Dissecting the roles of endothelin, TGF-β and GM-CSF on myofibroblast differentiation by keratinocytes

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Summary
Myofibroblasts are specialized fibroblasts that contribute to wound healing by producing extracellular matrix and by contracting the granulation tissue. They appear in a phase of wound healing when the dermis strongly interacts with activated epidermal keratinocytes. Direct co-culture with keratinocytes up-regulates TGF-β activity and also induces fibroblast to differentiate into α-smooth muscle actin (αSMA)-positive myofibroblasts. TGF-β activity alone cannot completely account for αSMA induction in these co-cultures, and here we analyze mechanical force generation, another potent inducer of myofibroblast differentiation in this model. Using deformable silicone substrates, we show that contractile activity of fibroblasts is already induced after 1-2-days of co-culture, when fibroblasts are generally αSMA negative. Endothelin-1 (ET-1), the most potent inducer of smooth muscle cell contraction, was up-regulated in co-cultures, while blocking ET-1 with the ET receptor inhibitor PD156252 inhibited contraction in these early co-cultures. In 4-5 days of co-culture, however, fibroblast contractile activity correlated with an increased expression of αSMA expression. Stimulation of fibroblast mono-cultures with ET-1 in a low serum medium did not induce αSMA expression; however, ET-1 did synergize with TGF-β. Surprisingly, GM-CSF, another mediator stimulating myofibroblast differentiation in granulation tissue, inhibited αSMA expression in fibroblasts, co-stimulated with TGF-β and ET-1. GM-CSF activated NFκB, thus interfering with TGF-β signaling. Blocking TGF-β and ET-1 largely impaired αSMA induction in co-cultures at day 7 and, in combination, almost completely prevented αSMA induction. Our results dissect the roles of TGF-β and ET-1 on mechanical force generation in keratinocyte-fibroblast co-cultures, and identify GM-CSF as an inducer of myofibroblasts acting indirectly.

Keywords
Wound healing, myofibroblasts, endothelin, TGF-β, GM-CSF

Introduction
Wound healing is a complex response of the organism aiming to restore tissue integrity and organ function. In skin, vascular and inflammatory changes predominate for the first 3-5 days after wounding. The following granulation phase is characterized by new connective tissue production and re-epithelialization (1). In the mid-phase of cutaneous tissue repair, fibroblasts that have invaded the granulation tissue differentiate into myofibroblasts by acquiring smooth muscle cell (SMC) characteristics (2, 3). In
particular, they express α-smooth muscle actin (αSMA), the actin isofom found in vascular SMC, but not in normal fibroblasts (4). It is being increasingly accepted that expression of αSMA confers a high contractile activity and the capacity to contract the wound tissue to myofibroblasts; in addition, myofibroblasts are the principal producers of extracellular matrix (ECM) components in granulation tissue (5).

TGF-β has been identified as the primary mediator to induce myofibroblast differentiation and αSMA expression (6). However, this TGF-β action depends on the presence of a specific ECM, such as ED-A fibronectin (7), and on mechanical tension (8). The level of myofibroblast differentiation increases with increasing stiffness of two-dimensional culture substrates (9, 10) and of three-dimensional collagen gels (9, 11). Increasing mechanical tension in vivo by splinting the edges of open rat wounds with a plastic frame, increases αSMA expression in the granulation tissue (12). In contrast, relaxing fibroblast-populated stressed collagen gels (13) and splinted wounds (12) decreases the level of myofibroblast differentiation. Furthermore, relaxation of stressed collagen gels was shown to induce programmed cell death of fibroblasts (14, 15), which is a proposed mechanism to remove myofibroblast from the healed wound (16), resulting in a mature scar. A mutual interaction between TGF-β signaling and mechanical stimulation has been demonstrated for fibroblasts grown in the presence of TGF-β on collagen substrates with varying stiffness; TGF-β was less effective in inducing α-SMA expression on compliant substrates compared to rigid substrates (9). On the other hand, injecting mechanically stressed granuloma pouches (a myofibroblast-rich model tissue for fibrosis) with TGF-β antagonists reduced the number of myofibroblasts (12). Thus, myofibroblast differentiation appears to require both, TGF-β and mechanical tension and the lack of one factor leads to reduced levels of αSMA expression (8).

