Genetically Modified Dermal Keratinocytes Express High Levels of Transforming Growth Factor-β1

Aziz Ghahary,* Edward E. Tredget,* Lung-Ji Chang,† Paul G. Scott,*‡ and Qiong Shen*

*Department of Surgery, Wound Healing Research Group, Edmonton, Alberta, Canada; †Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada; ‡Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

In an attempt to genetically modify cultured keratinocytes with transforming growth factor-β1 (TGF-β1), which has been proven to be one of the most important cytokines involved in wound healing, two constructs were made. One, designated pG3Z:K14-TGF-β1, is a plasmid in which the expression of TGF-β1 is driven by the keratin 14 promoter. The other, designated pLin-TGF-β1, is a retroviral vector in which the retroviral 5′ long-terminal repeat promoter drives expression. In both constructs, the deletion of a small fragment of the noncoding region of the TGF-β1 gene was made to differentiate the transcript from that for endogenously expressed TGF-β1. Different types of cells were transfected with the pG3Z:K14-TGF-β1 construct using the calcium phosphate method. The pLin-TGF-β1 construct was propagated in a retroviral packaging cell line and conditioned medium that contained high titers of the virus was used to transduce keratinocytes or other types of cells grown in standard culture. Northern analysis, used to evaluate the expression of TGF-β1 mRNA in the pG3Z:K14-TGF-β1 transfected keratinocyte Cl-177 cell line, showed a smaller TGF-β1 transcript compared with that endogenously expressed by dermal fibroblasts. The level of TGF-β1 protein evaluated by enzyme-linked immunosorbent assay was significantly higher in medium conditioned by either the K14-TGF-β1 transfected or the pLin-TGF-β1 transduced keratinocytes, compared with that obtained from control cells; however, the level of TGF-β1 protein was unchanged in cultures of pG3Z:K14-TGF-β1 transfected nonkeratinocyte cells such as fetal and adult fibroblasts. Using the mink lung epithelial cell growth inhibition assay, we found an increase in TGF-β1 activity in conditioned medium from the pG3Z:K14-TGF-β1 transfected cells. To evaluate possible paracrine effects of the keratinocyte derived TGF-β1, a coculture system was established with pLin-TGF-β1 transduced keratinocytes grown in the upper chamber and dermal fibroblasts in the lower chamber. The results showed that TGF-β1 released from keratinocytes diffused to the lower chamber where it stimulated collagen production by dermal fibroblasts. In summary, we demonstrate here that primary cultured keratinocytes can be genetically modified to express high levels of TGF-β1 and suggest that this offers a potential approach for the therapy of dermal lesions such as nonhealing wounds. ***Key words: co-culture/fibroblasts/gene therapy/keratinocyte/K14/retroviral vector/TGF-β1. J Invest Dermatol 110:800–805, 1998***

Transforming growth factor β1 (TGF-β1) is a multifunctional cytokine that influences many important physiologic processes, such as cellular proliferation and differentiation (Sporn *et al*, 1986). This cytokine is secreted as a latent high molecular weight complex (L-TGF-β), consisting of a 25 kDa dimeric mature protein with 112 amino acids in each subunit and the dimeric *N*-terminal precursor peptide known as the latency associated peptide (Tsuji *et al*, 1990). TGF-β1 is a member of a large family of proteins consisting of five TGF-β isoforms (TGF-β1–5), bone morphogenetic proteins, inhibins, activins, and mullerian inhibiting substance. TGF-β isoforms share 70–80% sequence identity and the sequences of the mature, proteolytically processed forms of each member are highly conserved between species (Roberts and Sporn, 1989). TGF-β1, 2, and 3 have been found in mammals and their tissue specific differential expression may have important biologic consequences (Ghahary *et al*, 1996).

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Reprint requests to: Dr. Aziz Ghahary, 161 HMRC, 8440–112 Street, University of Alberta, Edmonton, Canada, T6G 2B7.

Abbreviation: LTR, long-terminal repeat.

It is well known that TGF-β1 is one of the most effective growth factors stimulating the synthesis and deposition of various extracellular matrix proteins important in differentiation, morphogenesis, and wound healing (Goldstein *et al*, 1989; Roberts and Sporn, 1989; Hill *et al*, 1992). TGF-β1 stimulates fibroblasts to synthesize collagen, fibronectin, and glycosaminoglycans (Ignotz and Massague, 1986; Sporn *et al*, 1987); it enhances neovascularization (Allen *et al*, 1993) and modulates the production of several proteases and their inhibitors (Edwards *et al*, 1987; Overall *et al*, 1989). TGF-β1 is a very potent chemoattractant for monocytes (Wakefield *et al*, 1987) and fibroblasts (Postlethwaite *et al*, 1987). Subcutaneous injection into newborn mice stimulates granulation tissue formation (Roberts *et al*, 1986) and it has also been shown to accelerate healing of incisional wounds in rats (Mustoe *et al*, 1987).

