

Keratinocytes Inhibit Expression of Connective Tissue Growth Factor in Fibroblasts *In Vitro* by an Interleukin-1 α -Dependent Mechanism

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The wound healing process concludes with down-regulation of fibroblast activity. Clinical observations suggest that the regenerating epidermis suppresses this activity. An important regulator of fibroblast activity is the fibrogenic cytokine connective tissue growth factor. We hypothesized that epidermal keratinocytes may affect fibroblast activity via this cytokine. We demonstrate keratinocyte-mediated suppression of connective tissue growth factor at both the mRNA and protein levels by around 50% or more when fibroblasts were cultured in multiwell plates with keratinocyte cultures in accompanying semipermeable cell culture inserts, or stimulated by keratinocyte-conditioned media. Both basal and transforming-growth-factor- β 1-stimulated levels of connective tissue growth factor were inhibited. A 3 h coculture period with keratinocytes was sufficient to suppress connective tissue growth factor expression by fibroblasts, but the inhibition developed over a

time period of around 16 h. The putative keratinocyte-derived factor(s) responsible for these effects was found to be soluble and stable. By analyzing cytokines secreted by keratinocytes we identified interleukin-1 α as a potent inhibitor of connective tissue growth factor mRNA expression in fibroblasts. Involvement of this cytokine in keratinocyte-mediated connective tissue growth factor suppression was confirmed by using anti-interleukin-1 α antibodies. Tumor necrosis factor α or prostaglandins did not appear to be involved. In conclusion, our results indicate that interleukin-1 α secretion by keratinocytes provides a mechanism for the downregulation of connective tissue activity during the end-stage of wound healing, when epithelia coverage has developed over the wound area. **Key words:** coculture/re-epithelialization/transforming growth factor β 1/wound healing. *J Invest Dermatol* 119:449–455, 2002

Wound healing is dependent on the recruitment of several cell types that appear in the wound area in a temporally and spatially defined manner. Re-epithelialization largely coincides with the recruitment of dermal fibroblasts and it is likely that crosstalk between epidermal keratinocytes and fibroblasts is important during the rebuilding of tissue integrity (Fusenig *et al*, 1994). Healing of extensive wounds often results in excessive scarring, disfiguring, and functional impairment of the affected area (Haverstock, 2001). This is a particular problem in the healing of large burn wounds, and it appears that an early re-epithelialization or coverage of the wounded area with autologous skin grafts limits excessive deposition of connective tissue. Experiments in which keratinocytes were cocultured with fibroblasts have demonstrated establishment of paracrine loops of cytokines between the two cell types (Maas-Szabowski *et al*, 2000; Szabowski *et al*, 2000), a phenomenon that most probably also operates *in vivo* to regulate cellular function (Goretsky *et al*, 1996). Epidermal keratinocytes has been demonstrated downregulate the production of the major matrix component of the dermis, collagen I, by fibroblasts (Lacroix *et al*, 1995; Garner, 1998). Several

fibrogenic cytokines have been described. Transforming growth factor β 1 (TGF- β 1) is a potent stimulator of various extracellular matrix molecules, as well as of inhibitors of matrix degrading enzymes (Roberts *et al*, 1990). Connective tissue growth factor (CTGF) is another regulatory protein acting downstream of TGF- β 1, and promoting proliferation and collagen synthesis of mesenchymal cells. It has been hypothesized that CTGF is a TGF- β -stimulated regulator specific for the mesenchymal compartment (Grotendorst, 1997). TGF- β 1 stimulates expression of CTGF, but not all effects ascribed to TGF- β 1 are mediated by CTGF (Grotendorst, 1997). Conversely, CTGF may act independently of TGF- β signaling or expression (Dammeier *et al*, 1998; Chambers *et al*, 2000; Holmes *et al*, 2001). Both TGF- β 1 and CTGF are upregulated during wound healing (Igarashi *et al*, 1993; Frank *et al*, 1996) and overexpressed in various fibrotic conditions (Border and Noble, 1994; Lasky *et al*, 1998; Blobel *et al*, 2000; Shiuwen *et al*, 2000; Williams *et al*, 2000).

In this work, using *in vitro* models, we tested the hypothesis that epidermal keratinocytes act to suppress expression of the fibrogenic cytokine CTGF in fibroblasts.

MATERIALS AND METHODS

Antibodies and cytokines Polyclonal antihuman CTGF antibodies and recombinant CTGF ligands were obtained from Fibrogen (San Francisco, CA). Polyclonal anti- β -tubulin antibodies came from Santa Cruz Biotechnology (Santa Cruz, CA). Neutralizing interleukin-1 α (IL-

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1 α) antibodies were obtained from R&D Systems (Minneapolis, MN). Recombinant IL-1 α and TGF- β 1 were obtained from Sigma (St. Louis, MO).

Cells Cells were isolated from samples of skin from young individuals undergoing plastic surgery at our department. Epidermal keratinocytes were isolated from human skin as described previously (Rheinwald, 1980). Briefly, skin samples were treated with dispase and epidermis was mechanically separated from the underlying dermis; this was followed by mechanical fragmentation and treatment of the separated epidermal sheets with trypsin. Keratinocytes were propagated on irradiated 3T3 feeder cells in Dulbecco's modified Eagle's medium (DMEM)/Ham's F₁₂ (4:1) supplemented with 5 μ g per ml Zn-free insulin (donated by Lilly Research Laboratories), 2 nM 3,3',5-triiodo-D-thyronine (Sigma), 0.4 μ g per ml hydrocortisone (Sigma), 0.1 nM cholera toxin (Sigma), 10 ng per ml epidermal growth factor (Austral Biologicals, San Ramon, CA), 24 μ g per ml adenine, 10% fetal bovine serum (HyClone, Logan, UT), and 50 μ g per ml gentamicin. Fibroblasts were isolated from the dermal compartment by collagenase treatment and subcultured in DMEM supplemented with 10% bovine calf serum (HyClone) and 50 μ g per ml gentamicin. Subconfluent cells were washed with phosphate-buffered saline and detached by treatment with trypsin. Cells at population doubling level 5–10 were used for the experiments.