During wound repair two peaks of TGF-β activity have been demonstrated, occurring early during the inflammatory response and, later, in conjunction with the appearance of contractile myofibroblasts in the wound, respectively (17). The phase of granulation tissue contraction correlates with activated keratinocytes, migrating from the wound edges in order to re-epithelialize the wound. Thus, it is conceivable that keratinocyte-fibroblast interactions play an important role in myofibroblast induction. We have recently observed that in direct co-culture with keratinocytes, fibroblasts acquire a myofibroblastic phenotype. After 4 days of co-culture, fibroblasts start to express αSMA and the majority of the mesenchymal cells differentiate into αSMA-positive myofibroblasts after 7 days. In co-cultures, the levels of latent and activated TGF-β are both up-regulated. Inhibition of TGF-β activity with neutralizing antibodies reduces αSMA expression significantly, but not completely (Shepard et al., in press).

In this study, we were interested in linking growth factor pathways with contractile activity in our co-culture model. We used deformable silicone substrates which, upon contraction by cells, produce wrinkles in phase contrast microscopy (18). Surprisingly, we found early stimulation of contractile activity in fibroblasts co-cultured with keratinocytes at a time point when most of the fibroblasts have not yet acquired αSMA expression. We identified endogenous production of endothelin activity in co-cultures as one of the principal stimulators closely in line with its potent vasoconstrictive properties (19). Endothelins are also involved in αSMA regulation as in hepatic stellate cells during liver fibrosis (20). When analyzing contraction at time points when most of the fibroblasts differentiated into myofibroblasts, we found that ET-1 synergizes with TGF-β on αSMA induction, albeit less efficiently when compared with keratinocyte co-culture. To this end, GM-CSF was also included in this study since it was shown to enhance granulation tissue formation in vivo (21, 22). Furthermore, local application of GM-CSF augments tissue repair in wounds which are difficult to heal (23, 24), and it was shown to be highly up-regulated in keratinocyte-fibroblast co-cultures (25). Most surprisingly, GM-CSF was shown to have an αSMA-suppressing effect on fibroblasts stimulated with TGF-β, ET-1 or a combination of both, probably by activating NFkB and interfering with TGF-β signaling.

Materials and methods

Cell isolation and culture

Human dermal fibroblasts (HDF) were obtained from adult skin explants and isolated as previously described (26). HDF were grown in DMEM (Gibco BRL, Eggenstein, Germany) containing 10% FCS, 50 μg/ml ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. HDF were used between passages 6 and 8. Growth arrested HDF were obtained by γ-irradiation with 70 Gy from a cobalt source. The human keratinocyte cell line, HaCaT (27) was kindly provided by Prof. Dr. N. E. Fusenig (DKFZ, Heidelberg, Germany) and cultured in the same medium as HDF between passages 40 and 60. For immunofluorescence studies, cultures were established on gelatin coated glass slides (Greiner, Solingen, Germany) or coverslips.

Antibodies, growth factors and inhibitors

The mouse anti αSMA monoclonal antibody (clone 1A4) (4), ET-1, the endothelin receptor A and B antagonist hexapeptide PD156252 (28) and FITC coupled phalloidin were purchased from Sigma (Deisenhofen, Germany), human TGF-β1 (platelet derived and recombinant) and the neutralizing mouse anti-TGF-β1, 2, 3 mAb (clone 1D11) were obtained from R&D systems (Wiesbaden-Nordenstadt, Germany). Polyclonal rabbit anti-keratin serum was purchased from Dako (Hamburg,
Germany). Recombinant GM-CSF (Leukomax®) was obtained from Novartis (Nürnberg, Germany) and used at 100 ng/ml unless otherwise indicated. Hybridoma cells expressing the mouse anti-human integrin α1 monoclonal antibody (clone TS 2/7/1.1, (29)) were obtained from ATCC (Manassas, VA, USA). As secondary antibodies, we used Cy3 coupled sheep anti-mouse IgG (Sigma, Deisenhofen, Germany), hors eradish peroxidase-coupled rabbit anti-mouse IgG (Dako Diagnostika, Hamburg, Germany) and Alexa 488nm coupled goat anti mouse IgG (Molecular Probes, Göttingen, Germany).

Co-culture stimulation and inhibition experiments
Keratinocyte-fibroblast co-cultures were initiated by plating normal or irradiated HDF at 1.5 × 10⁶ cells/cm² in 15 cm tissue culture dishes or in 6 wells plates in DMEM, 10% FCS. After overnight attachment, 2.5 × 10³ HaCaT keratinocytes/cm² were added (yielding a ratio of 1:6 keratinocytes:fibroblasts). DMEM containing 10% or 0.5% FCS, 50 µg/ml ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin was used as indicated and then changed daily. For growth factor stimulation or inhibition experiments, cultures were washed three times with plain DMEM without FCS followed by addition of fresh DMEM/0.5% or DMEM/10% FCS again as indicated containing growth factors or inhibitors at the indicated concentration. Medium was subsequently replaced every two days.