The process of cultivating keratinocytes *in vitro* to confluency for grafting onto patients with severe thermal injuries has been well established (Green *et al*, 1979). O'Connor *et al* were the first to use autologous cultured keratinocytes for grafting on to a burn wound (O'Connor *et al*, 1981). The use of cultured epithelial autografts has not only been beneficial for patients with massive burns, but has also been successfully used for the treatment of giant congenital nevi (Gallico *et al*, 1989) and junctional epidermal bullosa (Carter *et al*,

1987) and in post-burn scar revision (Kumagai *et al*, 1988). Although cultured epithelial autografts have a recognized limitation for wound healing in burn patients, the technique offers the advantage of minimal donor site requirements and the potential for the molecular biologic manipulation of target cells. Keratinocyte gene therapy therefore offers enormous potential for novel therapeutic approaches not only following thermal injury, but for other congenital and acquired disorders of the skin (Fabre and Cullen, 1989). We describe here the construction and evaluation of two vectors, pG3Z:K14-TGF-β1 and pLin-TGF-β1, carrying the TGF-β1 gene. The K14 promoter-driven TGF-β1 is specifically targeted to cultured keratinocytes whereas the retroviral long-terminal repeat (LTR) promoter-driven TGF-β1 should be expressed in any cell type, including cultured keratinocytes. It is the aim of this study to provide evidence that keratinocytes that may be used as wound coverage can be genetically modified to express high levels of TGF-β1.

MATERIALS AND METHODS

Construction of the pG3Z:K14-TGF-β1 and pLin-TGF-β1 vectors Two vectors were constructed to express human TGF-β1 in keratinocytes. To specifically target the expression of this cytokine to keratinocytes, the pG3Z:K14 cassette, in which the keratin 14 promoter drives the expression of TGF-β1, was used. The K14 cassette contains a keratin-14 promoter/enhancer in the Ava 1 restriction site that is upstream of a K14 polyadenylation signal. In this cassette, there is a BamH1 restriction site located between the K14 promoter and polyadenylation sequences in which the cDNA of interest may be cloned; however, we could not use this site because the TGF-β1 cDNA has a BamH1 restriction site within its coding region. To overcome this problem, the overhanging ends created by Pst I and EcoR1 in the TGF-β1 cDNA and by BamH I in the vector were filled with the Klenow fragment. The blunt-ended Pst1-EcoR1 fragment of TGF-β1 cDNA, containing 76 nucleotides of the 5′ untranslated region, an ATG start codon, a sequence coding for the 390 amino acids of L-TGF-β1, and 176 nucleotides of the 3′ untranslated region, was then subcloned into the blunt-ended restriction site downstream of the K14 promoter in the pG3Z:K14 cassette (**Fig 1*A***). This construct is designated pG3Z:K14-TGF-β1. The K14 promoter has been successfully used to drive the expression of IL-6 cDNA in keratin-14 expressing epithelial cells in the skin of transgenic mice (Turksen *et al*, 1992).

A second construct, in which a moloney murine leukemia virus 5′ LTR promoter drives the expression of TGF-β1, was also made. The proviral structure of the L-TGF-β1 retroviral vector, referred to as pLin-L-TGF-β1 (**Fig 1*B***), carries the 5′ and 3′ viral LTR, a neomycin-resistance gene, and a latent TGF-β1 construct that consists of 76 nucleotides of the 5′ untranslated region, an ATG start codon, a sequence coding for the 390 amino acids of L-TGF-β1, and 68 nucleotides of the 3′ untranslated region.

Gene transfection and transduction The pG3Z:K14:TGF-β1 construct was used to transfect cultured Hela cells, the XB-2 keratinocyte cell line (ATCC, CL-177), fetal and adult fibroblasts, and primary human keratinocytes. To optimize conditions for transfection with calcium phosphate or liposomes, the XB-2 keratinocyte cell line was used with ^{14}C-labeled chloramphenicol in a chloramphenicol acetyltransferase assay. The result showed greater chloramphenicol acetyltransferase activity with the calcium phosphate precipitation and therefore this technique was used in all subsequent experiments (data not shown). The success of this transfection was also evaluated by northern analysis for TGF-β1 mRNA and by enzyme-linked immunosorbent assay (ELISA) and the mink lung epithelial cell growth inhibition assay of conditioned medium for protein.

The pLin-TGF-β1 retroviral vectors lack the gag, pol, and env genes required for viral packaging and are therefore replication incompetent and cannot be propagated in any cell with a normal genome. Therefore, calcium phosphate precipitation was employed to introduce this construct into a PA317 packaging cell line that possesses viral structural proteins for vector. Packaging cells were enriched by growth in the presence of 0.5 mg G-418 for 6–10 d. A total of 53 packaging cell colonies resistant to the antibiotic were screened and eight colonies with high TGF-β1 production titers (3–4 ng per ml) were selected and subcultured. At confluency, conditioned medium was collected, assayed for TGF-β1 protein, and used for transduction of primary keratinocytes.

