Keratinocytes suppress transforming growth factor-β1 expression by fibroblasts in cultured skin substitutes

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Summary   Transforming growth factor (TGF)-β1 is a multipotent growth factor with an important role in tissue homeostasis. This growth factor regulates cell proliferation, adhesion, migration and differentiation, as well as extracellular matrix deposition. The temporal secretion and activation of latent TGF-β1 is thus of major importance to physiological and pathological processes and in wound healing and tumour formation. Cultured skin substitutes, as used to treat extensive acute or chronic skin wounds, offer an attractive model to investigate cellular interactions in cytokine and growth factor expression and response *in vitro*. In the present investigation, expression of TGF-β1 was analysed in keratinocyte, fibroblast and melanocyte monolayer cultures, as well as in the dermal vs. epidermal components of reconstituted human skin. Immunohistology, enzyme-linked immunosorbent assay (ELISA) and Northern blotting were used to demonstrate expression at the RNA and protein level. In the monolayer cultures, levels of TGF-β1 synthesized by melanocytes were observed to be considerably elevated when compared with keratinocytes. Most TGF-β1, however, was secreted by fibroblasts. The relative contribution of the epidermal and dermal components of the skin substitutes to overall TGF-β1 levels was determined by comparing results obtained for either component in the presence and absence of fibroblasts and keratinocytes. From results obtained by ELISA it was apparent that TGF-β1 levels generated predominantly by fibroblasts within the skin substitutes were greatly reduced over time in the presence of keratinocytes. Suppression of fibroblast TGF-β1 expression in the presence of keratinocytes was also demonstrable at the RNA level by Northern blotting. Results obtained by immunohistochemistry suggest that most, if not all, of the growth factor was present in the latent form. It is therefore most likely that the observed effect results from a factor secreted by keratinocytes, which is capable of suppressing TGF-β1 synthesis by fibroblasts. These results suggest that expression of TGF-β1 by fibroblasts is downregulated by paracrine actions of keratinocytes in healing skin.

Key words: cultured skin substitutes, transforming growth factor-β1, wound healing

Transforming growth factor (TGF)-β1 is a 25 kDa homodimeric protein of 112 amino acids per monomer.[1,2] These amino acids constitute the C-terminus of a 391 amino acid precursor encoded by a 2.5 kb mRNA species. The remainder of the precursor constitutes the latency associated peptide (LAP) that is non-covalently bound to TGF-β1 intracellularly, remains associated with the growth factor during secretion and dissociates extracellularly upon activation of TGF-β1.[3,4] In most *in vitro* systems, TGF-β1 appears in the latent form. Several *in vivo* conditions will contribute to TGF-β1 activation, including tissue injury, inflammation and tissue repair.[5,6]

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Following activation, TGF-β1 differentially affects proliferation in multiple cell types, stimulating proliferation in most cells of mesenchymal origin yet inhibiting keratinocyte mitosis. Moreover, TGF-β1 promotes migration of keratinocytes, fibroblasts and macrophages, stimulates myofibroblast conversion and induces scar formation. TGF-β1 thus profoundly affects wound healing.[7] Differential mRNA display has recently contributed to further elucidation of the mechanisms of action and molecular targets of TGF-β1.[5]

Within human skin, TGF-β1 production has been reported for keratinocytes, melanocytes, fibroblasts, endothelial cells, macrophages and platelets.[8–13] Some of these cell types are present in cultured skin substitutes used to treat severe chronic and burn wounds. The

rapid expansion of these cells in culture allows for coverage of large wound areas using autologous cells. Improvements to earlier developments of the keratinocyte grafting method presently include the use of a dermal substitute consisting of autologous fibroblasts attached to a collagen–glycosaminoglycan (C-GAG) matrix.[14] Application of cultured skin substitutes in the clinic may be further optimized following a thorough investigation of secreted levels of cytokines involved in the wound healing process. The activity of these cytokines can influence the take of the grafts as well as the healing of the grafted wounds.