Separation of fibroblasts from co-cultures
Fibroblasts were harvested from co-cultures using paramagnetic particle immunoseparation or selective detachment. For immunoseparation, fibroblasts were labeled with paramagnetic beads (Dynal, Hamburg, Germany), coated with immobilized mouse anti-α1-integrin mAb in PBS/1% BSA (6:1 ratio beads: fibroblast). Cultures were washed three times, inspected microscopically for specific binding and then trypsinized. Fibroblasts were retained from the keratinocyte-fibroblast suspension by applying magnetic force and extensive washings. For selective detachment, co-cultures were incubated in PBS-EDTA (0.05% w/v) followed by gentle pipetting. Dislodged fibroblasts were recovered from the still tightly-attached keratinocytes, and concentrated by centrifugation at 200 g for 10 min. (26).

Northern blotting
Fibroblasts were selectively recovered from co-cultures by paramagnetic separation; total RNA was isolated using Qiagen RNA columns (Qiagen, Hilden, Germany) and 10 µg were size fractionated on 1% agarose gels containing 1% formaldehyde running in MOPS buffer (0.02 M MOPS, 5 mM sodium acetate, 0.1 mM EDTA, 1 M formaldehyde). After blotting onto nylon membranes, hybridization was performed using Hybrimax solution (Ambion, Huntingdon, UK) according to the manufacturer’s instructions. cDNA probes used for hybridization were an ET-1 1,200 bp EcoRI cDNA fragment (19), and a GAPDH 1,400 EcoRI fragment, kindly provided Dr. E. Schwarz, DFKZ, Heidelberg, Germany. Random radioactive labeling of the cDNA hybridization probes followed the manufacturer’s instructions using the readiprime II random labeling system (Amersham Pharmacia Biotech, Freiburg, Germany). Radioactive signals were visualized on X-ray films with intensifying screens.

Electric mobility shift assays
Confluent fibroblast cultures were stimulated with 100 ng/ml GM-CSF in DMEM/10% FCS, and nuclear extracts were prepared after 1 and 2 hours (30). Each nuclear extract containing 5 µg protein was preincubated with poly (dl-dC) for 15 min. To test for NF-κB binding activity the mixture was incubated with a 32P-labeled double-stranded 30-bp probe from the mouse kappa light chain enhancer containing one NF-κB site (31). Samples were run on a 4% polyacrylamid gel and analyzed by autoradiography.

Protein detection and quantification
For immunostaining, cells cultured on glass slides were fixed in 3% PBS-buffered paraformaldehyde for 10 min and permeabilized with Triton X-100 in PBS. Blocking was achieved by preincubation in PBS/10% BSA for 1 hour at room temperature. Primary antibodies were diluted in PBS/1% BSA and added for 1 hour at room temperature and slides were mounted using FA mounting fluid. For Western blotting, normal or irradiated HDF were isolated from co-cultures by PBS-EDTA treatment. Co-cultured as well as control fibroblasts were lysed in RIPA buffer and protein concentration determined by the BCA kit (Pierce, Rockford, USA). Reducing 10% SDS-PAGE electrophoresis was carried out according to (32) with 5 µg protein loaded per lane. Figure 6 was done with normal fibroblasts directly lysed in sample buffer. Gels were run in duplicate: one gel was stained with SERVA blue (Serva, Heidelberg, Germany), the other was blotted and transfer efficiency checked by Ponceau Red staining (Sigma, Deisenhofen, Germany). After blocking in PBS/5% (w/v) non-fat milk powder and 0.5% Tween (v/v) for 1 hour at room temperature, primary antibodies were incubated for 1 hour at room...
temperature. Following three washes in PBS-0.5% Tween (v/v), bound primary antibody was developed by horseradish peroxidase-coupled rabbit anti-mouse IgG, diluted 1:1,500 in blocking buffer, and subsequent detection with chemiluminescence using the ECL kit (Amersham/Pharmacia Biotech, Freiburg, Germany); chemiluminescent signals were recorded on X-ray films.

ELISA measurements of ET-1
For ET-1 determinations in culture supernatants an ELISA-kit was obtained from Amersham (Amersham/Pharmacia Biotech, Freiburg, Germany). Conditioned medium was clarified by centrifugation and used in the assay performed according to the supplier’s recommendations. This ELISA cross-reacts with endothelin-2, but not endothelin-3. Furthermore, precursor peptides such as big ET-1 and -2 are not recognized and react with less than 0.07%.