Cell cultures The procedure of Rheinwald and Green (1975) was used for cultivation of human foreskin keratinocytes using serum-free keratinocyte medium (GIBCO, Grand Island, NY) supplemented with bovine pituitary extract (25 μg per ml) and epidermal growth factor (0.5 ng per ml). Primary cultured keratinocytes at passages 3–5 were used. Keratinocytes were then grown alone or in the upper chamber of a coculture system with dermal fibroblasts in the lower chamber. In this system TGF-β1 released from the pLin-TGF-β1 transduced keratinocytes can diffuse through the 0.4 μm porous membrane separating the two chambers. To establish the fibroblast cultures, either fetal foreskin or normal skin punch biopsies obtained from patients undergoing elective reconstructive surgery, were established in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum as previously described (Ghahary *et al*, 1994). Strains of dermal fibroblasts at passages 3–7 were used in this study. Other cells, such as Hela cells, XB-2 keratinocytes, and pA317 packaging cells, were obtained from the American Type Culture Collection (12301-Parklawn Drive, Rockville, MD) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Extraction of cellular RNA and northern analysis To demonstrate whether the K14 promoter drives the expression of TGF-β1 mRNA, XB-2 keratinocytes were transfected with either pG3Z:K14-TGF-β1 or pG3Z:K14 construct alone and 72 h later the culture medium was removed and cell layers were lysed in 6 ml of guanidinium thiocyanate as previously described (Ghahary *et al*, 1994). Total RNA was extracted by the guanidinium isothiocyanate/CsCl procedure of Chirgwin *et al* (1979), separated by electrophoresis, and blotted onto nitrocellulose filters. Filters were then baked under vacuum for 2 h at 80°C and prehybridized in a solution containing 50% formamide, 0.3 M sodium chloride, 20 mM Tris HCl (pH 8.0), 1 mM ethylenediamine tetraacetic acid, 1X Denhardt's solution (1X = 0.02% bovine serum albumin, Ficoll and polyvinylpyrrolidone), 0.005% salmon sperm DNA, and 0.005% poly (A) for 2–4 h at 45°C. Hybridization was performed in the same solution at 45°C for 16–20 h using cDNA probes for either human TGF-β1 or β-actin. The probes were labeled with ^{32}P-α-dCTP (DuPont Canada, Streetsville, Mississauga, Ontario, Canada) by nick-translation. Filters were initially washed at room temperature with 2 × sodium citrate/chloride buffer (1X = 0.15 M sodium chloride, 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate for 30 min, then for 20 min at 65°C in 0.1 × sodium citrate/chloride buffer and 0.1% sodium dodecyl sulfate solution. Autoradiography was performed by exposing Kodak X-Omat film to the nitrocellulose filters at –70°C in the presence of an enhancing screen. The human TGF-β1 cDNA was a gift from Dr. G.I. Bell (Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology and Medicine, University of Chicago, IL). β-actin cDNA was obtained from the American Type Culture Collection (Rockville, MD).

Enzyme-linked immunosorbent assay for TGF-β1 To determine the amounts of TGF-β1 produced by pG3Z:K14-TGF-β1 transfected or pLin-TGF-β1-transduced cells, a sandwich ELISA for TGF-β1 was used (Danielpour, 1993). Briefly, 96 well plates were coated with 100 μl per well of monoclonal antibody to human TGF-β (Genzyme, Cambridge, MA) at a concentration of 1 μg per ml in phosphate-buffered saline (PBS). The plates were incubated for 2 h at room temperature followed by 16 h at 4°C. After washing twice with PBS-Tween-20 (PBS-T), the plates were blocked with 1% bovine serum albumin (crystallized, Sigma, St Louis, MO) for 60 min at room temperature and washed three times with PBS-T. One milliliter of conditioned medium from each culture sample was acidified with 24 μl of 5N HCl for 15 min at room temperature and neutralized with 40 μl of 1 M HEPES/5 N NaOH (5/2). One hundred microliters of the acidified/neutralized samples was added to each well of the plates, which were then incubated at room temperature for 60 min. After washing, the plates were incubated with 100 μl per well of chicken anti-human TGF-β (R&D Systems, Minneapolis, MN) at a concentration of 2.5 μg per ml for 60 min at room temperature with shaking. After washing five times with PBS-T, the plates were incubated with alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma) at room temperature for 60 min followed by five washings with PBS-T. After adding substrate (o-nitrophenyl phosphate, 1 mg per ml, Sigma), the plates were incubated at room temperature for 60 min and the optical density was read using a THERMOmax (Molecular Devices, Menlo Park, CA) microplate reader at a wave length of 405 nm. Serial dilutions (0, 125, 250, 625, 1250, to 2500 pg per ml) of recombinant human TGF-β1 (Genzyme) were used to prepare a standard curve.