Expression of TGF-β1 in a model system representing cultured skin substitutes was investigated at several time points following inoculation of cultured skin substitutes with cultured cells. Because expression was investigated in the epidermal and dermal components separately, contributions of each cell type to expression of TGF-β1 could be studied. By enzyme-linked immunosorbent assay (ELISA), expression in the dermal and epidermal compartments of cultured skin substitutes was compared with expression in normal and wounded skin as well as with expression by cells in monolayer cultures. Both cell homogenates and culture supernatants of monolayer cultures were investigated. Northern blots were performed to correlate protein and mRNA levels and determine whether alterations in protein levels are reflected in levels of transcription. To localize TGF-β1 protein in cultured skin substitutes further, immunostaining was performed using antibodies to TGF-β1 as well as to LAP.

Materials and methods

Culture methods

Keratinocytes were cultured at 37 °C in 5% CO_2 at all times. Human epidermal keratinocytes, isolated as previously described[15] and stored in liquid nitrogen in first passage were thawed and propagated in MCDB153 medium containing 1 ng/mL epidermal growth factor (EGF), 0·5 μg/mL hydrocortisone, 5 μg/mL insulin, 0·5% (v/v) bovine pituitary extract and antibiotics. Media were changed at 2-day intervals. Cells were harvested at 80% confluency to initiate skin insert cultures. For this model, keratinocytes were inoculated at 10^6 cells/cm^2 in Falcon 6 well culture inserts of 0·4-μm pore size in keratinocyte culture medium 1 day prior to initiating skin substitutes. Fibroblasts were isolated from human skin as previously described[16] and stored in liquid nitrogen until use. Upon thawing, cells were cultured in

Dulbecco's modified Eagle's medium (DMEM) with 10 ng/mL EGF, 0·5 μg/mL hydrocortisone, 5 μg/mL insulin, 5% fetal bovine serum and antibiotics.

Melanocytes were isolated from human epidermis essentially as previously described.[17] Briefly, dermatomed adult human breast skin was exposed to 1% trypsin in phosphate-buffered saline (PBS) at room temperature overnight. An equal volume of fetal bovine serum was added and the resulting cell suspension was washed and plated in MCDB153 with 4% fetal bovine serum, 0·6 ng/mL basic fibroblast growth factor, 10^{-8} mol/L α-melanocyte stimulating hormone, 10^{-9} mol/L endothelin-1, 10 μg/mL vitamin E and 5 μg/mL insulin. Residual fibroblasts were removed using 0·1 mg/mL geneticin treatment for 3 consecutive days as previously described.[18]

For the preparation of skin substitutes, fibroblasts were harvested and subsequently inoculated on both sides of a C-GAG substrate, prepared as previously described and preincubated in fibroblast culture medium.[19] Following fibroblast inoculation at 10^6 cells/cm^2, insert filters containing adherent epidermal cells were harvested and placed on top of fibroblasts on C-GAG. Cocultures of keratinocytes on filters and fibroblasts on C-GAG were incubated in medium as described by Chen et al.[20] Fibroblasts and keratinocytes from a single donor were used to generate inserts. Cells derived from either of two donors were used in the present studies. Both donors were female, and aged 8 months or 3 years at the time of skin donation. Cells were stored in liquid nitrogen for several years following confluency in first passage and replated at > 90% plating efficiency. Insert samples were frozen at 2, 8 and 14 days following keratinocyte inoculation (on day 2, skin equivalents were lifted to the air–liquid interface, to promote epidermal differentiation). Epidermal cell and fibroblast layers were separated using forceps and individually frozen at − 80 °C until use.

Organotypic culture of normal human skin was performed as previously described with minor modifications.[21] Briefly, 4-mm biopsies were prepared from dermatomed normal human adult skin and cultured on 3-μm pore size filter inserts (Falcon, Franklin Lakes, NJ, U.S.A.) in DMEM with 10% normal human complement-inactivated serum. Biopsies were snap frozen in OCT compound using liquid nitrogen until use.

