Resistance of keratinocytes to TGFβ-mediated growth restriction and apoptosis induction accelerates re-epithelialization in skin wounds

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Summary

The pleiotropic growth factor TGFβ plays an important role in regulating responses to skin injury. TGFβ targets many different cell types and is involved in all aspects of wound healing entailing inflammation, re-epithelialization, matrix formation and remodeling. To elucidate the role of TGFβ signal transduction in keratinocytes during cutaneous wound healing, we have used transgenic mice expressing a dominant negative type II TGFβ receptor exclusively in keratinocytes. We could demonstrate that this loss of TGFβ signaling in keratinocytes led to an accelerated re-epithelialization of full thickness excisional wounds accompanied by an increased proliferation in keratinocytes at the wound edge. Furthermore, we show that impaired TGFβ signaling in keratinocytes reduces apoptosis in re-epithelialized wounds of transgenic animals.

A cDNA array identified the transcription factor early growth response factor 1 (Egr1) as a target gene for TGFβ in late phases of the wound healing process. As a member of the immediate-early gene family, Egr1 is upregulated shortly after injury and induces the expression of growth factor genes. We could demonstrate that Egr1 expression is also upregulated in skin wounds which have already undergone re-epithelialization. In conclusion, we attribute the enhanced re-epithelialization in our transgenics to the resistance of keratinocytes to TGFβ-mediated growth restriction and apoptosis induction. We also propose a new role for TGFβ induced Egr1 in late phase wound repair.

Key words: TGFβ, Egr1, Wound healing, Re-epithelialization, Apoptosis, Mouse

Introduction

Growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), keratinocyte growth factor (KGF) and transforming growth factor beta (TGFβ) coordinate the process of wound healing (for a review, see Martin, 1997). Among these, TGFβ, the prototype of the transforming growth factor family, has most probably the broadest range of activities (for a review, see Blobe et al., 2000). The three isoforms of TGFβ, TGFβ1, TGFβ2 and TGFβ3 have been shown to be involved in the major phases of wound healing, i.e. inflammation, re-epithelialization, matrix formation and remodeling (for a review, see O'Kane and Ferguson, 1997; Roberts and Sporn, 1996). Re-epithelialization is a crucial step towards wound healing. Impairment of this process results in the development of chronic wounds. The reconstitution of the epithelial barrier is achieved by keratinocytes migrating from the wound edge into the wound site. These keratinocytes originate in a proliferative burst at the wound edge (Garlick and Taichman, 1994b; Matoltsy and Viziam, 1970). Both, proliferation and migration of keratinocytes, are influenced by TGFβ (Garlick and Taichman, 1994a; Hebda, 1988; Tsuboi et al., 1992). However, the role of TGFβ in re-epithelialization is unclear. On the one hand, in vitro experiments with keratinocytes have shown that TGFβ1 stimulates the expression of integrins, primarily α5β1, αvβ6 and αvβ5, which enable epidermal cells to migrate over the provisional wound bed (consisting mainly of fibrin, fibronectin and vitronectin) (Gailit et al., 1994; Zambruno et al., 1995); on the other hand TGFβ inhibits keratinocyte proliferation in vitro and in vivo (Coffey et al., 1988; Sellheyer et al., 1993). To further complicate the situation, topical application of TGFβ on skin wounds has different effects on re-epithelialization, depending on the dose and model chosen (Garlick and Taichman, 1994a; Hebda, 1988; Mustoe et al., 1991).

A recently published wound healing study using *Smad3* null mice (Ashcroft et al., 1999) has shed some light on this paradox. Re-epithelialization in these mice, which lack a component of the TGFβ cascade, was accelerated. The elevated BrdU labeling index of keratinocytes at the wound edge demonstrated that TGFβ signaling can indeed inhibit keratinocyte proliferation in vivo and thereby inhibit re-epithelialization. Nevertheless, owing to the fact that in this knockout mouse the TGFβ signaling pathway via Smad3 is abrogated in all cells but may take place via Smad2, it is difficult to distinguish primary and secondary effects on keratinocytes (Ashcroft et al., 1999). To circumvent this problem, we have investigated wound healing of full thickness excisional wounds in a mouse model with an interrupted TGFβ signaling pathway specifically in keratinocytes. We and others have previously shown that the expression of a dominant negative type II TGFβ receptor in keratinocytes impairs the TGFβ signal-transduction pathway in the epidermis (Amendt

et al., 1998; Wang et al., 1997). Owing to the use of the bovine keratin 5 promoter, the signal transduction pathway is disrupted in keratinocytes with proliferative potential. This defined interruption of TGFβ signaling gives us the unique opportunity to differentiate between direct and secondary effects of TGFβ on keratinocytes.

