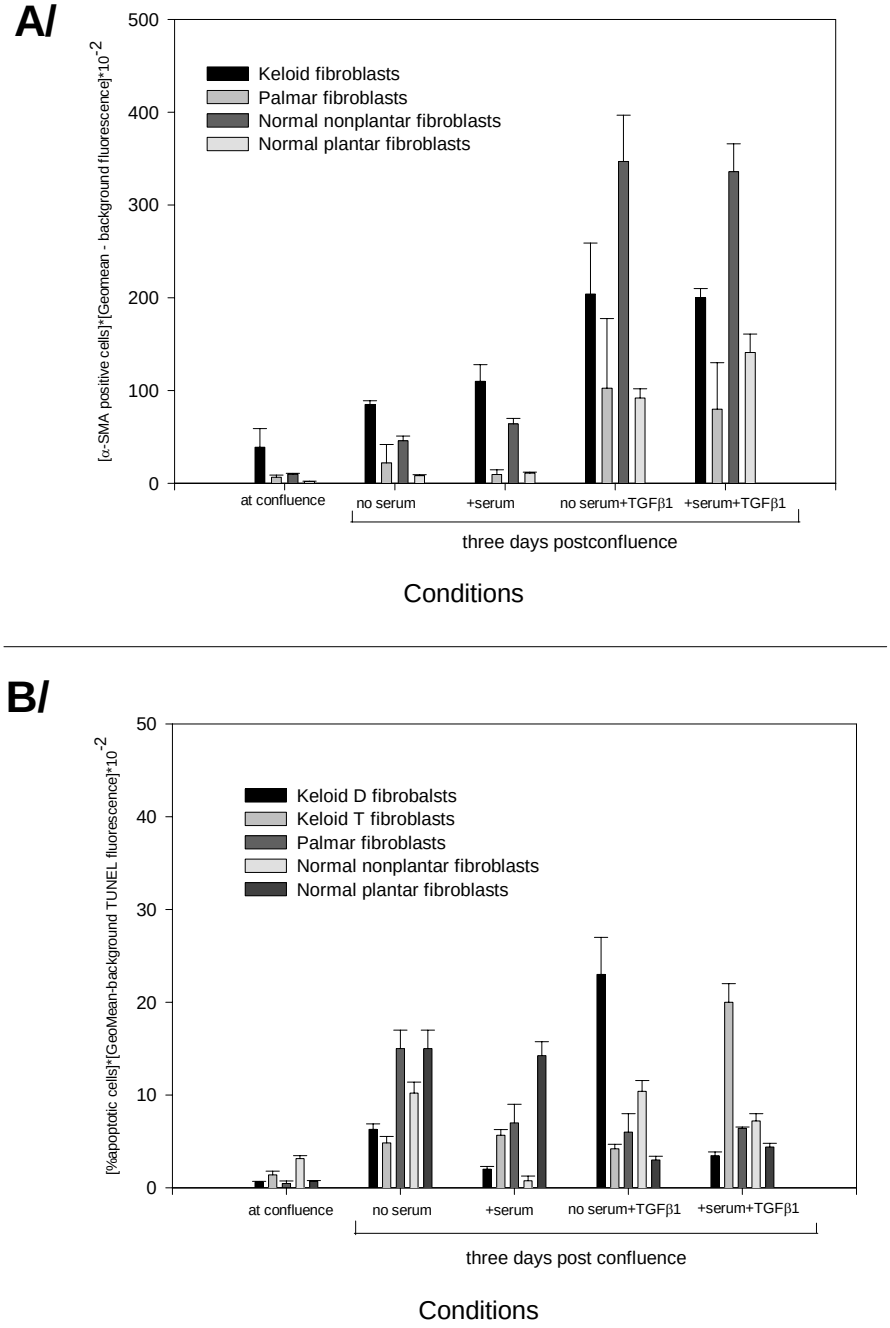

Figure 5

Effect of serum and TGFβ1 on α-SMA and on apoptosis in fibroblasts. Fibroblasts were analyzed as described in Figure 4. (for examples of actual FACS scans, please, see additional file 3: Additional data #3.ppt). Also **A/ α-SMA**: The product of [Percentage immunopositive cells]*[GeoMean of the fluorescence distribution – background] was used to estimate the α-SMA content and includes contributions from both viable myofibroblasts (UL quadrant, see Fig. 4) and apoptotic myofibroblasts (UR quadrant, see Fig. 4). The data are average of three experiments with two keloid-palmar pairs and one nonplantar-plantar pair of fibroblast cultures. **B/ Apoptosis:** The product: [Percentage apoptotic fibroblasts]*[GeoMean TUNEL fluorescence – background] was used to estimate the apoptotic status of the culture with contributions from both fibroblasts (LR) and myofibroblasts (UR). Average of data from three experiments with: two keloids (plotted separately), two palmar cultures (the overall average is plotted) and one normal nonplantar and plantar cultures.