Immunohistochemistry

Six-micrometre frozen sections were cut using a cryomicrotome and placed on slides coated with organosilane

(Sigma, St Louis, MO, U.S.A.). Sections were fixed for 10 min in cold acetone and stored at −20 °C until use.

Thawed sections were prewashed in PBS and incubated in 0·03% H_2O_2 in PBS for 30 min to inactivate internal peroxidase. Washed sections were incubated in optimal dilutions of primary antiserum in 1 g/L bovine serum albumin in PBS for 3 h. Primary antibodies used were neutralizing antibody MAS792, reactive with dimeric (25 kDa) and monomeric (12–15 kDa) molecules of TGF-β1 (Accurate Chem. Sci. Corp., Westbury, NY, U.S.A.), and AF-246-NA anti-LAP antibody (R&D Systems, Minneapolis, MN, U.S.A.). After washing, sections were incubated in optimized dilutions of biotinylated secondary antibodies to the respective primary antibodies. Horse anti-goat was used for the LAP stainings, and goat anti-mouse (Southern Biotechnologies, Birmingham, AL, U.S.A.) for others. Subsequently, sections were washed and incubated with peroxidase-labelled streptavidin 1 : 400 (Southern Biotechnologies). Twenty-five µg/mL aminoethylcarbazole (Sigma) in acetate buffer 0·1 mol/L, pH 5·2, was used to detect antigenic sites. Mayer's haematoxylin was used as a counterstain.

Enzyme-linked immunosorbent assays

Fresh and cultured normal human skin was snap frozen in liquid nitrogen and stored at −70 °C until use. Cultured skin substitutes were separated into dermal and epidermal components using forceps and frozen and stored as described for normal human skin. Cells in monoculture were scraped in PBS, and cells as well as culture supernatants were frozen until use.

Prior to use, frozen tissue samples were pulverized using a Bessman stainless steel tissue pulverizer (Fisher Scientific, Pittsburgh, PA, U.S.A.). Cell and tissue samples were suspended in PBS and sonicated on ice for 45 s using a 550 Sonic dismembrator (Fisher Scientific). ELISAs were performed according to the manufacturer's instructions (R&D Systems). Briefly, samples were acid-treated to activate TGF-β1 present in the samples. Receptor-coated plates were subsequently incubated with neutralized samples under study, washed and incubated with horseradish peroxidase-conjugated polyclonal TGF-β1 antiserum. Washed wells were subsequently exposed to tetramethylbenzoate substrate in the presence of H_2O_2. After 10 min, the reaction was terminated using 0·8 mol/L sulphuric acid. The resulting yellow coloration was measured spectrophotometrically at 450 nm. Simultaneously, protein assays were performed on the same samples using a BioRad *DC*

colorimetric protein assay (BioRad, Hercules, CA, U.S.A.). This assay is based on reactivity of protein with copper under alkaline conditions. This is followed by reduction of Folin reagent by copper-treated protein. Results were analysed using a spectrophotometer at 750 nm. TGF-β1 concentrations were normalized to protein concentrations for cell cultures and skin substitutes or normal skin samples, or normalized to cell numbers for culture supernatants. Statistical analysis of the results was performed using Student's *t*-test.