Coculture 0.3×10^6 fibroblasts were cultured in plastic dishes [six-well Falcon multiwell plates, surface area 9.62 cm² (BD Labware, Franklin Lakes, NJ)], and 0.3×10^6 keratinocytes were seeded in Falcon polyurethane cell culture inserts (0.4 μ m pore diameter). The inserts were precoated with a mixture of 10 μ g per ml bovine plasma fibronectin (Gibco BRL/Life Technology, Paisley, U.K.), 30 μ g per ml bovine collagen (Vitrogen, Cohesion, Palo Alto, CA), and 10 μ g per ml bovine serum albumin (Sigma) for 2 h at 37°C. As controls, 0.2×10^6 fibroblasts were grown in inserts instead of keratinocytes. Cell cultures were prepared in separate compartments (wells and inserts) 1 d before start of the cocultures. The culture medium during this period was the standard growth medium for the two cell types as described above. Upon initiation of the cocultures, the medium was changed to DMEM/Ham's F₁₂ (4:1) supplemented with 0.5% fetal bovine serum (HyClone), and the inserts were combined with the wells. The total volume was 4 ml (2 + 2 ml). At this point cell densities were nearly confluent in both compartments. Coculture time was continued for various lengths of time as indicated. TGF- β 1 was added to both compartments at the start of the cocultures in some experiments.

Keratinocyte-conditioned medium Two $\times 10^6$ keratinocytes were seeded in 75 cm² flasks precoated with collagen/fibronectin/bovine serum albumin as described above. The next day cells were washed with phosphate-buffered saline and conditioned with 10 ml DMEM/Ham's F₁₂ (4:1) supplemented with 0.5% fetal bovine serum for 3 h. Cell density was approximately 50% at this point. Two milliliters of the conditioned medium was added to fibroblast cultures in six-well plates and the cultures were continued for various lengths of time, as indicated, before analysis of CTGF mRNA or protein levels. Conditioned medium from fibroblasts was run in parallel and used as a control for keratinocyte-conditioned medium. TGF- β 1 was added to the conditioned medium before the latter was added to the fibroblasts.

Northern blotting RNA was extracted from cells using TRIzol reagent (Life Technology). Total RNA was quantitated fluorometrically (SYBR Green II, FMC Bioproducts, Rockland, ME). The RNA was denatured in formaldehyde, separated by agarose gel electrophoresis, and transferred to nylon membranes (Hybond N+, Amersham Pharmacia Biotech, Buckinghamshire, U.K.). Fixation of the RNA was performed by UV-crosslinking (GS GeneLinker, Bio-Rad, Hercules, CA), and the membranes were prehybridized for 15 min at 65°C in Rapid Hyb Buffer (Amersham Pharmacia Biotech). cDNA for CTGF was from Fibrogen. As control probe for RNA loading, oligonucleotides coding for 18S or 28S ribosomal RNA (Barbu and Dautry, 1989; Carlson *et al*, 1993) were used. cDNAs were labeled with [α -³²P]dCTP using the Megaprime kit (Amersham Pharmacia Biotech) and the control oligonucleotides were labeled with [γ -³²P]ATP and polynucleotide kinase (Barbu and Dautry, 1989). cDNA probes were denatured and added to prehybridization buffer, and hybridization proceeded for 2 h at 65°C. Oligonucleotide probes were prehybridized and hybridized at 37°C. For cDNA probes, membranes were washed twice in 2 \times sodium citrate/chloride buffer (SSC)/0.1% sodium dodecyl sulfate (SDS) at room temperature and then in 0.2 \times SSC/0.1% SDS at 65°C. For oligonucleotide probes, membranes were washed in 2 \times SSC/0.1% SDS at room temperature followed by 0.2 \times SSC/0.1% SDS at 37°C. Signals were detected and

quantified by autoradiography and densitometry (SigmaGel Software, Jandel Scientific, Chicago, IL). Reprobing with 28S or 18S probes was performed after removal of RNA from the membranes by boiling in 0.1% SDS. The size of the CTGF transcript was estimated at 2.4 kb as reported previously (Bradham *et al*, 1991).

Western blotting The culture medium was removed and the cells were washed with phosphate-buffered saline. Cells were solubilized in RIPA buffer (0.15 M NaCl, 0.05 M Tris-HCl, 0.1% SDS, 1% Igepal CA-630 (Sigma), 5 mg per ml Na deoxycholate) containing protease inhibitors. Protein concentration was determined using the Bio-Rad ¹²⁵I protein assay (Bio-Rad, Hercules, CA). Equal amounts of proteins (40 μ g) were boiled in SDS sample buffer and separated by SDS polyacrylamide gel electrophoresis under nonreducing conditions. Proteins were electro-transferred to Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech). Immunoblotted proteins were incubated with rabbit polyclonal anti-CTGF antibodies and anti- β -tubulin antibodies followed by incubation with horseradish-peroxidase-conjugated secondary antibodies and chemiluminescence detection (ECL, Amersham Pharmacia Biotech). Recombinant CTGF was used as control. CTGF protein was detected as a doublet with a relative molecular weight of around 38 kDa (confirmed by personal communication with Dr. Patricia Segarini, Fibrogen, CA). β -Tubulin migrated with a relative molecular weight of around 52 kDa.