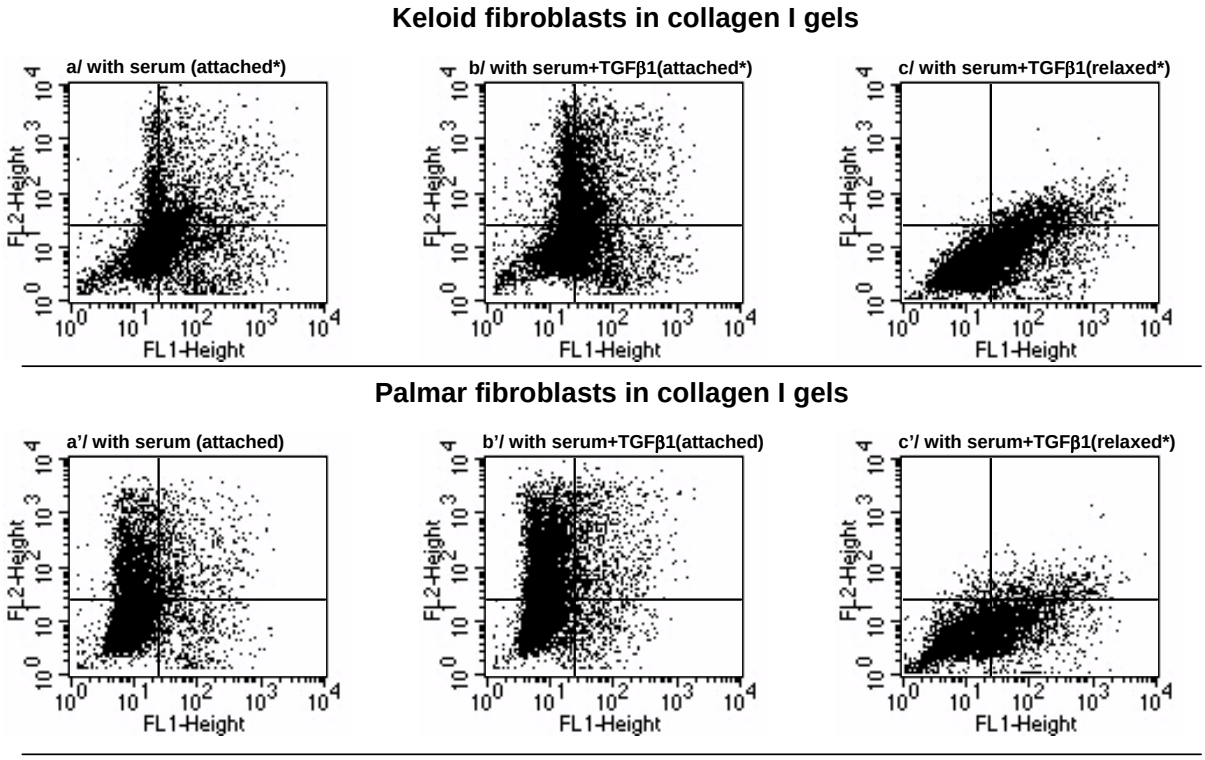

Figure 6
FACS analyses of keloid and palmar fibroblasts embedded in collagen I gels. Keloid (a-c) and palmar (a'-c') fibroblasts in gels incubated for three days either with serum (a, a') or with serum and TGFβ1 (b, b', c, c'). Attached* – gels with keloid fibroblasts (a, b) were partially detached, self shrinking and remaining attached only in the center, while the gels with the palmar fibroblasts were fully attached – (a' b'). Relaxed gels (c, c') were fully floating in the media.

of the gel remained attached) and apoptosis. In relaxed gels there was no detectable increase in α-SMA in palmar fibroblasts and a decrease in α-SMA in keloid fibroblasts. Most of the fibroblasts were apoptotic (e.g. LR in Figs. 6c, 6c'). Similar results were obtained with normal PP and normal non-PP fibroblasts.