In this study, we have confirmed that TGFβ impairs re-epithelialization by inhibiting keratinocyte proliferation. In addition, we could demonstrate that resistance to TGFβ reduces keratinocyte apoptosis. Furthermore, we demonstrate that the expression of the transcription factor, early growth response gene 1 (Egr1) is upregulated in late phases of wound healing. Moreover, we could show that this raised expression of Egr1 in late phase wounds is induced by TGFβ. Egr1 is a member of the immediate-early gene family and has been shown to be induced shortly after injury (Khachigian et al., 1996; Pawar et al., 1995). Egr1, also known as NGFI-A, zif268, tis8 and Krox24, is the prototype member of the early growth response gene family (Egr1-Egr3) (for reviews, see Gashler and Sukhatme, 1995). Members of this family are rapidly induced by a variety of extracellular stimuli including growth factors and cytokines, hypoxia, physical forces and injury (Khachigian et al., 1996; Lemaire et al., 1988; Schwachtgen et al., 1998; Yan et al., 1999). During injury, Egr1 is considered to be a major transcription factor for genes encoding crucial cytokines and growth factors in injury repair, such as interleukin 2 (IL2), tumor necrosis factor α (TNFα), platelet-derived growth factors A and B (PDGF-A and PDGF-B), basic fibroblast growth factor (bFGF) and TGFβ (Biesiada et al., 1996; Khachigian et al., 1996; Liu et al., 1996; Skerka et al., 1995; Yao et al., 1997). Our finding that Egr1 is upregulated by TGFβ in keratinocytes at late stages of the wound healing process provides new insights into the specific function of TGFβ signaling in keratinocytes: the induction of master regulators for essential factors of tissue repair and remodeling.

Materials and Methods

Wounding and preparation of wound tissue

The two previously characterized transgenic lines TgN(K5dkTbRII)114Mbl and TgN(K5dkTbRII)54Mbl were used (Amendt et al., 1998). Both lines express the human type II TGFβ receptor, which lacks the cytoplasmic domain, under the control of the bovine keratin 5 promoter, which drives the expression of the transgene in the epidermis and in follicular cells. Keratinocytes from these transgenics show resistance to TGFβ-mediated growth restriction, as demonstrated by [^{3}H]thymidine incorporation assays. However, homeostasis was unaltered in normal skin. The lines were maintained as heterozygotes on a FVB/N background. Littermates were used as controls. Full thickness excisional wounds were inflicted on the back of 8-week-old mice using a biopsy punch of 8 mm diameter. Biopsy specimens were obtained at days 3, 5, 7, 10, 11, 12, 13 and 14 after wounding. Biopsies were either embedded in tissue freezing medium (Tissue Tek, Miles, IN) and immediately frozen in liquid nitrogen or directly frozen in liquid nitrogen for RNA preparation.

BrdU-labeling and apoptosis

BrdU-labeling experiments were performed using the *In Situ* Cell Proliferation Kit, AP (Boehringer Mannheim, Mannheim, Germany) essentially as described (Amendt et al., 1998). In brief, mice were injected with 30 μg of BrdU per gram body weight and sacrificed after a labeling period of 1 hour. Fixation and processing of the samples were performed according to the manufacturers instructions. BrdU indices were determined by photographing sections and counting labeled nuclei in a 64 mm^2 field. Detection and quantification of programmed cell death in the epidermis was performed using the *In Situ* Cell Death Detection Kit, FLUOS (Boehringer Mannheim, Mannheim, Germany). Fresh cryosections were treated as recommended by the supplier, counterstained for keratin 14, and analyzed by fluorescence microscopy, essentially as described (Breuhahn et al., 2000). The number of apoptotic cells in the basal epidermal layer was determined and related to 100 total basal cells. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; Boehringer Mannheim, Mannheim, Germany). Numbers given for analysis of apoptosis are mean values obtained from at least four different animals and numbers given for BrdU-labeling experiments are mean values obtained from at least three different animals.