Reverse transcription–polymerase chain reaction

To demonstrate expression of TGF-β1 mRNA by fibroblasts and keratinocytes in monolayer cultures as well as in cultured skin substitutes, reverse transcription–polymerase chain reaction (RT–PCR) was performed. To this end, RNA was isolated from cultured cells and from components of cultured skin substitutes using Tri-Reagent (MRC, Cincinnati, OH, U.S.A.). Separate components of cultured skin substitutes were homogenized in Tri-Reagent using the Turrax tissue homogenizer. Subsequently, RNA was isolated according to standard protocols. RT was performed using a gene-specific downstream primer under the following conditions: 1−5 µg RNA and 2 pmol/L downstream primer were heated to 70 °C for 10 min, cooled to 4 °C and mixed with PCR buffer (Perkin Elmer Corp., Norwalk, CT), 3 mmol/L dNTPs (Gibco Life Technologies, Gaithersburg, MD, U.S.A.), 3·5 mmol/L $MgCl_2$ (Gibco Life Technologies) and 200 units Superscript II Reverse Transcriptase (Gibco Life Technologies) at 42 °C for 50 min. The mixture was cooled to 37 °C and 2 units of RNase H were added for 30 min. Subsequently, a PCR reaction was performed as follows: 25% of the RT reaction was mixed with 1 × PCR buffer, 1 mmol/L $MgCl_2$ and 20 pmol/L of primers. Primers used were 5′CAAGTGGACATCAACGGGTT3′ and 5′GCAGGAGCG CACGATCATGT3′ as previously described.[22,23] A hot-start PCR was performed for 35 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min followed by a single additional 10 min elongation step. TGF-β1-containing plasmid pRKβ1E, a kind gift from Dr R.Derynck,[24] served as a positive control and H_2O without DNA as a negative control. PCR products were subsequently run on a 1% agarose gel in Tris–borate–ethylenediamine tetraacetic acid buffer.

Northern hybridization

RNA isolated as described above under RT–PCR was

electrophoresed in Reliant gels using formaldehyde/ formamide denaturation and blotted on to Nytran maximum strength membranes (Schleicher and Schuell, Dassel, Germany) by capillary transfer using $10\times$ SSC. RNA was cross-linked to membranes by ultraviolet cross-linking. RNA was revealed by methylene blue staining to evaluate RNA transfer. Blots were stored in $5\times$ SSC at $4\,^{\circ}$C until use.

A probe to TGF-β1 was prepared from plasmid pRKβ1E. Briefly, the plasmid was amplified in XL1-Blue bacteria, reisolated and cut with *Eco*RI and *Hin*dIII to excise a 1·9 kb TGF-β1 cDNA fragment. The fragment was ^{32}P-dCTP labelled using an RTS Radprime DNA labelling system (Gibco Life Technologies). The resulting probe was separated from unincorporated labelled nucleotides using a Bio-Spin 30 Chromatography column (BioRad) and used directly for Northern blotting. To this end, blots were preincubated in hybridization buffer at $50\,^{\circ}$C for 3 h. Subsequently, the probe was added to 15 mL of fresh hybridization buffer containing 50% formamide and incubated at $58\,^{\circ}$C for 16 h. Washed blots were exposed to Kodak X-O-MAT AR film using an intensifying screen for 72 h at $-80\,^{\circ}$C.

Results

Immunohistochemistry

Figure 1 shows the detection of expression of TGF-β1 (A,B) and LAP (C,D) in normal human skin before and after *in vitro* cultivation. Specific staining is visible in red; haematoxylin counterstaining is blue. A slight increase in dermal expression of LAP is noted following cultivation of normal human skin. A greater increase is observed in staining by the MAS792 antibody performed on sections derived from the same skin. In snap frozen normal skin, perivascular localization of LAP is observed in the dermis. In the epidermis, the LAP antibody is reactive with the nucleated layers of the epidermis, whereas MAS792 is reactive only with the cornified layer of the epidermis prior to explant culture.