The impact of ECM (collagen) on α-SMA accumulation was also evaluated in two other experimental settings (data not shown): monolayer cultures of fibroblasts grown with ascorbic acid to permit collagen secretion, and monolayer cultures of fibroblasts grown on collagen I coated plates. In both settings α-SMA levels were reduced compared to controls grown on noncoated tissue culture plastic or without ascorbic acid (see similar results in [47]). Taken together these results suggest that the presence of collagen and the stressed to relaxed state transition of the ECM alters or dominates TGFβ1 signal transduction (see also [25]). The corresponding reduction in α-SMA is in agreement with recent in vivo data on splintered vs. unsplintered rat wounds [48], but differs from in vitro results on human gingival fibroblasts [49].

When apoptosis was evaluated, it was found to increase significantly in all fibroblast types within attached gels by comparison to the input fibroblasts (Fig. 7B). Many of the apoptotic cells were α-SMA positive (see UR in Fig. 6). Previous reports indicating a lack of apoptosis in attached gels were based on analyses using an ELISA that did not allow detection of events in apoptosis which precede the formation of a nucleosomal ladder [50]. Experiments aimed at evaluating keloid fibroblasts in attached gels were confounded by the ability of these fibroblasts to contract the gels and cause partial gel relaxation with concom-

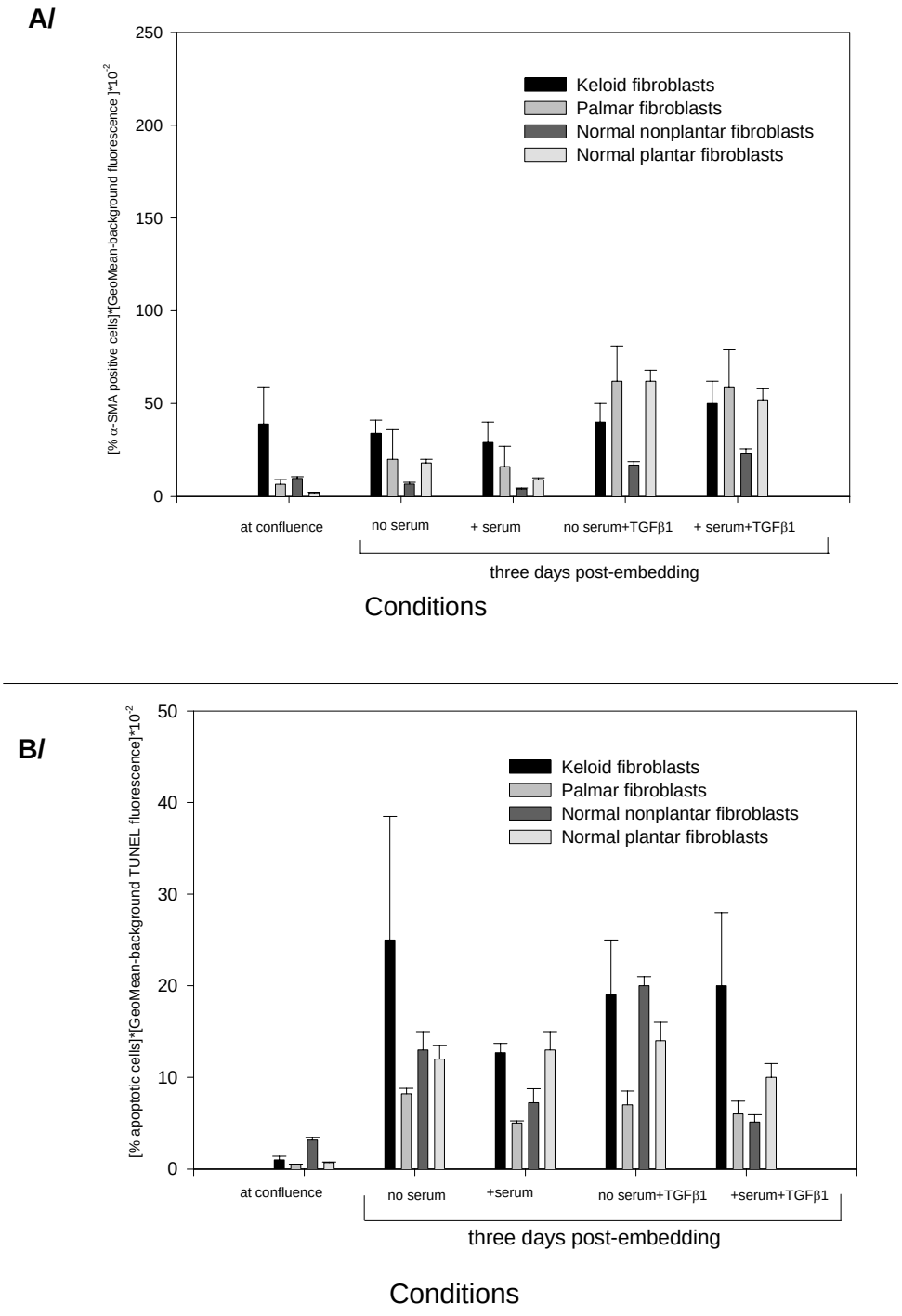

Figure 7

Myofibroblasts and apoptosis of fibroblasts embedded in attached collagen I gels. Summary of all results for fibroblasts grown up to confluence, embedded in collagen gels and harvested after three days. Analysis was as described in Fig. 4 and 5. **A/α-SMA**. The data for the keloid and palmar cultures are average from three experiments with two keloid-palmar pairs and three experiments with a nonlantar-plantar pair; **B/Apoptosis**. Results are averages of three experiments. The average for the keloid cultures are plotted separately. For palmar cultures the average is over experiments and over cultures from two different individuals.

itant apoptosis (Figs. 6a, 7B). Thus, while keloid fibroblasts did not readily undergo apoptosis in response to serum-starvation in monolayer cultures, they underwent the most apoptosis once embedded in attached gels, due to the self-relaxation of these gels. At the same time and under the same conditions the corresponding palmar fibroblasts showed lower apoptosis levels (Figs. 6b, 7B). TGFβ1 had minor and variable effect on apoptosis in attached gels (Fig. 7B).