Contraction experiments on deformable silicone substrates
Deformable silicone substrates were prepared essentially according to (18), with a stiffness restricting the formation of surface wrinkles to highly contractile cells (33). Briefly, 35 µl of silicone (poly-dimethyl-siloxane; 30,000 centistokes; Dow Corning, Midland, MI) were placed in the center of a 22 × 22 mm coverslip. After incubation for 30 minutes in vacuum to remove trapped air bubbles, the silicone surface on the coverslips was cross-linked by heating in a Bunsen flame for 1 second. The coverslips were sealed with a 20 mm diameter rubber ring using silicone glue (732, Dow Corning, Wiesbaden, Germany) to form a tissue culture chamber. The devices were sterilized by UV-irradiation and coated with 1% gelatin in double distilled water for 24 hours at 37°C. Fibroblasts were seeded at 5,000 cells/cm² and the next day 1.500 HaCaT keratinocytes/cm² were added to prepare co-cultures. Fibroblast contractile activity resulted in the formation of wrinkles in the silicone substrate and was controlled daily and documented after 1 day and again after 5 days. To block ET-1 activity, PD156252 was added at 9 µg/ml to co-cultures. All experiments were performed at least three times.

Results

Fibroblasts exhibit increased contractile activity in co-culture with keratinocytes
To analyze the contractile activity of fibroblasts in the presence or absence of HaCaT keratinocytes, we cultured the cells at low density on gelatin-coated deformable silicone substrates in DMEM with 10% FCS. Cultures were checked daily for appearance of surface wrinkles, which occur when cells exert high

Figure 1: Wrinkle formation on deformable silicone substrates. Fibroblasts monocultures (A, C) or HaCaT-fibroblast co-cultures (B, D) were seeded on deformable silicone substrates. After 1 day pictures A and B were taken. Wrinkle formation was largely confined to HaCaT-fibroblast co-cultures and there only observed with fibroblasts. In B, keratinocytes are marked by arrows. After 5 days (C and D) wrinkles were predominant in HaCaT-fibroblast co-cultures. The localization of wrinkle forming cells was most obvious in fibroblasts in close proximity to HaCaT colonies (encircled area). Bar: 100 mm.
contractile forces to their deformable substrate. Fibroblasts in mono-cultures exhibited few if any wrinkles at all investigated time points (Fig. 1A and C); in contrast, fibroblasts co-cultured with HaCaT cells (Fig. 1B, D) clearly produced wrinkles, which were particularly prominent in close vicinity to keratinocyte colonies (encircled area) after 5 days of co-culture (Fig. 1D). Keratinocytes never produced surface wrinkles at any condition tested (data not shown). Co-cultured fibroblasts appeared to show a biphasic pattern in their contractile activity over the whole observation period. A first activity peak was observed after 1-2 days (Fig. 1B), followed by a relaxation period at day 3 (not shown) and gradually increased again to a second contractile peak, most prominent after 5 days (Fig. 1D). Wrinkle formation by fibroblasts in mono-culture was also induced by adding conditioned media from co-cultures for 1 day, which was, however less efficient compared with co-cultures (Table 1). In contrast, conditioned media from mono-cultures of fibroblast and HaCaT cells did not induce wrinkle formation.

We were interested to see whether increased fibroblast contractile activity in co-culture with HaCaT cells correlated with expression of αSMA. After 1 day in mono- and in co-culture, αSMA positive myofibroblasts occurred only occasionally (Fig. 2A, D). Fibroblast developed α-SMA-negative stress fibers in both conditions; however, contraction was restricted to fibroblasts in co-cultures. Later, at day 4 and day 7 αSMA expression was clearly up-regulated in co-cultured fibroblasts starting in close proximity to keratinocyte colonies (Fig. 2B, C), whereas in mono-cultures few cells were αSMA positive (Fig. 2E, F). Thus, early co-cultures appear to contain factors that stimulate high fibroblast contractile activity in the absence of αSMA expression.

**ET-1 is produced in co-cultures of keratinocytes and fibroblasts**

Which endogenous mediator could induce contractile activity in keratinocyte fibroblast co-cultures? Preliminary cDNA chip

### Table 1: Wrinkle formation in fibroblast, HaCaT keratinocyte mono-cultures and HaCaT-fibroblast co-cultures.

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<thead>
<tr>
<th>Direct Cell-Cell Contact</th>
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| 5 ± 4 | **Figure 2: Stress fiber and αSMA expression in HaCaT-fibroblast co-cultures.**