Epithelial cell growth-inhibition assay The mink-lung epithelial cell (CCL-64, American Culture Tissue Type) growth-inhibition assay and preparation of conditioned media were used as described (Danielpour *et al*, 1989; Ghahary *et al*, 1998). Briefly, subconfluent cultures were trypsinized, washed in assay medium (Dulbecco's modified Eagle's medium, 0.2% fetal bovine serum, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, and 1% penicillin/streptomycin antibiotic), seeded at 5 × 10^5 per 0.5 ml per well in 24 well plates, and incubated for 3 h at 37°C in an atmosphere of 5% CO_2. Various volumes (0, 1, 2.5, 5, 10, 20, and 40 μl) of low-serum conditioned media obtained from the pG3Z:K14-TGF-β1 transfected and untransfected cells or a standard amount of TGF-β1 (Genzyme) was then added. Twenty-two hours later 0.25 mCi of ^{3}H-labeled thymidine was added to each well for

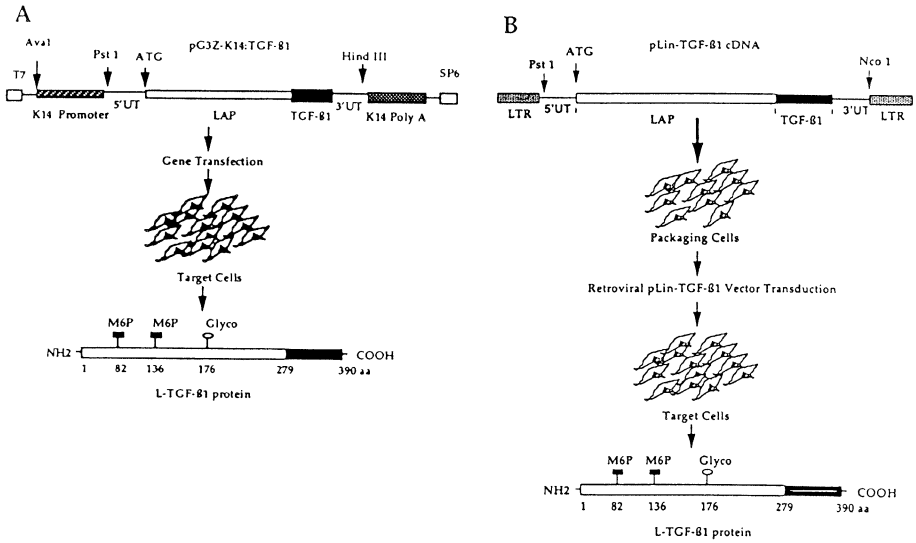

Figure 1. Scheme for the construction and amplification of the pG3Z-K14:TGF-β1 and pLin-TGF-β1 retroviral vectors. (*A*) The Pst1-EcoR1 fragment of TGF-β1 cDNA containing 76 nucleotides of the 5′ untranslated region, an ATG start codon, a cDNA coding sequence for 390 amino acid of L-TGF-β1, and 176 nucleotides of the 3′ untranslated region that has been excised and subcloned into the BamH1 restriction site located downstream of the K14 promoter of the pG3Z:K14 cassette as described in the *Materials and Methods*. The K14 cassette contains a keratin-14 promoter/enhancer in the Ava 1 restriction site that is upstream of a K14 polyadenylation signal at the 3′ end. (*B*) The structure of L-TGF-β1 retroviral vector referred to as pLin-L-TGF-β1 carrying a 5′ LTR and a DNA sequence corresponding to the latent TGF-β1 construct identical to that described in (*A*). Latent TGF-β1 with 390 amino acids released from either pG3Z-K14:TGF-β1 transfected or pLin-TGF-β1 transduced cells is also shown. ■, Mature TGF-β1; □, latency associated protein.

2 h and incubation continued. Cells were then fixed, washed, and the radioactivity was measured in the cell lysate collected from each well.

Hydroxyproline analysis by gas chromatography/mass spectrometry To examine whether TGF-β1 released from pLin-TGF-β1 transduced keratinocytes grown in the upper chamber of a coculture system can stimulate production of collagen by dermal fibroblasts grown in the lower chamber, the hydroxyproline content of the medium was measured as previously described (Tredget *et al*, 1993). Briefly, the medium was dried in a rotary evaporator after adding a known amount of hydroxypipecolic acid as internal standard. Samples were hydrolyzed in 0.5 ml of 6N HCl at 116°C for 16 h. The N,O-trifluoroacetyl N, methyl ester derivative of hydroxyproline was prepared after drying the hydrolysate. Gas chromatography/mass spectrometry analysis was performed on an HP 5890 GC linked to a HP 5970 MSD (mass selective detector) monitoring the ions m/z-164 and 278. Each sample was run in triplicate and the results expressed as pg hydroxyproline per ml of medium per 48 h obtained by reference to a standard curve of collagen analyzed under identical conditions.

Statistical analysis The differences in hydroxyproline and TGF-β1 protein production between either the pG3Z:K14-TGF-β1 transfected and untransfected or the pLin-TGF-β1 transduced and untransduced cells were calculated and compared. The statistical significance was evaluated using Student's t test; p values of < 0.05 were considered significant.