Upon gel relaxation (i.e. after full detachment) apoptosis significantly increased (LR in Fig. 6c, 6c'). In contrast to monolayer cultures exogenous TGFβ1 did not alter apoptosis (Fig. 6c, 6c', data not shown). This is consistent with other data on gel-embedded foreskin fibroblasts, which led to the conclusion that prior differentiation into myofibroblasts is not required for apoptosis in granulation tissue [41]. Our data on attached gels (when α-SMA positive fibroblasts were present even without exogenous TGFβ1) demonstrated that a considerable percentage of myofibroblasts underwent apoptosis (Fig. 6a, 6a', 6b, 6b', and data not shown). The decrease in percentage of myofibroblasts in the relaxed gels containing keloid fibroblasts (compare Fig. 6b, 6b' with Fig. 6c, 6c') may be due to a gradual turnover of α-SMA in the cells undergoing apoptosis. In the scheme presented in Fig. 4 this process would correspond to the phenotypic conversion of apoptotic myofibroblasts (UR) to apoptotic fibroblasts (LR) by proteolysis of α-SMA. However, during granulation tissue remodeling in vivo, decreased cellularity may be the result of apoptosis of fibroblasts and myofibroblasts [29].

Our findings showed that ***reduction of tension in the ECM provokes apoptosis and reduces α-SMA; as human dermal myofibroblasts readily undergo apoptosis in response to gel relaxation (or wound contraction) their absence from the neodermis of a healed wound is likely the result of apoptosis.***

Conclusions

There are several issues addressed for the first time in this paper relating apoptosis and myofibroblast formation in human dermal fibroblasts.

Phenotypic differences exist among fibroblasts from different dermal origin

- non-palmoplantar (non-PP) or palmoplantar (PP) – normal (nonplantar, plantar, palmar from keloid patient biopsies) or abnormal (from keloid biopsies). Our work demonstrated that keloid fibroblasts had higher cellular levels of several members of the TGFβ1 pathway (TGFβ1-RII and ED-A fibronectin; also thrombospondin-1 [36]). In addition TGFβ1 effects on apoptosis differed in non-PP vs. PP fibroblasts. TGFβ1 acted pro-apoptotically on non-PP fibroblasts and anti-apoptotically on PP fibroblasts. It had a pro-apoptotic effect on myofibroblasts. These results suggest that, at least in part, the difference between keloid and palmar fibroblasts may be due to particular levels of TGFβ1-pathway related proteins at nonglabrous vs. glabrous sites.

Effect of tension in the ECM

- We found that matrix tension was dominant over TGFβ1 in determining myofibroblast formation and apoptosis levels. Apoptotic cells predominated in relaxed gels, independent of TGFβ1 availability. Keloid fibroblasts, once embedded in collagen gels – an environment exerting less tension on the fibroblasts – were more apoptotic than the corresponding palmar fibroblasts. This difference may be attributed to the ability of keloid fibroblasts to contract the collagen gel.