Immunohistochemical analysis of skin substitutes cultured for 14 days and containing both fibroblasts and keratinocytes is shown in Figure 2. Interestingly,
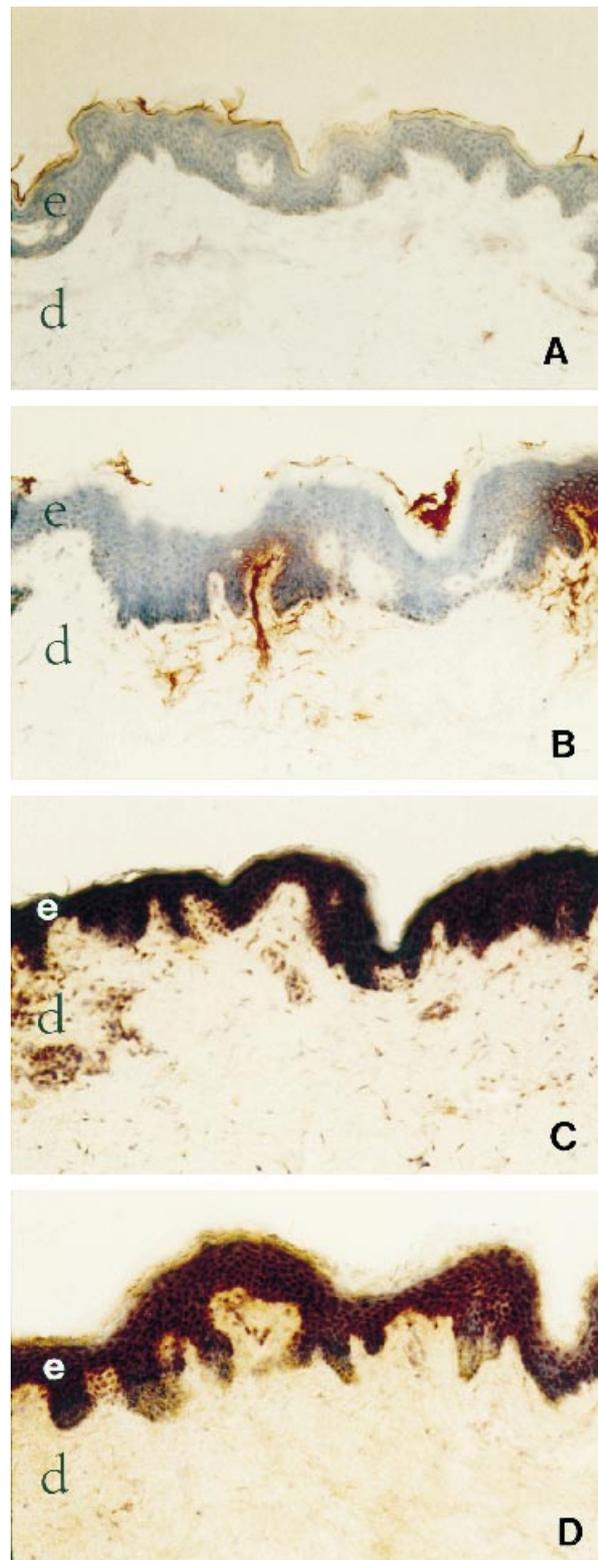
Figure 1. Transforming growth factor (TGF)-β1 immunostaining of frozen sections of control skin (A,C) as well as skin explants from the same donor cultured for 3 days (B,D). Antibodies used were directed against active (A,B) and latent (C,D) TGF-β1. Specific staining is red; haematoxylin counterstaining is blue; e, epidermis; d, dermis (original magnification ×95).
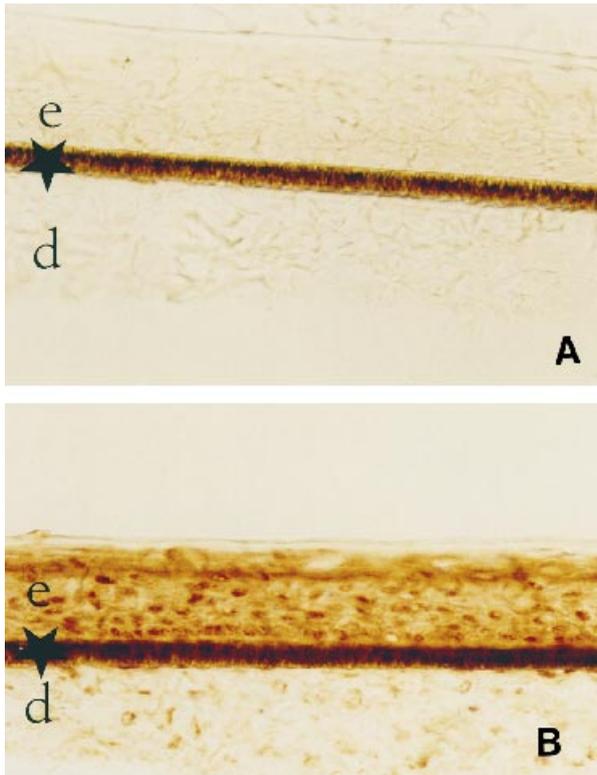
Figure 2. Immunoperoxidase staining of cultured skin substitutes cultured for 14 days using antibodies to active transforming growth factor-β1 (A) and to latency associated peptide (B). The immuno-peroxidase product is red; no counterstaining was used; e, epidermal compartment; d, dermal compartment; ★, filter (original magnification ×95).