HaCaT keratinocyte-fibroblast co-cultures (A, B, C) as well as fibroblast control mono-cultures (D, E, F) were stained for stress fibers with phalloidin (green), αSMA (red) and for visualization of keratinocyte colonies for keratins (blue). A and D correspond to 1 day of culture. αSMA positive cells were only seen occasionally, when wrinkle formation was already occurring with high frequency in co-cultures. In co-cultures at day 4 (B, E) αSMA positive cells were increasing and located mostly in close proximity to keratinocyte colonies (encircled) while at 7 days (C, F) most of the co-cultured fibroblasts differentiated into myofibroblasts. Bar corresponds to 100 µm.
hybridization experiments suggested an up-regulation of ET-1 transcripts in co-cultures (Shephard, et al., in press). Indeed, Northern blot experiments confirmed that ET-1 transcripts were induced in fibroblasts upon co-culture with keratinocytes (Fig. 3A), while fibroblast mono-cultures or HaCaT cells, both in mono and co-cultures expressed negligible levels of ET-1 transcripts (Fig. 3A and data not shown). In order to be biologically active, the ET-1 pro-form needs to be processed to the mature 21 AA peptide. We measured processed ET-1 in a conditioned medium using a 21 AA peptide-specific ELISA test. In line with the hybridization data, we observed a more than 3-fold increase in active ET-1 in co-cultures compared with the respective mono-culture controls at every 48h-interval tested throughout the 6-day observation period (Fig. 3B). The ELISA used, cross reacts with endothelin-2, and it cannot be ruled out that some of the signals may derive from endothelin-2.

**Inhibition of ET-1 activity reduces fibroblast contraction in early co-cultures**

We next questioned whether ET-1 activity is responsible for up-regulating fibroblast contraction in keratinocyte co-cultures or in conditioned co-culture medium by using the endothelin A and B receptor specific antagonist PD156252. PD156252 inhibited fibroblast contraction in 1 day of co-cultures (Fig. 4B; Table 1) and in transfer experiments with conditioned media from co-cultures, compared to the respective controls. However, PD156252 had no effect on fibroblast wrinkle formation after a 5-day co-culture, despite the inhibition of ET-1 (data not shown). At the concentrations employed (10 µM) we did not observe any indication of inhibition of proliferation or toxicity. Co-cultures as well as control mono-cultures treated with PD156252 reached confluence as did the untreated controls. Also, HaCaT keratinocyte colonies appeared with the same size and frequencies in co-cultures arguing against toxic effects by the inhibitory hexapeptide PD156252.

**ET-1 alone is not sufficient to induce α-SMA expression in fibroblasts**

Since contractile activity was previously shown to correlate with myofibroblast differentiation (33, 34), we wanted to study the effect of ET-1 on αSMA expression possibly explaining the later contractile activity peak in co-cultured fibroblasts. We tested whether ET-1 induces αSMA expression in mono-cultured fibroblasts in low serum medium for 7 days. To dissect the effects of ET-1 from TGF-β we performed these experiments in DMEM with 0.5% FCS to reduce serum-derived TGF-β activity. ET-1 at 5 ng/ml, 50 ng/ml and 250 ng/ml did not change αSMA expression levels (Fig 5, lanes 2-4) compared to untreated cells (Fig. 5, lane 8). Treating fibroblasts with TGF-β1 resulted in the expected increase of αSMA expression (Fig. 5, lane 1), which was maximal at a concentration of 1 ng/ml (data not shown) and did not increase further at the used concentration of 5 ng/ml. When fibroblasts were co-stimulated with TGF-β at 5 ng/ml and ET-1 at 50 ng/ml and 250 ng/ml, a synergistic increase of αSMA expression was observed well above the level of maximal TGF-β stimulation (Fig. 5, lanes 5 and 6). Interestingly, α-SMA expression was highest in HaCaT-fibroblast co-cultures (Fig. 5, lane 7), by far exceeding maximal stimulation with TGF-β alone and co-stimulation with ET-1 in mono-cultures. We tested whether other mediators known to stimulate myofibroblast differentiation and known to be expressed in keratinocyte-fibroblast co-cultures could augment

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**Figure 3: ET-1 induction in HaCaT-fibroblast co-cultures.**

(A) RNA was isolated from fibroblasts co-cultured with HaCaT cells (right lane) and fibroblast controls after 6 days (left lane). Northern blots were hybridized with the indicated probes. There was a clear increase in ET-1 transcripts in co-cultured fibroblasts compared to keratinocytes (Fig. 3A), while fibroblast mono-cultures or HaCaT cells, both in mono and co-cultures expressed negligible levels of ET-1 transcripts (Fig. 3A and data not shown). In order to be biologically active, the ET-1 pro-form needs to be processed to the mature 21 AA peptide. We measured processed ET-1 in a conditioned medium using a 21 AA peptide-specific ELISA test. In line with the hybridization data, we observed a more than 3-fold increase in active ET-1 in co-cultures compared with the respective mono-culture controls at every 48h-interval tested throughout the 6-day observation period (Fig. 3B). The ELISA used, cross reacts with endothelin-2, and it cannot be ruled out that some of the signals may derive from endothelin-2.