For the exuberant accumulation of collagen during keloid formation, matrix relaxation-induced apoptosis must be delayed. This suggests that body-site specific keloid formation may be dependent on the difference in the development and resolution of tension in the wound bed at nonglabrous vs. glabrous sites in keloid prone patients. Thus a necessary requirement for keloid formation will be maintenance of tension to prevent (delay) apoptosis. This gives sufficient time for the fibroblasts to express and deposit collagen in the ECM. With the accumulation of collagen the tension experienced by the fibroblasts in the keloid will be reduced and apoptosis initiated. According to our data reduced tension would lead to lowering of the α-SMA level in the keloid.

Although myofibroblasts were not detected in vivo [51], the propensity of keloid fibroblasts to express α-SMA in vitro and the loss of this protein upon matrix contraction (gel relaxation) do not allow one to dismiss a role for myofibroblasts in keloids. Rather it may be necessary to re-evaluate α-SMA expression in the early stages of keloid development, when tension within the wound bed is high. Usually the studied keloids are at least several months old and may have had enough time for tension relaxation and loss of α-SMA. Interestingly, keloids in some Caucasian patients were found to contain α-SMA positive fibroblasts [52]. Many of the α-SMA positive cells were spindle-shaped, which suggests that these cells experienced outside tension. Furthermore, persisting tension in the wound bed may explain why hypertrophic scars maintain the myofibroblast phenotype long after wound closure.

Fibroblasts at glabrous sites (palms, soles) do not promote keloid formation consistent with an earlier reduction in cellularity, necessary for a normal wound healing. It is noteworthy that tension/mechanical loading is exerted through the ECM by fibroblasts expressing a "synthet-

ic" fibroblast phenotype [53]. As TGFβ1 supports this phenotype, keloid formation at glabrous sites may be further limited by the PP fibroblast requirement for higher and/or more sustained levels of TGFβ1.

Clinical applications of pressure dressings that relieve wound bed tension are traditionally used to prevent aberrant scar formation during burn wound healing. Comparisons of non-pressure vs. pressure-treated hypertrophic scars have shown that pressure supports the disappearance of α-SMA expressing myofibroblasts [54]. The data presented in this work suggest that body site differences in fibroblasts and wound tension are critical determinants of the keloid-less healing of palmoplantar wounds of keloid-prone individuals.

List of abbreviations

ECM – extracellular matrix; non-PP – non-palmo-plantar, PP – palmo-plantar; Quadrants in the FACS plot: LL – lower left, UL – upper left; LR – lower right; UR – upper right; α-SMA – alpha smooth muscle actin; TCA – tri-chloro-acetic acid; PBS – Phosphate Buffered Saline; EDTA – Ethylene DiamineTetraacetic acid.

Competing interests

None declared.

Authors' contributions

All authors read and approved the final manuscript.

Additional material

Additional File 1

FACS analysis of fibroblasts reacted with primary antibodies, followed by FITC-conjugated secondary antibody. The X and Y axes show the fluorescence intensity and the cell count, respectively. The background fluorescence values (marked by the black bars) were around 20 (in arbitrary fluorescence units) for all secondary antibodies used. Fibroblasts cultures were grown with serum as monolayer cultures and were harvested three days postconfluence. Corresponding graph is presented as Fig. 3 in the main text.

Click here for file

[http://www.biomedcentral.com/content/supplementary/1471-5945-2-13-S1.ppt]

Additional File 2

Correlation of size and granularity with the levels of TGFβ1-Rec II, ED-A and α-SMA in keloid fibroblasts. The fluorescence ranges for each protein were chosen so as to represent fibroblasts with background fluorescence, with intermediate fluorescence and with highest fluorescence. The FACS results are for the samples represented in Additional files #1 and in Fig. 3.

Click here for file

[http://www.biomedcentral.com/content/supplementary/1471-5945-2-13-S2.ppt]

Additional File 3

Two color FACS analysis of apoptosis and myofibroblast formation. Graphs are given in Fig. 5A,5B of the main text. Fibroblasts were analyzed at confluence (a/ – palmar fibroblasts, a'/ – keloid fibroblasts) and three days after confluence under a variety of conditions – without serum (b, b'), – with serum (c, c'), – with exogenous TGFβ1 (10 ng/ml) without (d, d') or with (e, e') serum [See Fig. 4 for details]. Cells with advanced apoptosis have high fluorescence and were found to the right of the broken vertical bar in b, b'. During serum starvation about 10% of PP fibroblasts were found floating (data not shown, see also 36). FACS analysis demonstrated that these cells were all apoptotic with FL1 between 100–200 – i.e. around and to the right of the dash in (b, b'). Corresponding graphs are given in Fig. 5A,5B of the main text.