Determination of cytokine levels Analysis of the concentration of cytokines in keratinocyte-conditioned media was performed with commercial enzyme-linked immunosorbent assay (ELISA) kits for IL-1 α , IL-1 β , tumor necrosis factor α (TNF- α), and interferon- γ (IFN- γ) (Biotrak, Amersham Pharmacia Biotech).

Statistical analysis All results are presented as means \pm SEM unless otherwise stated. Student's *t* tests (unpaired) were used for statistical analysis. *n* values signify the number of independent experiments performed. *p*-values less than 0.05 were considered statistically significant.

RESULTS

Keratinocytes inhibit expression of CTGF in fibroblasts in the absence of physical contacts Human keratinocytes and dermal fibroblasts were cultured physically separated in cocultures, as described in *Materials and Methods*. CTGF mRNA and protein expression in fibroblasts were investigated by Northern blotting and Western blotting, respectively. mRNA levels were determined after 16 and 48 h of coculture, and protein levels were measured after 48 h of coculture. After 16 h the level of CTGF mRNA in fibroblasts from cocultures was 49% \pm 7.8% of the level in fibroblasts from control cultures (*n* = 3, *p* = 0.0006), and after 48 h CTGF mRNA levels were 64% \pm 5.0% in fibroblasts from cocultures compared to control cultures (*n* = 8, *p* < 0.0001) (**Fig 1A**). The same pattern was observed when cellular CTGF protein was measured: CTGF protein was 69% \pm 5.8% in cocultures compared to control cultures after 48 h (*n* = 5, *p* = 0.0008) (**Fig 1C**). Next, we investigated whether keratinocytes could also inhibit TGF- β 1-stimulated CTGF expression. In the presence of 40 pM TGF- β 1, CTGF mRNA and protein levels were increased in fibroblast monocultures, as expected (**Fig 1B, C**). Keratinocytes were still able to suppress fibroblast CTGF expression to the same extent as in the absence of TGF- β 1, however; when TGF- β 1 was added, the level of CTGF mRNA in fibroblasts was 41% \pm 9.3% in cocultures compared to control cultures after 16 h (*n* = 3, *p* = 0.0032) (**Fig 1B**), and after 48 h CTGF protein levels were 52% \pm 1.4% in cocultures compared to control cultures (*n* = 2) (**Fig 1C**). In summary, via soluble factors keratinocytes were able to inhibit basal or TGF- β 1-stimulated CTGF mRNA and protein in fibroblasts.

A short coculture time with keratinocytes, or conditioned medium from keratinocytes, is sufficient for suppression of CTGF in fibroblasts In order to investigate more closely the efficiency of keratinocyte-mediated CTGF suppression, fibroblasts were exposed to keratinocytes for short time periods. Fibroblasts were cocultured with keratinocytes in inserts for 3 or 6 h, inserts were then removed, and CTGF mRNA was analyzed 24 h after

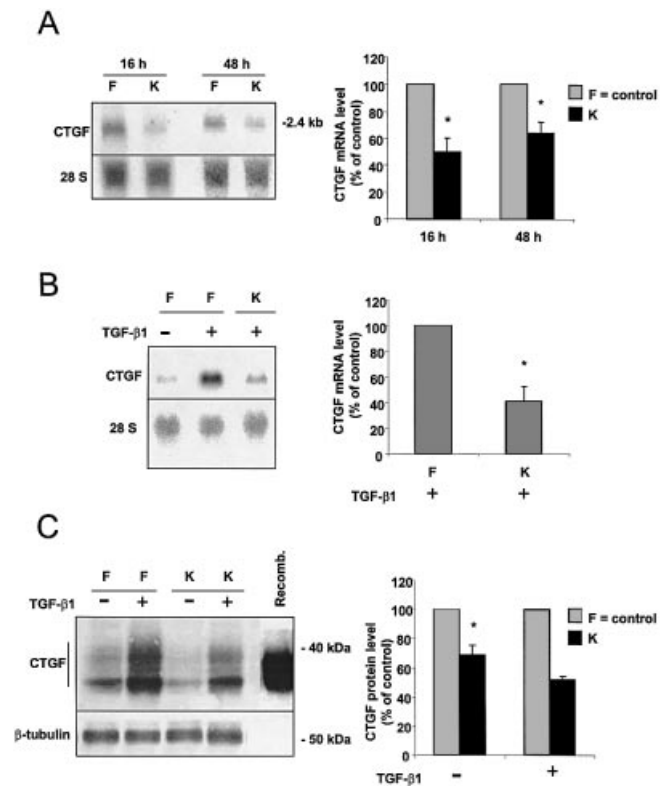


Figure 1. Keratinocytes downregulate CTGF expression in fibroblasts in the absence of physical contacts. Fibroblasts were cocultured with keratinocytes or fibroblasts (control) in cell culture inserts in the absence or presence of 40 pM TGF- β 1. After 16 or 48 h fibroblasts were subjected to Northern blotting for analysis of CTGF mRNA expression. In parallel cultures, cellular CTGF protein level was determined by Western blotting. Levels of 28S ribosomal RNA and β -tubulin were used as internal controls. (A) CTGF mRNA expression after 16 h ($n = 3$) and 48 h ($n = 8$) of coculture in the absence of TGF- β 1. (B) CTGF mRNA expression after 16 h in the presence of TGF- β 1 ($n = 3$). (C) CTGF protein level after 48 h of coculture in the absence ($n = 5$) or presence ($n = 2$) of TGF- β 1. Values are expressed as means \pm SEM, $n =$ number of separate experiments performed. (*Significant at $p < 0.01$.) F, fibroblasts in inserts (control); K, keratinocytes in inserts; Recomb., recombinant CTGF protein.