Figure 3. Transforming growth factor (TGF)-β1 levels in cultured skin substitute compartments as detected by enzyme-linked immunosorbent assay. At time 0, values shown were detected in monoculture homogenates. At time points 2, 8 and 14 days, levels were evaluated in homogenates of either the epidermal keratinocyte compartment or the dermal fibroblast compartment of skin substitutes initially inoculated with only fibroblasts (F alone), only keratinocytes (K alone) or both (F combined and K combined). Values represent the mean of four separately executed experiments ± SEM.

the pattern of immunostaining in the cultured skin substitutes resembles that found in uncultured skin even if sampled only 2 days after keratinocyte inoculation (results not shown). Although LAP is abundant in both the dermal and the epidermal components of cultured skin substitutes, no staining is observed with the MAS792 antibody at any time point, indicating that no active TGF-β1 is detectable in the inserts. Non-specific coloration of the insert filter is prominent.

Enzyme-linked immunosorbent assays

In native human skin before and after culture, the mean ± SEM amounts of TGF-β1 detected were 126 ± 83 pg/mg protein and 235 ± 37 pg/mg protein, respectively. This is consistent with induced synthesis of TGF-β1 by short-term organotypic culture of total human skin as noted under Immunohistochemistry. Figure 3 shows quantitative detection of TGF-β1 present in the dermal and the epidermal components of cultured skin substi-

tutes at serial time points. Day 0 represents TGF-β1 in homogenates of cultured cells used to inoculate skin substitutes. Incubation on the collagen substrate thus induces a rapid increase in TGF-β1 production by fibroblasts. In addition, a small increase in production by keratinocytes is noted in cultured skin substitutes as compared with undifferentiated, monolayer culture. Relatively high levels of TGF-β1 expression by fibroblasts persist in the absence of keratinocytes. In contrast, TGF-β1 levels in the dermal component decrease steadily with time in the presence of stratified cultures of keratinocytes. Results from four separate experiments were combined in Figure 3: this explains the experimental variation observed in the data. These results indicate downregulation by keratinocytes of TGF-β1 expression in fibroblasts.

A *t*-test performed to compare TGF-β1 levels in the dermal component in the absence (F alone) or in the presence (F combined) of keratinocytes demonstrated a significant decrease at 14 days with $t = 3 \cdot 293$ at 6 d.f. ($\alpha < 0 \cdot 01$). Table 1 presents values for TGF-β1 concentrations in homogenized monolayer cultured cells and the respective supernatants in one representative
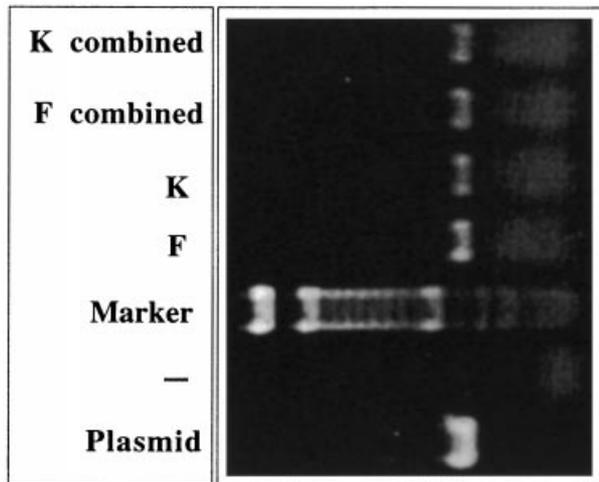
Figure 4. Reverse transcription–polymerase chain reaction of transforming growth factor-β1 mRNA isolated from keratinocyte (K) or fibroblast (F) monolayer cultures or from separated dermal fibroblast or epidermal keratinocyte compartments generated using both cell types in skin substitutes cultured for 14 days. Marker lane: 100 base pair ladder. Plasmid pRKβ1E was used as a positive control, H₂O as a negative control. The fragment generated by the primers is 445 nucleotides in length.