Click here for file

[http://www.biomedcentral.com/content/supplementary/1471-5945-2-13-S3.ppt]

Additional File 4

The difference in the α-SMA levels for two corresponding pairs non-palmoplantar and palmoplantar fibroblasts Absolute α-SMA levels may vary among fibroblasts from different individuals, but not the differences between paired keloid and palmar samples, and normal nonplantar and plantar samples, i.e. the predominance of α-SMA in non-palmoplantar fibroblasts is statistically significant (p = 0.05). Additional data #4 corresponds to Fig. 5A in the main text.

Click here for file

[http://www.biomedcentral.com/content/supplementary/1471-5945-2-13-S4.ppt]

Additional File 5

A measure for the concentration of a fluorescent marker in a population of FACS analyzed cells.

Click here for file

[http://www.biomedcentral.com/content/supplementary/1471-5945-2-13-S5.doc]

References

1. Tredget EE, Nedelec B, Scott PG: **Hypertrophic scars, keloids, and contractures.** *Surg Clin North Am* 1997, **77:**701-731
2. Urioste SS, Arndt KA, Dover JS: **Keloid and hyperthropic scars: Review and treatment strategies.** *Semin Cutan Med & Surg* 1999, **18:**159-171
3. Dalkowski A, Schuppan D, Orfanos CE, Zouboulis CC: **Increased expression of tenascin C by keloids in vivo and in vitro.** *Br J Dermatol* 1999, **141:**50-56
4. Neely AN, Clendening CE, Gardner J, Greenhalgh DG, Warden G: **Gelatinase activity in keloids and hypertrophic scars.** *Wound Repair Regen* 1999, **7:**166-171
5. Ishihara H, Yoshimoto H, Fujioka M, Murukami R, Hirano A, Fujii T, Ohtsuru A, Namba H, Yamashita S: **Keloid fibroblasts resist ceramide-induced apoptosis by overexpression of insulin-like growth factor I receptor.** *J Invest Dermatol* 2000, **115:**1065-1071
6. Yoshimoto H, Ishihara H, Ohtsuru A, Akino K, Murakami R, Kuroda H, Namba H, Masahiro I, Fujii T, Yamashita S: **Overexpression of insulin-like growth factor-1 (IGF-I) receptor and the invasiveness of cultured keloid fibroblasts.** *Am J Pathol* 1999, **154:**883-889
7. Lim TC, Moochhala SM, Tan Walter TL, Chhatwal VJS, Suhumaran S, Hon WM, Khoo HE: **Downregulation of inducible nitric oxide synthase expression in keloids.** *Plast Reconstr Surg* 1996, **98:**911-912
8. Nirodi CS, Devalaraja R, Nanney LB, Arrindell S, Russel S, Trupin J, Richmond A: **Chemokine and chemokine receptor expression in keloid and normal fibroblasts.** *Wound Repair Regen* 2000, **8:**371-382