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Figure 4: Endothelin receptor antagonist PD156252 impairs wrinkle formation at early time points. HaCaT-fibroblast co-cultures were incubated with PD156252 at 9 µg/ml (B) or without endothelin receptor inhibitor (A). The photographs were taken after 2 days. Wrinkle formation was less pronounced with the endothelin receptor antagonist at early time points up to day 3. Then the inhibitory effect of PD156252 on wrinkle formation disappeared. After 5 days wrinkle formation was indistinguishable in PD156252-treated as well as untreated co-cultures. Phase contrast photographs were taken at the same magnification, the bar represents 100 µm.

Figure 5: ET-1 requires TGF-β activity to induce α-SMA expression in fibroblast mono-cultures. (A) Fibroblast mono-cultures were stimulated with 5 ng/ml TGF-β and analyzed for induction of αSMA expression (lane 1) in DMEM with 0.5% FCS. ET-1 in doses ranging from 5 to 250 ng/ml had virtually no effect on αSMA expression (lanes 2-4). Only co-stimulation of 5 ng/ml TGF-β and ET-1 at 50 and 250 ng/ml resulted in αSMA expression levels above those obtained for TGF-β (lanes 5 and 6). For comparison αSMA expression levels in fibroblast control cultures as well as HaCaT-fibroblast co-cultures are shown (lane 7 and 8). For equal loading, Coomassie stained gel run in parallel is shown below. (B) Measurement of αSMA expression from three independent experiments. The HaCaT keratinocyte-fibroblast co-cultures were set as 100%.
the combined effects of ET-1 and TGF-β. GM-CSF was induced in co-cultures (25), and enhanced myofibroblast differentiation in the granulation tissue during wound healing (21, 22). We tested GM-CSF on fibroblast co-stimulated with or without TGF-β and ET-1 in DMEM/10% FCS. Compared with Figure 5, control fibroblasts expressed higher baseline levels of αSMA owing to the high serum levels (Fig. 6, left lane). TGF-β induced αSMA expression and ET-1 was also stimulatory in the high serum medium. Surprisingly, GM-CSF reduced the αSMA levels in TGF-β and ET-1 co-stimulated fibroblasts as well as baseline expression in DMEM/10% FCS (Fig. 6). Following up on this observation we analyzed whether GM-CSF was able to activate NFκB, which is known to interfere with TGF-β signaling (35, 36). Indeed, GM-CSF at 100 ng/ml activated NFκB in fibroblasts after only 1 hour and, surprisingly, was only slightly less efficient than IL-1 (Fig. 7).

Myofibroblast differentiation in keratinocyte-fibroblast co-cultures requires both, ET-1 and TGF-β activity

Finally, we examined the individual roles of TGF-β and endogenous ET-1 activity on myofibroblast differentiation in our co-culture model. Again, using α-SMA as a surrogate marker for myofibroblast differentiation, we blocked endogenous TGF-β activity in keratinocyte-fibroblast co-cultures with the TGF-β neutralizing antibody and/or ET-1 activity with PD156252. PD156252 blocked α-SMA induction in co-cultured fibroblasts almost to the same extent as TGF-β neutralizing antibody 1D11. In co-cultures, treated with PD156252 or neutralizing anti-TGF-β antibody, we observed a strong decrease of αSMA induction; however, low αSMA induction was always measured when compared with controls (Fig. 8, lanes 1 to 4). Only the combination of both, PD156252 and TGF-β-neutralizing antibody almost completely prevented α-SMA up-regulation in co-cultured fibroblasts (Fig. 8, lane 5 compared to lane 7 and 8).
Figure 7: GM-CSF activates NFκB in fibroblasts. Fibroblasts were stimulated with GM-CSF (100 ng/ml) for the indicated times. Nuclear extracts were prepared and analyzed for activated NFκB in EMSA assays. As control, IL-1 stimulated fibroblasts were analyzed in the right lane after stimulation for 2 hours. There was a clear induction of NFκB in GM-CSF stimulated fibroblasts albeit slightly weaker compared with IL-1 stimulation.