Table 1. Transforming growth factor-β1 concentrations in monolayer cultures

	Cell homogenates (pg/mg protein)	Culture supernatants (pg/mL per mg cellular protein)
Fibroblasts	54	710
Keratinocytes	14	35
Melanocytes	137	128

fragment is absent from the negative control without DNA and prominent in the plasmid positive control sample.

Northern blotting

Figure 5 depicts levels of TGF-β1 mRNA in the dermal and epidermal components of cultured skin substitutes before (a) and after (b) reconstitution. The levels of hybridization to the TGF-β1 probe are compared with the amounts of RNA loaded per lane. It appears that the levels of mRNA message are similar for both skin compartments at early time points. At day 14, however, suppressed mRNA levels in fibroblasts in the presence of keratinocytes as opposed to fibroblasts cultured in the absence of keratinocytes can be clearly observed, supporting the notion that regulation of protein levels has extended to transcriptional regulation at that time.

Discussion

Data in this report show that expression of TGF-β1 in cultured skin substitutes prepared with fibroblasts, keratinocytes and passenger melanocytes, results predominantly from fibroblasts. TGF-β1 present within

experiment. Data presented in the table suggest that melanocytes can contribute to epidermal TGF-β1 production but do not secrete it as much as fibroblasts.

Reverse transcription–polymerase chain reaction

Figure 4 demonstrates the presence of mRNA encoding TGF-β1 as shown by the presence of a band of correct molecular weights as specified by the primers used (445 nucleotides). Cytokine mRNA can thus be found in both fibroblasts and keratinocytes, in monolayer cultures as well as in cultured skin substitutes. The 445 nucleotide

Figure 5. Northern analysis of transforming growth factor (TGF)-β1 mRNA levels in (a) monolayer cultured cells used for reconstitution and (b) compartments of cultured skin substitutes cultured for 2, 8 or 14 days: evaluations were made in homogenates of either the epidermal keratinocyte compartment or the dermal fibroblast compartment of skin substitutes initially inoculated with only fibroblasts (F alone), only keratinocytes (K alone) or both (F combined and K combined). In the bottom frames 28S rRNA bands are shown prior to hybridization to depict the total amount of RNA loaded; the top frames represent the bands specifically hybridizing to the TGF probe at an approximate mRNA molecular weight of 2.5 kb.

the epidermis may be generated at least in part by melanocytes, as suggested by analysis of monolayer cultures of cell types present in cultured skin substitutes.

Cultured skin substitutes were prepared containing fibroblasts attached to a C-GAG substrate, and epidermal cells separated from the dermal substrate by a synthetic porous membrane. This model system permitted analysis of individual tissue compartments in the presence and absence of the reciprocal compartment under identical culture conditions. By this method it was shown that fibroblasts retain high levels of TGF-β1 synthesis if grown on a collagen-based matrix in the absence of keratinocytes. Moreover, the results support the contention that collagen induces TGF-β1 synthesis by fibroblasts. Conversely, in the presence of keratinocytes, fibroblast levels of the growth factor dropped steadily over a time period of 14 days. In contrast, low levels of epidermal TGF-β1 remained relatively constant. As the amount found in the epidermal compartment did not significantly vary in the presence or absence of fibroblasts, it is probable that the decline found in the dermal substitute in the presence of keratinocytes can be ascribed to suppression of fibroblast TGF-β1 synthesis in the presence of keratinocytes.