9. Messadi DV, Le A, Berg S, Jewett A, Zhuang W, Kelly P, Bertolami CN: **Expression of apoptosis-associated genes by human dermal scar fibroblasts.** *Wound Repair Regen* 1999, **7:**511-517
10. Chin GS, Liu W, Steinbrech D, Hsu M, Levinson H, Longaker MT: **Cellular signaling by tyrosine phosphorylation in keloid and normal human dermal fibroblasts.** *Plast Reconstr Surg* 2000, **106:**1532-1540
11. Akasaka Y, Ishikawa Y, Ono I, Fujita K, Masuda T, asuwa N, Inuzuka K, kiguchi H, Ishii T: **Enhanced expression of caspase-3 in hypertrophic scars and keloid: Induction of caspase-3 and apoptosis in keloid fibroblasts in vitro.** *Lab Invest* 2000, **80:**345-357
12. Shah M, Revis D, Herrick S, Baillie R, Thorgeirson S, Ferguson M, Roberts A: **Role of elevated plasma TGFβ1 levels in wound healing.** *Am J Pathol* 1999, **154:**1115-1124
13. Frank S, Madlener M, Werner S: **TGF β1 β2 and β3 and their receptors are differentially regulated during normal and impared wound healing.** *J Biol Chem* 1996, **271:**10188-10193
14. Streuli CH, Schmidhauser C, Kobrin M, Bissell MJ, Derynck R: **Extracellular matrix regulates expression of the TGFβ1 gene.** *J Cell Biol* **120:**253-260
15. Mikulec AA, Hanasono MM, Lum J, Kadleck JM, Kita M, Koch RJ: **Effect of tamoxifen on transforming growth factor β1 production by keloid and fetal fibroblasts.** *Arch Facial Plast Surg* 2001, **3:**111-114
16. Nowak KC, McCormack M, Koch RJ: **The effect of superpulsed carbon dioxide laser energy on keloid and normal dermal fibroblast secretion of growth factors: a serum free study.** *Plast Reconstr Surg* 2000, **105:**2039-2048
17. Narani N, Arora PD, Lew A, Luo L, Glogauer M, Ganss B, McCulloch CAG: **TGF-β induction of α-smooth muscle actin is dependent on the deformability of the collagen matrix.** *Curr Top Pathol* 1997, **93:**47-60
18. Chau D, Mancoll JS, Lee S, Zhao J, Phillips LG, Gittes GK, Longaker MT: **Tamoxifen downregulates TGFβ production in keloid fibroblasts.** *Ann Plast Surg* 1998, **40:**490-493
19. Younai S, Venter G, Vu S, Nichter L, Nimni M, Tuan T-L: **Role of growth factors in scar formation: an in vitro analysis.** *Ann Plast Surg* 1996, **36:**495-501
20. Lee TY, Chin GS, Kim WJH, Chau D, Gittes GK, Longaker MT: **Expression of TGFβ1, 2 and 3 proteins in keloids.** *Ann Plast Surg* 1999, **43:**179-184
21. Chin GS, Liu W, Peled Z, Lee TY, Steinbrech DS, Hsu M, Longaker MT: **Differential expression of TGFβ receptor I and II and activation of Smad 3 in keloid fibroblasts.** *Plast Reconstr Surg* 2001, **108:**423-429
22. Nakajima H, Kishi T, Tsuchiya , Yamada H, Tajima S: **Exposure of fibroblasts derived from keloid patients to low-energy electromagnetic fields.** *Ann Plast Surg* 1997, **39:**536-541
23. Grinnell F: **Fibroblasts, myofibroblasts, and wound contraction.** *J Cell Biol* 1994, **124:**401-404
24. Desmoulière A: **Factors influencing myofibroblast differentiation during wound healing and fibrosis.** *Cell Biol Int* 1995, **19:**471-476
25. Grinnell F: **Fibroblast-collagen-matrix contraction: growth factor signaling and mechanical loading.** *Trends Cell Biol* 2000, **10:**362-5
26. Serini G, Bochaton-Piallat M-L, Ropraz P, Geinoz A, Borsi L, Zardi L, Gabbiani G: **The fibronectin domain ED-A is crucial for the myofibroblastic phenotype induction by TGFβ1.** *J Cell Biol* 1998, **142:**873-881
27. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA: **Myofibroblasts and mechanoregulation of connective tissue remodeling.** *Nature Rev Moll Cell Biol* 2002, **3:**349-363
28. Greenhalgh DG: **The role of apoptosis in wound healing.** *Int J Biochem Cell Biol* 1998, **30:**1019-1030
29. Desmoulière A, Redard M, Darby I, Gabbiani G: **Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar.** *Am J Pathol* 1995, **146:**56-66
30. Appleton I, Brown NJ, Willoughby DA: **Apoptosis, necrosis and proliferation:Possible implications in the etiology of keloids.** *Am J Pathol* 1996, **149:**1441-1447
31. Sayah DN, Soo C, Shaw WW, Watson J, Messadi D, Longaker MT, Zhang X, Thing K: **Downregulation of apoptosis-related genes in keloid tissues.** *J Surg Res* 1999, **87:**209-216
32. Ladin DA, Hou Z, Patel D, McPhail M, Olson JC, Saed GM, Fivenson DP: **p53 and apoptosis alterations in keloids and keloid fibroblasts.** *Wound Repair Regen* 1998, **6:**28-37