RESULTS

Keratin 14 promoter drives the expression of TGF-β1 mRNA in keratinocytes To examine whether the pG3Z:K14-TGF-β1 was constructed correctly and whether the K14 promoter drives the expression of the TGF-β1 transcript in keratinocytes, XB-2 keratinocytes (Cl-177 cell line) were transfected using the calcium phosphate precipitation method and the total RNA was extracted and evaluated by northern analysis. In all experiments, the pG3Z:K14 vector with no TGF-β1 insert was used as a negative control. Total RNA from two different strains of dermal fibroblasts was also extracted and analyzed as a control for the endogenously expressing TGF-β1 transcript. As shown in **Fig 2**, the TGF-β1 transcript is highly expressed in the pG3Z:K14-TGF-β1 transfected keratinocytes (**Fig 2**, keratinocytes, lane +) and this transcript is smaller than that expressed by two strains of dermal fibroblasts (fibroblast lanes) examined. Rehybridization of the same blot with a probe specific for β-actin revealed that the apparently high expression of TGF-β1 in transfected keratinocytes and its smaller apparent size are not due to variations in loading or in mobility of RNA during electrophoresis (**Fig 2*a***, β-actin band). These results show that the K14 promoter can drive the expression of mRNA for TGF-β1. To examine whether this TGF-β1 is expressed at the protein level and released from transfected cells, conditioned medium was evaluated by ELISA. The results (**Fig 2*b***) revealed a 2-fold increase in TGF-β1 in conditioned medium derived from transfected cells relative to the control.

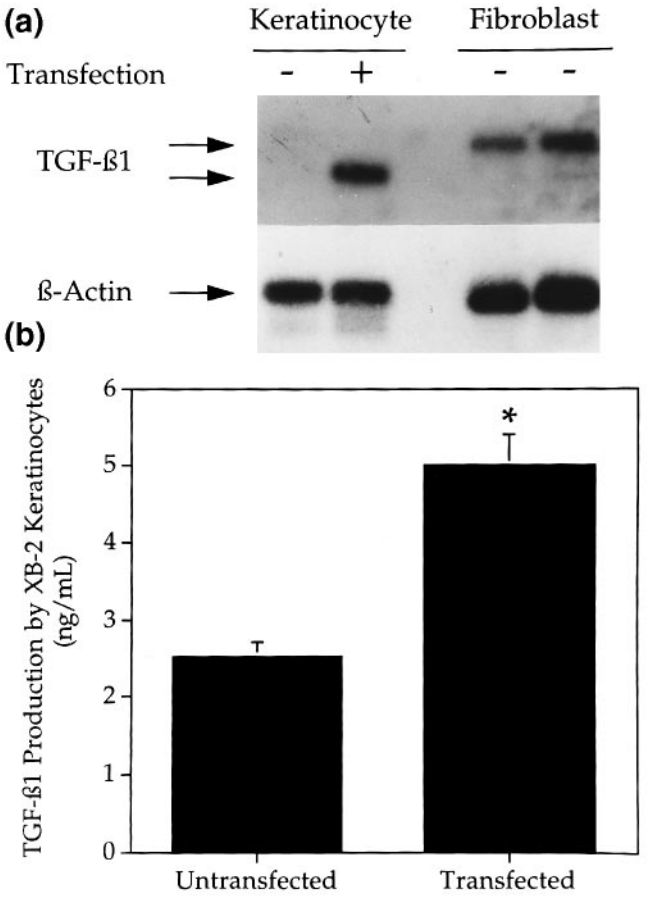

Figure 2. The keratin 14 promoter drives the expression of TGF-β1 mRNA and protein in pG3Z:K14:TGF-β1 transfected XB-2 keratinocytes. (*a*) Total RNA from either pG3Z:K14-TGF-β1 (+), pG3Z:K14 (–) transfected XB-2 keratinocytes or two strains of untransfected dermal fibroblasts hybridized initially with human TGF-β1 cDNA and subsequently with a cDNA specific for β-actin used as a control for RNA loading. (*b*) The quantitative analysis of TGF-β1 in conditioned medium of transfected and untransfected keratinocytes, measured by ELISA as described in the *Materials and Methods*. Data are shown as means ± SEM for three separate experiments done in duplicate. The asterisk denotes a significant difference ($p < 0.05$) in production of TGF-β1 between the pG3Z:K14:TGF-β1 and pG3Z:K14 transfected cells.