Figure 8: ET-1 and TGF-β are required for αSMA expression in HaCaT-fibroblast co-cultures. (A) HaCaT keratinocytes and fibroblasts were co-cultured for 7 days in DMEM/0.5% FCS. αSMA expression in fibroblasts was measured by western blotting after isolation of fibroblasts from co-cultures. There was a clear increase of αSMA expression in co-cultured compared with control fibroblasts (lanes 7 and 8). Adding neutralizing anti-TGF-β-antibody reduced αSMA expression in co-cultured fibroblasts (lanes 1 and 2) as did PD156252 (lanes 3 and 4). Only the combination of neutralizing anti-TGF-β-antibody and PD156252 blocked αSMA expression in co-cultured fibroblasts almost to basal levels observed in control fibroblasts (lanes 5 and 6). A Coomassie stained gel run in parallel shows equal loading. (B) Measurement of signal intensities of three independent experiments. Again, αSMA expression in co-cultures was set 100%.
Discussion

Wound healing is a complex response of the organism aiming at restoring tissue integrity. Several cell types are involved and communicate via diffusible mediators. Recent work has advanced our understanding of the nature and the time of action of the mediators and cytokines that are involved in tissue repair (37). Combining in vitro and in vivo models has helped in dissecting individual regulatory pathways (26, 38, 39). Keratinocyte-fibroblast interactions become most prominent in the mid-phase of wound healing when new connective tissue is formed, and keratinocytes migrate towards the wound center to facilitate wound closure. Most studies have analyzed epithelial-mesenchymal cell-cell interactions with a focus on the epithelial phenotype (40).

However, less is known about the interaction of both cell types and the influence of keratinocytes on the mechanisms and events that govern the fibroblast phenotype. Recently, we demonstrated that keratinocyte co-culture induces the transition of fibroblasts towards a myofibroblastic phenotype (Shephard et al., in press). Myofibroblasts are characterized by the expression of αSMA (2), production of extracellular matrix (5) and the development of high tensile forces that are thought to be responsible for granulation tissue contraction (3). TGF-β seems to be the most potent inducer of fibroblast-to-myofibroblast differentiation (6, 11), which however requires additional factors, such as mechanical stress (8). If fibroblasts are subjected to mechanical tension, e.g. when wounds are splinted with a plastic frame (12), the level of αSMA expression and the percentage of myofibroblasts increases substantially. On the other hand, if the surrounding ECM does not provide a mechanical feedback, tension does not develop, and myofibroblast differentiation is reduced even in the presence of active TGF-β (8, 9, 12). This suggests that TGF-β and mechanical tension act in synergy as myofibroblast differentiating stimuli.

We have recently shown that co-culture with keratinocytes up-regulates αSMA expression in fibroblasts, and here we aimed to dissect how growth factors and mechanical forces synergize to induce myofibroblast differentiation in these co-cultures. We show for the first time enhanced contractile activity of fibroblasts upon co-culture with keratinocytes on wrinkling silicone substrates (18, 33). Interestingly, wrinkle formation, i.e. up-regulated contractile activity was most pronounced in fibroblasts neighboring keratinocyte colonies. Generation of high contractile activity and force has been suggested to correlate with αSMA expression in myofibroblasts (9, 11, 33) and this
indeed correlates with an increased number of αSMA expressing myofibroblasts in 4-5 day old co-cultures. However, there was also early contractile activity in co-cultures when most of the fibroblasts were αSMA negative. Stress fibers were present in both co-cultured and control fibroblasts, and there was no apparent difference in the stress fiber pattern between the two culture conditions at days 1-2. Transfer experiments with conditioned media also rapidly induced fibroblast contraction in mono-culture, suggesting the involvement of diffusible mediator(s).

An initial clue to the nature of these factors comes from a comparison of the mRNA expression pattern of fibroblasts co-cultured or without HaCaT keratinocytes (Shephard et al., in press), demonstrating the apparent up-regulation of ET-1 mRNA in microarray studies; here, we confirmed this initial finding by Northern blotting. Endothelins are the most potent vasoconstrictive mediators and rapidly induce SMC contraction (19) and contraction of myofibroblasts (12, 41, 42). Fibroblasts, co-cultured with keratinocytes, expressed higher levels of ET-1 transcripts compared with control fibroblasts or keratinocytes in mono-culture. Moreover, the ET-1 prepro-form was processed to the biologically active 21 AA ET-1 peptide which was detected in the conditioned medium of early co-cultures by means of ELISA. These novel findings suggest that the conversion from the prepro-ET-1 to the mature ET-1 occurred in our co-cultures. The high variability of the ELISA measurements between different experiments could reflect differences in ET-1 production as well as processing efficiencies; in addition, the ELISA used here potentially cross-reacts with ET-2. In inhibitor experiments, we observed that PD156252 was ineffective in reducing contraction of fibroblasts in later co-cultures despite elevated levels of ET-1. At present, we have no explanation for this finding, and we can only speculate that co-cultured fibroblasts are either decreasingly sensitive to ET-1 stimulation or that other mechanisms inducing fibroblast contraction override the influence of ET-1 at this time point. How could these findings relate to wound healing? In granulation tissue, stimulation with ET-1 results in enhanced contraction by myofibroblasts, further increasing mechanical tension within this tissue (12, 41, 42).