initial exposure to keratinocytes. Twenty picomoles TGF- β 1 was added immediately after removal of the inserts. A 3 h keratinocyte interaction resulted in a drop in the CTGF mRNA level in fibroblasts to $38\% \pm 6.2\%$ of control levels ($n = 6$, $p < 0.0001$), and after a 6 h coculture time the level was $46\% \pm 6.8\%$ compared to control levels ($n = 2$) (Fig 2A). The inhibition of the CTGF mRNA level was followed by a corresponding decrease in cellular CTGF protein; a 3 h keratinocyte interaction resulted in a decrease to $30\% \pm 11\%$ relative to control levels 24 h after initial exposure to keratinocytes ($n = 2$) (Fig 2B). To investigate if suppression of CTGF depended on a paracrine loop between the two cell types, i.e., if the presence of fibroblasts was necessary in order for keratinocyte-derived CTGF inhibitory factor(s) to be produced, fibroblasts were subjected to conditioned medium from keratinocytes. Medium that had been incubated with keratinocytes or fibroblasts (control) for 3 h was added to fibroblasts. At this point 20 pM TGF- β 1 was also added to the cultures. After 16 h the level of CTGF mRNA in fibroblasts cultured in keratinocyte-conditioned medium was $31\% \pm 13\%$ compared to control levels ($n = 3$, $p = 0.0062$) (Fig 3A). Similarly, CTGF protein expression decreased to $29\% \pm 20\%$ ($n = 2$) in fibroblasts cultured in keratinocyte-conditioned medium compared to control levels (Fig 3B). Thus, the presence of fibroblasts was not necessary for production of CTGF inhibitory factor(s) by keratinocytes.

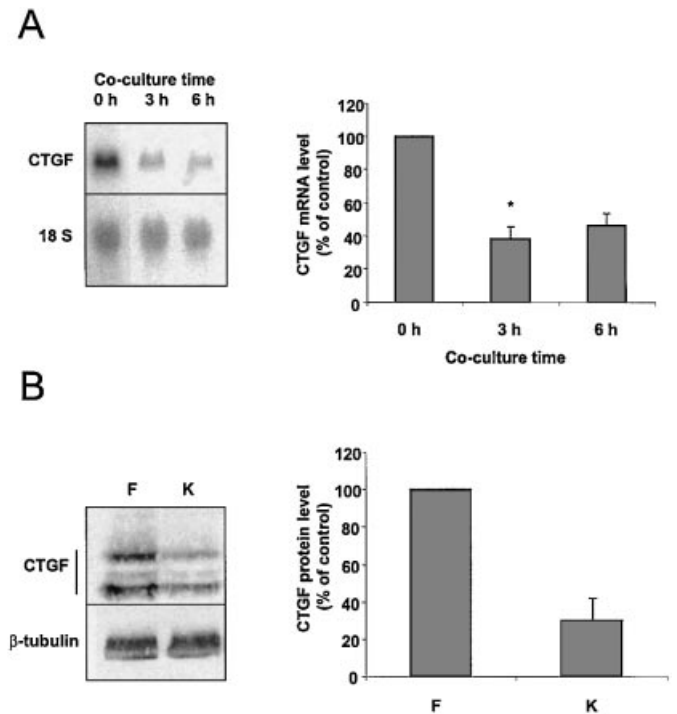


Figure 2. A 3 h coculture with keratinocytes is sufficient for suppression of CTGF mRNA and protein expression in fibroblasts. Fibroblasts were cultured for 3 or 6 h with keratinocytes in cell culture inserts. Inserts were removed, 20 pM TGF- β 1 was added, and fibroblasts were cultured for a total of 24 h following initial exposure to keratinocytes. CTGF mRNA was analyzed by Northern blotting and cellular CTGF protein by Western blotting. (A) CTGF mRNA expression in fibroblasts exposed to keratinocytes for 3 h ($n = 6$) or 6 h ($n = 2$). (B) CTGF protein expression in fibroblasts exposed to keratinocytes for 3 h ($n = 2$). A 3 h coculture with fibroblasts in inserts was used as the control. Values are expressed as means \pm SEM, $n =$ number of separate experiments performed. (*Significant at $p < 0.01$.) F, fibroblasts in inserts (control); K, keratinocytes in inserts.

Inhibition of keratinocyte-mediated CTGF suppression is developed over time, and sustained in fibroblasts In order to study the dynamics of the keratinocyte-mediated suppression of CTGF, we investigated CTGF mRNA and protein expression 6 and 20 h after a coculture period of 3 h. Twenty picomoles TGF- β 1 was added to the cultures after removal of inserts. At 6 h the level of CTGF mRNA was not changed significantly, whereas at 20 h there was a strong suppression of CTGF mRNA in fibroblasts cocultured with keratinocytes (Fig 4A). CTGF protein levels followed a similar pattern; at 6 h the level of cellular CTGF protein was unchanged compared to control levels, but at 20 h CTGF was strongly downregulated (Fig 4B). To further investigate the temporal pattern of CTGF downregulation, we cultured fibroblasts in keratinocyte-conditioned medium and analyzed mRNA levels at different time points. Twelve hours after addition of keratinocyte-conditioned medium there was still little change in CTGF mRNA expression, whereas after 24 or 48 h there was a strong relative downregulation of CTGF mRNA in fibroblasts (Fig 5). To further confirm that the keratinocyte-derived suppression of CTGF is sustained in fibroblasts, the fibroblasts were exposed to keratinocyte-conditioned medium for 16 or 40 h before addition of 20 pM TGF- β 1. CTGF mRNA was analyzed at different time periods after addition of TGF- β 1. Although the response to TGF- β 1 diminished with time, relative suppression of CTGF mRNA was strong at all time points in fibroblasts exposed to keratinocyte-conditioned medium. If TGF- β 1 was added late, i.e., after 40 h of culture, suppression of CTGF mRNA by keratinocyte-conditioned medium was still of the same