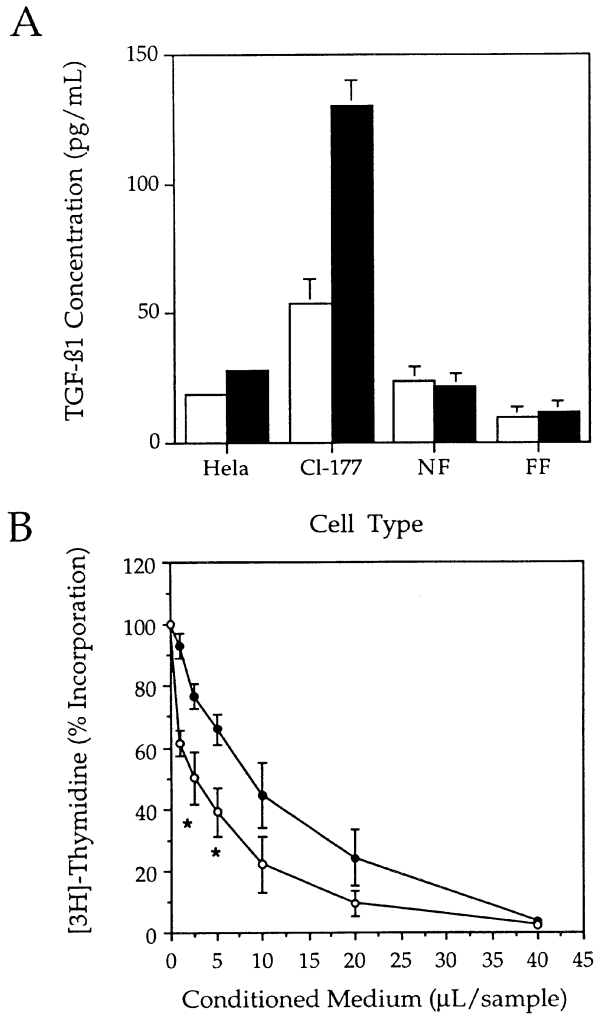

Figure 3. The keratin 14 promoter specifically drives the expression of TGF-β1 in keratinocytes but not in mesenchymal cells. Conditioned medium from either pG3Z:K14:TGF-β1 transfected Hela cells, Cl-177 keratinocytes, adult fibroblasts, or fetal foreskin fibroblasts (■) and the pG3Z:K14-transfected negative control cells (□) was collected and the TGF-β1 was measured by ELISA (*A*). Data are expressed as means ± SEM for two separate experiments done in triplicate. (*B*) A dose response curve for the biologic activity of TGF-β1 in the CL-177 cell conditioned medium, evaluated by the mink lung epithelial cell growth inhibition assay using [^{3}H]thymidine incorporation as an index of DNA synthesis. Data represent the means ± SEM of three different experiments done in triplicate. The asterisk denotes a significant difference ($p < 0.05$) in the inhibition of thymidine incorporation between the pG3Z:K14:TGF-β1 and pG3Z:K14-transfected cells. ○, condition medium derived from pG3Z:K14:TGF-β1 transfected cells; ●, condition medium derived from pG3Z:K14 transfected cells.

Activity of the keratin 14 promoter is specific for keratinocytes To address the question of how specific the K14 promoter is for keratinocytes, conditioned medium derived from pG3Z:K14:TGF-β1 transfected Hela cells, adult fibroblasts, or fetal foreskin fibroblasts was assayed by ELISA, using the pG3Z:K14 transfected cells as negative controls. The results shown in **Fig 3**(***A***) revealed that the K14 promoter is highly active only in the CL-177 keratinocytes. No significant difference in TGF-β1 production was found between pG3Z:K14:TGF-β1 transfected and untransfected for any of the other cell strains examined. In general, the level of endogenous TGF-β1 production was also markedly higher in the CL-177 cells.

The biologic activity of TGF-β1 released from CL-177 cells was evaluated in the mink lung epithelial cell growth inhibition assay using [^{3}H]thymidine incorporation as an index for inhibition of DNA synthesis. As TGF-β1 is secreted by many different cell strains in an inactive (latent) form (Sporn *et al*, 1986), conditioned medium derived from transfected and untransfected cells was acidified and then neutralized prior to being added to the mink lung epithelial cells. This experiment revealed that conditioned medium from pG3Z:K14:TGF-β1 transfected CL-177 cells had a greater inhibitory effect on the thymidine incorporation into the DNA of the mink lung epithelial cells than that obtained from untransfected control cells (**Fig 3*B***). This finding indicates that the TGF-β1 released from transfected cells can interact (after activation) with the receptors on the mink lung epithelial cells.

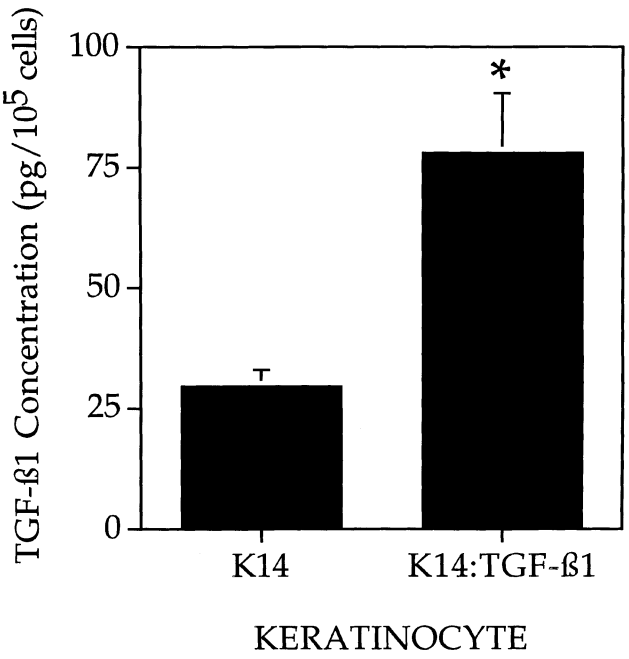

Figure 4. The keratin 14 promoter drives the expression of TGF-β1 in cultured keratinocytes. Primary cultured keratinocytes were established from fetal foreskin tissues as described in the *Materials and Methods*. Cells were transfected with either the pG3Z:K14:TGF-β1 or the pG3Z:K14 constructs (control), conditioned medium was collected and TGF-β1 was measured by ELISA. The data represent the means ± SEM for three separate experiments done in duplicate. The asterisk denotes a significant difference ($p < 0.05$) in TGF-β1 protein between pG3Z:K14:TGF-β1 transfected and control keratinocytes.