Both endothelin A and B receptors are expressed in granulation tissue, but only blocking of endothelin A receptors inhibits contraction by ET-1 (42). ET-1 stimulates expression of αSMA in hepatic stellate cells (20, 43), which concomitantly become more sensitive to further ET-1 stimulation (43).

ET-1 production is usually confined to endothelial cells or αSMA positive SMC. Which factor could induce ET-1 in keratinocyte-fibroblast co-cultures and which cell type is responsible for its secretion? IL-1 plays a central role in the fibroblast-keratinocyte co-culture model. In co-culture with fibroblasts, IL-1 is synthesized by keratinocytes (38) and was shown to induce IL-6 (26, 44) and KGF synthesis (26, 38). ET-1 itself is rapidly induced by IL-1, TNFα and LPS (45-48) and these agents activate NFκB, which was also shown to be involved in ET-1 mRNA induction (49). We have previously shown that keratinocyte-derived IL-1 is responsible for NFκB activation in fibroblasts of early co-cultures, reaching a maximum after 6 hours. Furthermore, it was a surprising finding that GM-CSF, induced in co-cultures (25, 26), also activates NFκB and almost as efficiently and quickly as IL-1. Direct interaction of the GM-CSFR α-chain with the IκB kinase-β has recently been shown to play a role in the activation of NFκB in endothelial cells (50). However, whether the same mechanism occurs in fibroblasts remains to be shown.

While endothelin activity seems to be important for the contractile activity of fibroblasts in early co-cultures, TGF-β-mediated expression of αSMA appears to dominate in up-regulating fibroblast contractile activity in later co-culture. αSMA expression was strongly induced in our co-cultures, i.e. the majority of fibroblasts differentiated into myofibroblasts. Interestingly, active TGF-β is present in co-cultures already during the first 1 or 2 days, however, TGF-β effects become apparent much later. In previous studies NFκB activation was shown to block TGF-β signaling (35, 36) and our present observation together with previous findings are in line with this mechanism. Both, GM-CSF induced in co-cultures (25) and keratinocyte-derived IL-1 activate NFκB (Shepard et al., in press) which in turn inhibits TGF-β signaling and downstream αSMA expression. How this inhibition is overcome in our co-culture model when αSMA is strongly induced is still unclear at present. For GM-CSF, our findings on inhibition of αSMA expression do not correlate well with previous observations in vivo showing an enhanced myofibroblast differentiation. GM-CSF locally applied or expressed alone or in combination with other mediators, results in the appearance of myofibroblasts and fibrosis (21, 22, 51). Furthermore, GM-CSF or GM-CSF-treated keratinocytes are effective in promoting wound healing in difficult to heal wounds (23, 24). In the present study, GM-CSF was tested as a myofibroblast differentiation stimulus possibly synergizing with ET-1 and TGF-β. How can these opposing properties of GM-CSF be reconciled? From the data available we propose that the effect of GM-CSF on myofibroblast differentiation may be indirect. GM-CSF application in vivo results in the infiltration and accumulation of monocytes/macrophages/dendritic cells at the site of injection (21, 52, 53), and these cells could then orchestrate granulation tissue formation in the context of tissue repair. In vitro, however, the pro-inflammatory activity of GM-CSF may dominate in fibroblasts, thus negatively regulating TGF-β and ET-1-dependent stimulation of αSMA expression.

For the link of growth factor pathways and mechanical force generation in our co-culture model we propose the model outlined in Figure 9. Early contraction is largely driven by ET-1 in αSMA negative fibroblasts. TGF-β activity is blocked by NFκB, activated by up-regulated GM-CSF and keratinocyte-
derived IL-1. Later, mechanical forces are generated by αSMA positive myofibroblasts. TGF-β and ET-1 are important inducers for αSMA, while the effects of IL-1 and GM-CSF seem to become attenuated. Endogenous ET-1 appears to be a co-factor required for αSMA induction but is dispensable for wrinkle formation in this model. *In vivo*, however, ET-1 stimulates contractile activity at pharmacological doses.

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### Abbreviations

αSMA alpha smooth muscle actin; GM-CSF granulocyte monocyte colony stimulating factor; ECM extracellular matrix; ET-1 endothelin-1; HDF human dermal fibroblast; SMC smooth muscle cell; TGF-β transforming growth factor beta.

### References