RT–PCR methodology demonstrated expression of TGF-β1 mRNA in both compartments of the inserts as well as in fibroblast and keratinocyte monolayer cultures. Further quantification of mRNA levels by Northern blotting showed no major differences in mRNA levels for TGF-β1 in the first 8 days of culture. These results suggest that the primary level of regulation in this time period is mRNA translation. However, 14 days after inoculation of keratinocytes, changes in mRNA levels are clearly observed between fibroblasts cultured in the presence or absence of keratinocytes. In the absence of keratinocytes, TGF-β1 mRNA increases in fibroblast cultures with time. In the presence of keratinocytes, fibroblast TGF-β1 mRNA decreases with time. This agrees with findings at the protein level as mentioned above.

Low levels of keratinocyte-derived TGF-β1 detected in this study are not consistent with results presented in an earlier report.[9] Such a discrepancy may be accounted for, in part, by the culture model used in this investigation, which allows for significant epidermal differentiation. Moreover, immunohistology performed on normal skin has previously led to seemingly conflicting results regarding the localization of TGF-β1 in the epidermis.[3] Such discrepancies may be explained both by differences in the origin of the skin and by the use of antibodies reactive with different antigenic epitopes for TGF-β1,

which appear to be exposed under different conditions.[25] In this respect, the availability of antisera to the active vs. the latent form of TGF-β1 is significant. In the present study, separate antibodies to TGF-β1 and LAP have been employed. Although lack of reactivity of the MAS792 antibody with latent TGF-β1 has not yet been reported, the present results, as well as preliminary data comparing expression patterns detected with this antibody with those detected with antiserum detecting only active TGF-β1 (results not shown), indicate that recognition by the MAS792 antibody is limited to the active form. From this investigation it thus appears that most TGF-β1 is present in skin in the latent form, particularly in the epidermis. In the dermis, active TGF-β1 is found in increased amounts following organotypic culture. This may represent activation by oxygen radicals as proposed by Barcellos-Hoff and Dix.[26]

In cultured skin substitutes, TGF-β1 appears to be present only in the latent form. However, it may be anticipated that TGF-β1 will be activated by factors in the wound bed following grafting. The presence of TGF-β1 in the grafts can subsequently contribute to wound healing and to graft acceptance by modulating inflammation and angiogenesis. Expression of TGF-β1 by allogeneic skin substitutes may thus account for its therapeutic activity in chronic wounds.

Extrapolating *in vitro* results to *in vivo* physiology, re-epithelialization during wound healing may contribute to subsequent downregulation of TGF-β1 synthesis within the skin. As TGF-β1 can inhibit mitosis of many skin cell types, it is believed that defects in such feedback mechanisms can lead to uncontrolled cell growth, as observed in skin tumours. In addition, TGF-β1 appears to be a determining factor in scar formation. Thus, timely downregulation of the growth factor may prevent extensive scarring. The temporal regulation of growth factor synthesis is therefore of major interest to improve wound care. Suppression of burn scars by sheet skin grafts and by primary engraftment of cultured skin substitutes is a clinical benefit that is well understood.[14,27] Conversely, interstices of widely meshed split-thickness skin grafts produce raised scars by granulation and secondary healing of the epidermis.[28] These clinical outcomes are consistent with the observed upregulation of TGF-β1 by fibroblasts in the absence of keratinocytes, and its downregulation in the presence of stratified epithelium as demonstrated here. The observed paracrine regulation of cellular physiology *in vitro* can account for regulation of scar formation in healing of skin wounds *in vivo*.

Besides absolute levels, activation of TGF-β1 deter-

mines the magnitude of biological response to the growth factor. In future investigations it will be important to determine factors leading to activation of latent TGF-β1 present within cultured skin substitutes. Understanding regulation of TGF-β1 and other growth factors in cultured skin substitutes will contribute to greater efficacy and improved outcome in the treatment of acute and chronic cutaneous wounds.

Acknowledgments

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