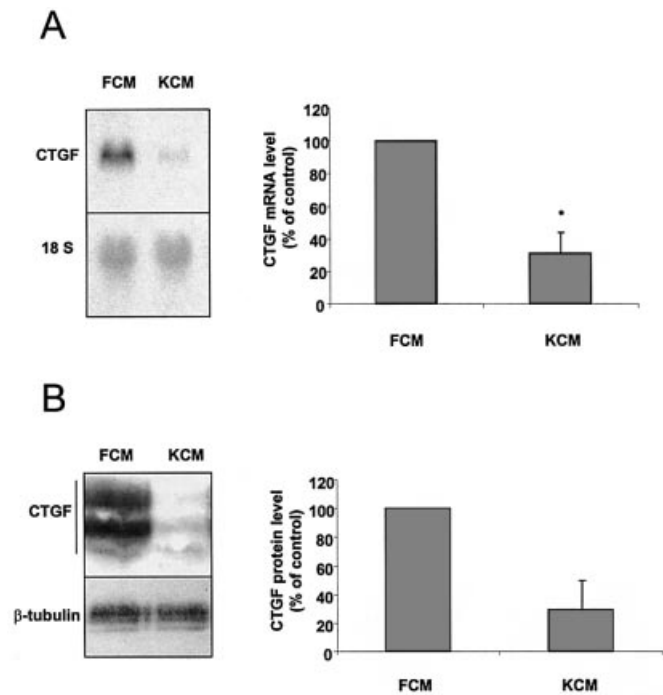


Figure 3. Conditioned medium from keratinocytes can replace inserts with keratinocytes for suppression of CTGF mRNA and protein in fibroblasts. Fibroblasts were subjected to conditioned medium from keratinocytes or fibroblasts (control) containing 20 pM TGF- β 1, and CTGF expression was analyzed after 16 h. (A) CTGF mRNA expression as determined by Northern blotting ($n = 3$). (B) Cellular CTGF protein level as determined by Western blotting ($n = 2$). Values are expressed as means \pm SEM, $n =$ number of separate experiments performed. (*Significant at $p < 0.01$.) FCM, fibroblast-conditioned medium (control); KCM, keratinocyte-conditioned medium.

magnitude (Fig 6). Taken together, the results show that stimulation of fibroblasts with keratinocyte-derived factors induces a suppression of CTGF expression that is developed over time and fully established after around 16–24 h. The fibroblasts so induced to inhibit CTGF expression sustain this phenotype for at least 24 h.

The putative keratinocyte-derived factor(s) suppressing CTGF expression is stable and insensitive to treatment with indomethacin In an effort to characterize the keratinocyte-derived factor(s) responsible for suppression of CTGF, we determined its resistance to inactivation after incubation at 37°C for 24 h. Fibroblasts were cultured in keratinocyte-conditioned medium that had been incubated at 37°C for 0 or 24 h. After 16 h in these media, 20 pM TGF- β 1 was added and CTGF mRNA levels were analyzed after an additional 3 h. The magnitude of suppression was similar in media that had been incubated for 0 or 24 h at 37°C, and conditioned media that had been stored at -70°C for 4 wk also showed a similar potency in suppressing CTGF expression (Fig 7A). Previous work has demonstrated the ability of prostaglandins to downregulate the expression of CTGF in fibroblasts (Ricupero *et al*, 1999; Stratton *et al*, 2001). We therefore investigated if the keratinocyte-mediated CTGF suppression could be reversed by the addition of indomethacin, a potent inhibitor of prostaglandin synthesis. Fibroblasts were cultured for 16 h in keratinocyte-conditioned medium containing 10 μ M indomethacin. Twenty picomoles TGF- β 1 was added and CTGF mRNA was measured after another 3 h. There was no difference in the suppression of CTGF with or without the addition of indomethacin (Fig 7B). This

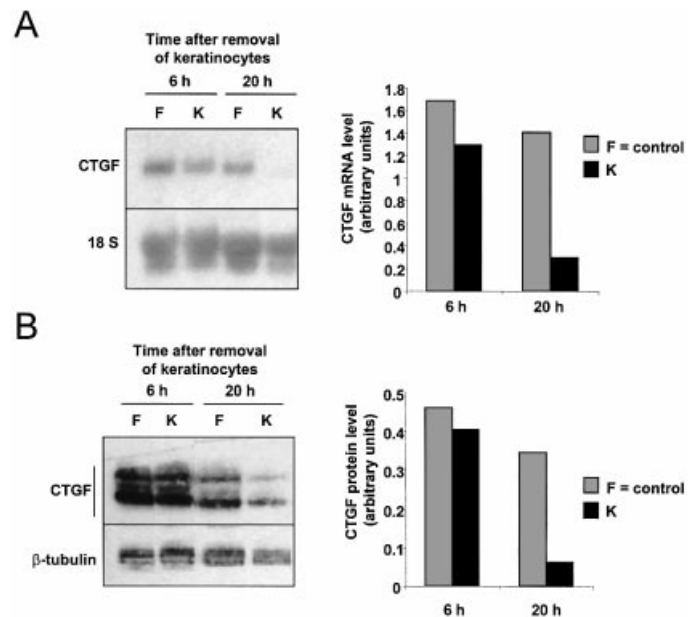


Figure 4. Keratinocyte-mediated CTGF suppression is developed over time in fibroblasts. Fibroblasts were cocultured for 3 h with keratinocytes or fibroblasts (control) in inserts. Inserts were removed, 20 pM TGF- β 1 was added, and CTGF mRNA and protein were analyzed by (A) Northern and (B) Western blotting after an additional 6 and 20 h of culture. The experiments were repeated with qualitatively identical results. F, fibroblasts in inserts (control); K, keratinocytes in inserts.