33. Teofoli P, Barduagni S, Ribuffo M, campanella A, De Pitta O, Puddu P: **Expression of Bcl-2, p53, c-jun and c-fos protooncogenes in keloids and hypertrophic scars.** *J Dermatol Sci* 1999, **22:**31-37
34. Luo S, Benathan M, Raffoul W, Panizzon RG, Egloff DV: **Abnormal balance between proliferation and apoptotic cell death in fibroblasts derived from keloid lesions.** *Plast Reconstr Surg* 2001, **107:**87-96
35. Choudon T, Sugihara T, Igawa HH, Funayama E, Furukawa H: **Keloid-derived fibroblasts are refractory to fas-mediated apoptosis and neutralization of autocrine TGFβ1 can abrogate this resistance.** *Am J Pathol* 2000, **157:**1661-1669
36. Chipev CC, Simman R, Hatch G, Katz AE, Siegel DM, Simon M: **Myofibroblast phenotype and apoptosis in keloid and palmar fibroblasts in vitro.** *Cell Death Differ* 2000, **7:**166-176
37. Zhang H-Y, Phan SH: **Inhibition of myofibroblast apoptosis by TGFβ1.** *Am J Respir Cell Mol Biol* 1999, **21:**658-665
38. Zhang H-Y, Gharaee-Kermani M, Phan SH: **Regulation of Lung fibroblast α-smooth muscle actin expression, contractile phenotype and apoptosis by IL-1β.** *J Immunol* 1997, **158:**1392-1399
39. Funato N, Moruyama K, Baba Y, Kuroda T: **Evidence for apoptosis induction in myofibroblasts during palatal mucoperiosteal repair.** *J Dent Res* 1999, **78:**1511-1517
40. Fluck D, Querfeld C, Cremer A, Niland S, Krieg T, Sollberg S: **Normal human primary fibroblasts undergo apoptosis in three-dimensional contractile collagen gels.** *J Invest Dermatol* 1998, **110:**153-157
41. Grinnell F, Zhu M, Carlson MA, Abrams JM: **Release of mechanical tension triggers apoptosis of human fibroblasts in a model of regressing granulation tissue.** *Exp Cell Res* 1999, **248:**608-619
42. Sato H, Suzuki A, Funahashi M, Takezawa T, Ogawa Y, Yoshizato K: **Characterization of growth, morphology, contractility, and protein expression of fibroblasts derived from keloid.** *Wound Repair Regen* 1996, **4:**103-114
43. Kikuchi K, Kadono T, Takehara K: **Effects of various growth factors and histamine on cultured keloid fibroblasts.** *Clin Lab Invest* 1995, **190:**4-8
44. Morishima Y, Nomura A, Uchida Y, Noguchi Y, Sakamoto T, Ishii Y, Goto Y, Masuyama K, Zhang MJ, Hirano K, Mochizuki M, Ohtsuka M, Sekizawa K: **Triggering the induction of myofibroblast and fibrogenesis by airway epithelial shedding.** *Am J Respir Cell Mol Biol* 2001, **24:**1-11
45. Gentilhomme E, Neveux Y, Lebeau J, Desmouliere A, Bergier J, Schmitt D, Haftek M: **Modulation of a fibrotic process induced by transforming growth factor beta-1 in dermal equivalents.** *Cell Biol Toxicol* 1999, **15:**229-39
46. Grinnell F, Chin-Han Ho: **Transforming Growth factor β stimulates fibroblast-collagen matrix contraction by different mechanisms in mechanically loaded and unloaded matrices.** *Exp Cell Res* 2002, **273:**248-255
47. Ehrlich HP, Cremona O, Gabbiani G: **The expression of α2β1 integrin and α smooth muscle actin in fibroblasts grown on collagen.** *Cell Biochem Funct* 1998, **16:**129-137
48. Hinz B, Mastrangelo D, Iselin CE, Chaponnier C, Gabbiani G: **Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation.** *Am J Pathol* 2001, **159:**1009-1020
49. Arora PD, Narani N, McCulloch CAG: **The compliance of collagen gels regulates TGFβ induction of α-smooth muscle actin in fibroblasts.** *Am J Pathol* 1999, **154:**871-882
50. Niland S, Cremer A, Fluck J, Eble JA, Krieg T, Solberg S: **Contraction-dependent apoptosis of normal dermal fibroblasts.** *J Invest Dermatol* 2001, **116:**686-692
51. Ehrlich HP, Desmouliere A, Diegelmann RF, Cohen IK, Compton CC, Garner WL, Kapanci Y, Gabbiani G: **Morphological and immunochemical differences between keloid and hypertrophic scar.** *Am J Pathol* 1994, **145:**105-113
52. Santucci M, Borgognoni L, Reali UM, Gabbiani G: **Keloids and hypertrophic scars of caucasians show distinctive morphologic and immunophenotypic profiles.** *Virchows Arch* 2001, **438:**457-463
53. Kessler D, Dethlefsen S, Haase I, Plomann M, Hirche F, Krieg T, Eckes B: **Fibroblasts in mechanically stressed collagen lattices as-**

sume a "synthetic" phenotype. *J Biol Chem* 2001, **276:**36575-36585

54. Costa AMA, Peyroll S, Porto LC, Comparin J-P, Foyatier J-L, Desmoulière A: **Mechanical forces induce scar remodeling: study in non-pressure-treated versus pressure-treated hypertrophic scars.** *Am J Pathol* 1999, **155:**1671-1679

Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-5945/2/13/prepub