The activity of the K14 promoter has also been examined in primary cultured keratinocytes. Results of an ELISA assay revealed a more than 2-fold increase (77.8 ± 12.4 *vs* 29.3 ± 8.8 pg per 10^5 cells, $p < 0.05$) in TGF-β1 production by pG3Z:K14:TGF-β1 transfected keratinocytes relative to pG3Z:K14 transfected control cells (**Fig 4**). This further confirmed that the K14 promoter drives the expression of TGF-β1 specifically in keratinocytes rather than mesenchymal cells.

A retroviral LTR promoter drives the expression of TGF-β1 in primary cultured keratinocytes As another approach to genetically modifying the expression of TGF-β1 in keratinocytes, a retroviral vector bearing pLin-TGF-β1 containing an aminoglycoside 3′ phosphotransferase antibiotic resistant gene has been constructed. A total of eight of 53 packaging cell colonies resistant to G-418 were screened, selected on the basis of high TGF-β1 production (3–4 ng per ml), and subcultured. Conditioned medium collected at confluency was evaluated for TGF-β1 protein and used for transduction of primary keratinocytes, cultured alone or with dermal fibroblasts. As shown in **Fig 5**(***A***), the concentration of TGF-β1 detected by ELISA is higher in conditioned medium from pLin-TGF-β1 transduced keratinocytes relative to that from control cells. Co-culture of these keratinocytes with dermal fibroblasts led to an increase in collagen production by the latter (**Fig 5*B***). These findings demonstrate that keratinocytes can be readily modified with the pLin-TGF-β1 retroviral vector to produce a high level of TGF-β1 that can stimulate collagen production by dermal fibroblasts.

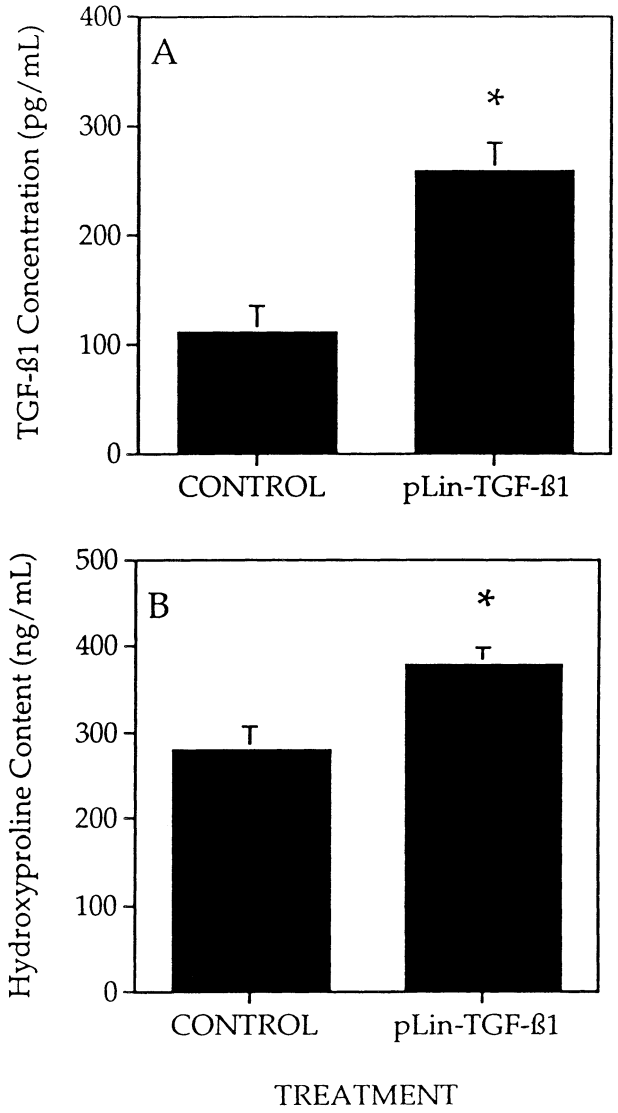

Figure 5. TGF-β1 released from primary keratinocytes increases the collagen production by dermal fibroblasts. The retroviral vector pLin-TGF-β1 was constructed and propagated in a PA317 packaging cell line in the presence of geneticin (G-418) as shown in **Fig 1**. Conditioned medium from the pLin-TGF-β1 transduced and untransduced packaging cells was then collected and used to transduce primary cultured keratinocytes. (*A*) Comparison of the concentration of TGF-β1 evaluated by ELISA in conditioned medium of pLin-TGF-β1 transduced and untransduced keratinocytes. These cells (keratinocytes) were then grown in the upper chamber of a coculture system in which dermal fibroblasts were grown in the lower chamber. (*B*) The effect of diffusible keratinocyte derived TGF-β1 on hydroxyproline used as an index for collagen production by dermal fibroblasts. Data represent means ± SEM for three separate experiments done in duplicate. The asterisk denotes a significant difference ($p < 0.05$) in TGF-β1 protein and hydroxyproline either between the pLin-TGF-β1 transduced and untransduced keratinocytes or between fibroblasts cocultured with the pLin-TGF-β1 transduced and untransduced keratinocytes, respectively.