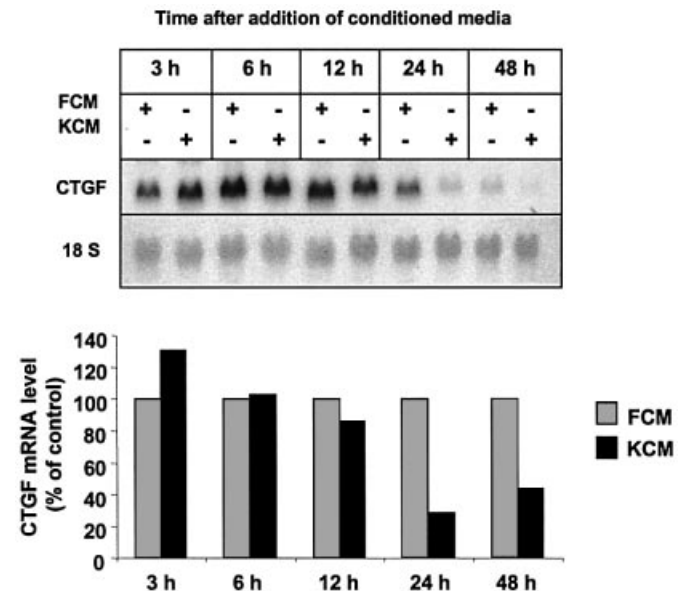


Figure 5. CTGF suppression is developed over time in fibroblasts in the presence of keratinocyte-conditioned medium. Fibroblasts were subjected to conditioned medium from keratinocytes or fibroblasts (control). Twenty picomoles TGF- β 1 was added and CTGF mRNA levels were analyzed by northern blotting after different time periods. The experiments were repeated with qualitatively identical results. FCM, fibroblast-conditioned medium (control); KCM, keratinocyte-conditioned medium.

finding is supported by the observation that the putative keratinocyte-derived factor(s) is stable, as prostaglandins typically have a very short half-life.

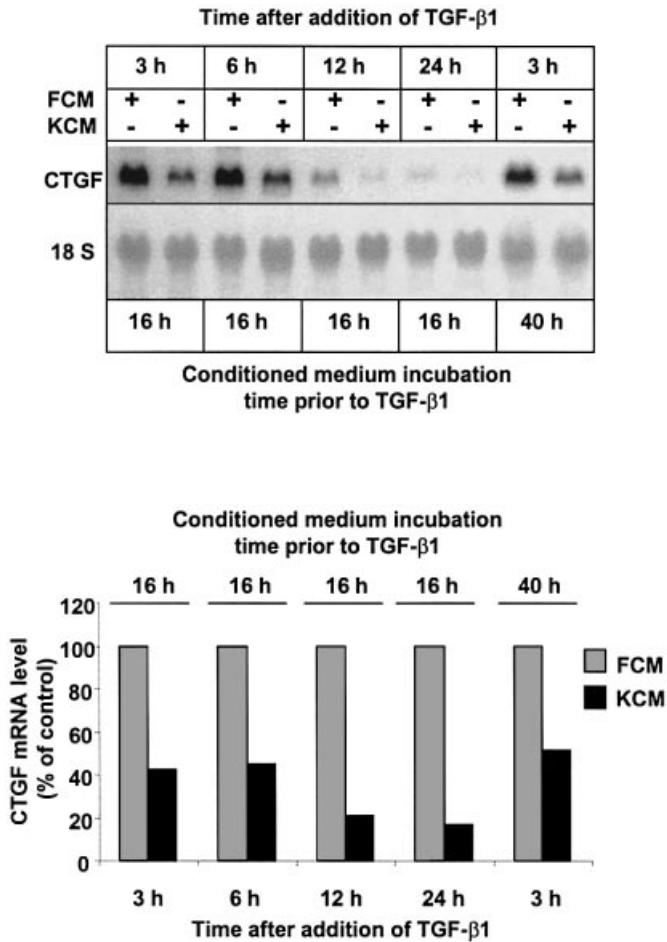


Figure 6. Keratinocyte-mediated CTGF suppression is sustained in fibroblasts. Fibroblasts were incubated with conditioned medium from keratinocytes or fibroblasts (control) for 16 or 40 h. Twenty picomoles TGF-β1 was added and CTGF mRNA levels were analyzed by Northern blotting after different time periods as indicated. The experiment was repeated with qualitatively identical results. FCM, fibroblast-conditioned medium (control); KCM, keratinocyte-conditioned medium.

Identification of IL-1α as a major keratinocyte-derived mediator of CTGF suppression in fibroblasts In order to try to identify a keratinocyte-mediated factor responsible for inhibition of CTGF expression in fibroblasts, we first analyzed the level of cytokines secreted by keratinocytes in our culture system. For this purpose we selected IL-1α, IL-1β, TNF-α, and IFN-γ, which have been demonstrated to be produced by keratinocytes in various conditions, and/or have been demonstrated to have effects on fibroblast activity (Jimenez *et al*, 1984; Burbach *et al*, 2001). As measured by ELISA, only IL-1α was detected at a significant level. The amount produced by keratinocytes in a 3 h culture was 23.7 ± 7.7 pg per ml (n = 5) (Fig 8A). This prompted us to test the effect of ectopically added IL-1α on CTGF expression. IL-1α was added to fibroblasts in various concentrations together with 20 pM TGF-β1, and CTGF mRNA was analyzed after 16 h. We found that IL-1α was a potent inhibitor of TGF-β-stimulated CTGF expression, with an IC₅₀ of around 10 pg per ml in our system (Fig 8B). To test if IL-1α was a true mediator of suppression of CTGF in our system, we added neutralizing anti-IL-1α antibodies to keratinocyte-conditioned medium before treatment of fibroblasts. Interestingly, the majority of the effect of conditioned medium was abrogated by the addition of 0.4 μg per ml of the antibody (Fig 9). Addition of control IgG did not have an effect (not shown). Thus, keratinocyte-derived IL-1α is a potent

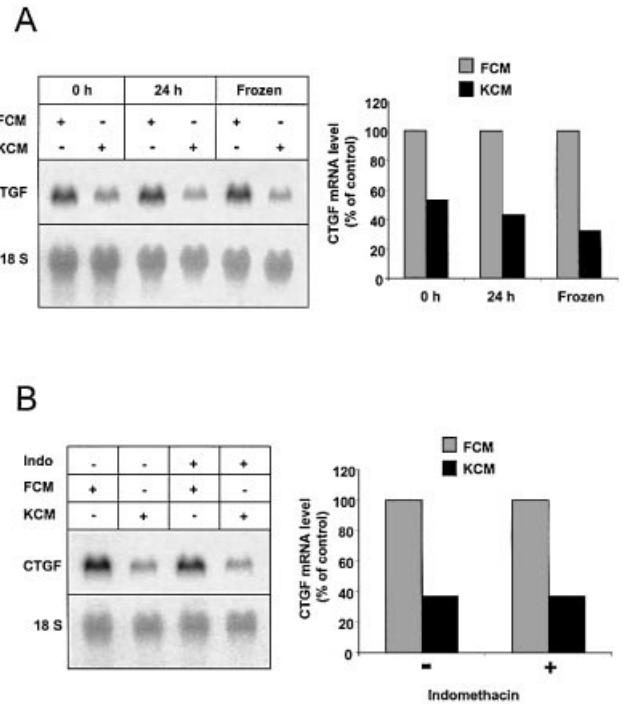


Figure 7. The putative keratinocyte-derived factor(s) suppressing CTGF expression is stable and insensitive to treatment with indomethacin. (A) Keratinocyte-conditioned medium was incubated at 37°C for 0 or 24 h, and then added to fibroblasts for 16 h. Twenty picomoles TGF-β1 was added and CTGF mRNA levels were analyzed by Northern blotting after an additional 3 h. Similarly, keratinocyte-conditioned medium that had been stored at -70°C for 4 wk was tested to evaluate its potency in inhibiting TGF-β-stimulated CTGF mRNA expression. Fibroblast-conditioned media that were treated identically were used as controls. (B) Keratinocyte- or fibroblast-conditioned medium containing 10 μM indomethacin was added to fibroblasts. After 16 h 20 pM TGF-β1 was added and CTGF mRNA levels were analyzed by Northern blotting after an additional 3 h. The experiments were repeated with identical results. Indo, indomethacin; FCM, fibroblast-conditioned medium (control); KCM, keratinocyte-conditioned medium.

suppressor of CTGF and is a major inhibitor of CTGF expression in our models.

DISCUSSION

We demonstrate the ability of epidermal keratinocytes to suppress the expression of CTGF in dermal fibroblasts by secretion of soluble factors, and this occurs in the absence of physical contact between the two cell types. A major mediator of this suppression was identified as IL-1α, whereas TNF-α and prostaglandins did not appear to be involved. Suppression of CTGF mRNA and protein was effective both in cocultures and when conditioned medium from keratinocytes was used to stimulate the fibroblasts. The CTGF suppressive fibroblast phenotype was developed over about 16 h and it was sustained.

Paracrine loops between keratinocytes and fibroblasts have been suggested to be important for basement membrane formation and fibroblast-supported keratinocyte proliferation (Maas-Szabowski *et al*, 1999). These paracrine interactions were found to involve IL-1 produced by keratinocytes, which stimulate expression of IL-1 type I receptors and keratinocyte growth factor in fibroblasts (Maas-Szabowski *et al*, 1999, 2000). For the generation of the CTGF suppressive fibroblast phenotype, however, we found that such a mutual interaction was not necessary, as conditioned medium from keratinocytes was at least as effective as the coculture approach. TGF-β1 was added to stimulate CTGF expression as a