DISCUSSION

The multifunctional characteristics of TGF-β1 make it a unique cytokine for possible treatment of nonhealing wounds or to accelerate the normal process of healing in severe thermal injury. This is because it modulates the synthesis and deposition of various extracellular matrix proteins such as fibronectin, collagenase, type I collagen, and type III collagen by dermal fibroblasts (Goldstein *et al*, 1989; Roberts and Sporn, 1989; Hill *et al*, 1992). This cytokine also enhances the production of protease inhibitors such as tissue metalloproteinase inhibitor-1 (Edwards *et al*, 1987; Overall *et al*, 1989), glycosaminoglycans (Ignotz and Massague, 1986; Sporn *et al*, 1987), and neovascularization (Allen *et al*, 1993).

The efficacy of any growth factor, including TGF-β1 in the stimulation of extracellular matrix *in vivo*, depends on the route by which it is delivered to the wound. Its efficacy is likely to be markedly depressed when administered topically or intravenously due to instability of the mature TGF-β1. Additionally, this approach may not be cost effective. Another approach might be to deliver the growth factor through cultured keratinocytes used as wound coverage. This approach is feasible because several studies have demonstrated that cultured epithelial autografts can successfully be used for wound coverage in patients with large thermal injuries, giant congenital nevi (Green *et al*, 1979; Gallico *et al*, 1989), and junctional epidermal bullosa (Carter *et al*, 1987) and in post-burn scar revision (Kumagai *et al*, 1988). This technique offers the advantage of minimal donor site requirements and the potential for molecular biologic manipulation of the keratinocytes during culture where they are readily accessible.

This study was conducted to examine the hypothesis that keratinocytes can be genetically modified to express TGF-β1. Data presented here indicate that gene modification can specifically be targeted to keratinocytes through the K14 promoter or nonspecifically through a retroviral promoter, each of which has potential advantages and disadvantages. As we have demonstrated in this study, the K14 promoter specifically drives the expression of the gene of interest, such as TGF-β1, in both primary and CL-177 keratinocyte cells but not in mesenchymal cells. The K14 promoter was chosen for this study because it has successfully been used in a transgenic model and its activity in stratified squamous epithelial cells has been reported (Vassar *et al*, 1989). It has also been used successfully to drive the expression of IL-6 cDNA in the skin of transgenic mice (Turksen *et al*, 1992).

Although the K14 promoter directs the expression of TGF-β1 to keratinocytes in a specific way, using this promoter in a plasmid vector with low transfection efficiency and low stability might be considered disadvantageous. Retroviral mediated gene transfer leads to stable integration of the gene of interest into the genome of keratinocytes. Eming *et al* (1996) recently used this approach to introduce the human IGF-1 gene with a rate of gene transduction greater than 50% in primary cultured keratinocytes. Genetically modified keratinocytes then showed a significant increase in the secretion of IGF-1 both *in vivo* and *in vitro* relative to untransduced cells. Another potential advantage of this technique is that transduced cells can be selected by culture in the presence of antibiotic. In contrast to the previous approach, retroviral mediated gene transfer lacks specificity and virus can enter all cells carrying viral receptors. This may be an obstacle when *in vivo* transduction is required but this lack of specificity may not be an important issue when delivering a gene product to the wound site through sheets of cultured keratinocytes. This is because the viral vectors used in our study were genetically modified to make them replication incompetent. Development of fibrotic condition due to overexpression of TGF-β1 from sheets of either K14-TGF-β1 or pLin-TGF-β1 transduced keratinocytes is also unlikely to be a problem in our system for two reasons. First, the genetically modified keratinocyte sheet used as a wound coverage is temporary and is dislodged following natural epithelialization, and second, TGF-β1 is released in a latent form and becomes activated when activation factors such as proteases are present in the wound environment. As in a normal condition, the mechanism of TGF-β1 activation may not be functional following epithelialization.

TGF-β1 released from pLin-TGF-β1 transduced keratinocytes grown in the upper chamber of a coculture system was able to stimulate synthesis of collagen by dermal fibroblasts (**Fig 5*B***), even though it was found to be inactive in the traditionally used mink lung epithelial cell growth inhibition assay (data not shown). We have recently reported that treatment of dermal fibroblasts with IGF-1 induces production of latent TGF-β1 and that this effect is probably mediated at least in part by auto-induction of TGF-β1 because it can be blocked by TGF-β1 neutralizing antibody (Ghahary *et al*, 1998). Both these observations suggest that dermal fibroblasts may have a mechanism to activate the latent TGF-β1.

In summary, data presented here clearly demonstrate that primary cultured keratinocytes can be genetically modified to express high levels of a multifunctional growth factor such as TGF-β1. This approach has potential for the development of novel therapies for the treatment of a broad range of dermal diseases.

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