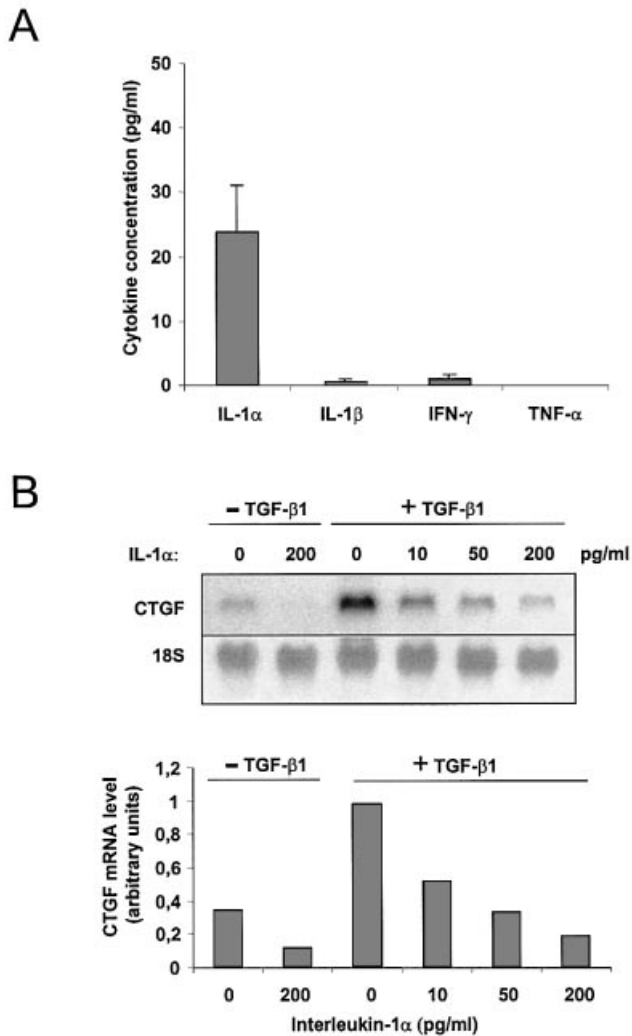


Figure 8. IL-1 α is secreted by keratinocytes and is suppressing CTGF mRNA in fibroblasts. (A) Keratinocyte-conditioned medium, prepared as described in *Materials and Methods*, was subjected to analysis of the cytokines IL-1 α , IL-1 β , IFN- γ , and TNF- α by ELISA. Values are expressed as mean \pm SEM of duplicates of five (IL-1 α) or three (IL-1 β , IFN- γ , and TNF- α) independent cultures. (B) CTGF mRNA levels in fibroblasts were analyzed by Northern blotting after treatment with various concentrations of IL-1 α for 16 h in the presence or absence of 20 pM TGF- β 1. The experiment was repeated with identical results.

standard procedure in our experiments, because the basal CTGF expression is very low and TGF- β 1 is normally present in reparative processes when re-epithelialization is ongoing (Frank *et al*, 1996). We identified IL-1 α as a major mediator of keratinocyte-derived CTGF suppression on the basis of the following results: (i) keratinocytes produce significant amounts of IL-1 α ; (ii) IL-1 α inhibits basal and TGF- β 1-stimulated CTGF expression; (iii) neutralizing IL-1 α antibodies reverse a major part of keratinocyte-mediated suppression of CTGF expression; and (iv) the putative keratinocyte-derived factor(s) is stable. In agreement with earlier reports (Delaporte *et al*, 1989; Kratz *et al*, 1991) we detected a slight increase (around 25%) in the number of fibroblasts cocultured with keratinocytes (not shown), indicating that CTGF suppression is not caused by a decreased viability of the fibroblasts. In addition, overall protein synthesis does not change as a result of coculture for 48 h in the presence of 0.5% fetal bovine serum (not shown).

IL-1 stimulates production of prostaglandins (Albrightson *et al*, 1985; Dukovich *et al*, 1986), and prostaglandins have been shown to inhibit CTGF expression in fibroblasts (Ricipero *et al*, 1999;

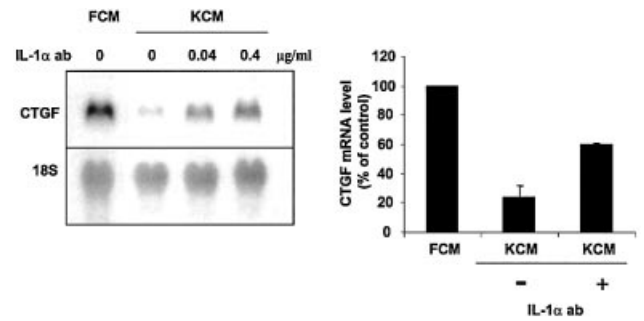


Figure 9. Neutralizing anti-IL-1 α antibodies reverse keratinocyte-mediated suppression of CTGF in fibroblasts. CTGF mRNA levels in fibroblasts were analyzed by Northern blotting after treatment with keratinocyte-conditioned medium in the absence or presence of neutralizing anti-IL-1 α antibodies. Twenty picomoles TGF- β 1 was added to the keratinocyte-conditioned medium. Values are expressed as means \pm SEM of three independent experiments (using 0.4 μ g per ml antibody). FCM, fibroblast-conditioned medium (control); KCM, keratinocyte-conditioned medium.

Stratton *et al*, 2001). In our hands, however, the IL-1 α effect is most probably not mediated via prostaglandins as indomethacin, a potent inhibitor of prostaglandin synthesis, could not reverse keratinocyte-mediated CTGF suppression. Importantly, our findings suggest that the amount of IL-1 α in keratinocyte-conditioned media can explain the full effect of keratinocyte-mediated CTGF suppression. If other factors secreted by keratinocytes have an effect on CTGF expression, this is probably minor in our experimental model. We did not detect significant amounts of IL-1 β in keratinocyte-conditioned media. Keratinocytes may produce significant amounts of pro-IL-1 β , however, which is converted to active IL-1 β *in vivo* and may affect CTGF expression during tissue repair (Black *et al*, 1988; Hazuda *et al*, 1990). Indeed, IL-1 β also demonstrates CTGF suppressive activity (unpublished observation). Being an immediate early gene product, CTGF mRNA expression in fibroblasts is induced rapidly by, for example, TGF- β 1 stimulation (Kucich *et al*, 2001, and unpublished observation). The suppression of CTGF observed in our work developed over time, however, and was sustained. This implies that a complex machinery is initiated by keratinocyte-conditioned medium/IL-1 α to sustain an inhibitory phenotype. Further studies will be required to elucidate the precise intracellular mechanism mediating the effects of IL-1 α on the expression of CTGF.

Our results are of relevance to wound healing, and it is possible that keratinocytes downregulate CTGF expression in dermal fibroblasts during re-epithelialization *in vivo*. Future investigations will show if the lack of viable keratinocytes, and the delayed re-epithelialization seen in large burns, result in overexpression of CTGF. Such an overexpression could be due to a deficiency of IL-1 α , and could be, at least partly, responsible for the formation of hypertrophic scars in these situations. Indeed, it was recently demonstrated that hypertrophic scarring after surgery is accompanied by a decreased epidermal expression of IL-1 α (Niessen *et al*, 2001). An interesting extension of this is the possibility that a deficiency in the response to keratinocyte-derived factors/IL-1 α could contribute to a continued activity of fibroblasts/production of extracellular matrix, despite an early re-epithelialization, resulting in excessive scarring or keloid formation. In conclusion, our results support the view that epidermal keratinocytes have a suppressive effect on dermal fibroblast activity.

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