Histology and immunohistology

Cryosections were cut at 5 μm thickness and fixed in 4% paraformaldehyde in neutral buffered saline for 20 minutes at room temperature. Neutrophils and mast cells were detected by histochemical staining of the sections with naphthol-ASD-chloroacetate-esterase. Treatment of samples for immunohistological procedures has been described previously (Breuhahn et al., 2000). In this study, an antibody directed to Mac-1 (CD11b, 1:100; Pharmingen, Germany, Catalog No. 01711D) was used for the detection of macrophages. Immunolocalization of Egr1 was carried out by using anti-mouse anti-Egr1 antibody (C-19, 1:100, Santa Cruz Biotechnology, Catalog no. sc-19) (Ghanem et al., 2000). Incubation with the primary antibodies was performed overnight at 4°C.

RNA isolation and northern blot

Total RNA was isolated using Tri Reagent (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Aliquots (~30 μg) of RNA were electrophoresed on 1% agarose formaldehyde gels and subsequently blotted onto nylon membranes (Hybond N, Amersham, Braunschweig, Germany). Filters were processed at high stringency as described (Church and Gilbert, 1984). For analysis of wound specimens, RNA obtained from three different animals was pooled. Probes used were either cDNA-fragments or generated by RT-PCR with the following primers.

GAPDH: GAPDH-1, 5′-CAA CTA CAT GGT CTA CAT GTT C-3′ (position 159-181; GenBank Accession Number M32599); and GAPDH-2, 5′-ACC AGT AGA CTC CAC GAC-3′ (position 340-322; GenBank Accession Number M32599)

Egr1: EGR-1, 5′- AGC ACC TGA CCA CAG AGT CC-3′ (position 578-597); and EGR-2, 5′- AGG TCT CCC TGT TGT TGT GG-3′ (position 1078-1059; GenBank Accession Number NM_007913)

cDNA probes were used for detection of the human dominant negative type II TGFβ receptor (human TGFβ type II receptor, Accession Number NM_003242) and for keratin 5 (bovine cytokeratin III, Accession Number K03536). Expression levels were quantified using a PhosphorImager System (Molecular Dynamics STORM 860 System) and the Image Quant Software (Molecular Dynamics, Sunnyvale, USA). The band intensities of different mRNA species were related to the band intensities of the housekeeping gene *Gapdh*.

Genomic expression arrays

cDNA array analysis was performed by using Atlas^{TM} Mouse 1.2 Arrays (CLONTECH, Heidelberg, Germany) that contain a total of 1176 cDNA segments spotted on a nylon membrane. Probing of cDNA arrays was performed as described in the CLONTECH Atlas cDNA Expression Arrays User Manual (PT3140-1). Briefly, RNA was

extracted from 7-day-old wounds of three different animals using the Atlas™ Pure Total RNA Labeling System according to the User Manual (PT3231-1). The RNA was pooled and radiolabeled with α-P^{32}-dATP from Amersham. After hybridization, the array membrane was washed and analyzed by using PhosphorImager System (Molecular Dynamics, Sunnyvale, USA). For normalization, cDNAs that did not show variable intensities and were located near the cDNA of interest were used.

Cell culture and transfection of HaCaT cells

HaCaT cells (Boukamp et al., 1988) were maintained in GC-Medium supplemented with 0.5% FCS (Vitromex, Weirdo, Germany). Stable DNA transfections were carried out using the calcium phosphate procedure (Graham and van der Eb, 1973). The expression vector pHbAPr-1 consists of a 4.3 kb fragment of the human β-actin gene promoter, a polylinker derived from pSP64 and the vector backbone derived from pcDV1, including the AmpR gene and NeoR gene (Leavitt et al., 1984; Melton et al., 1984; Okayama and Berg, 1983). The cDNA of the dominant negative type II receptor was cloned into the polylinker (Brand et al., 1993). The transfectants were selected in medium containing 1 mg/ml G418. For RNA-preparation, cells were lysed directly in Tri Reagent 45 minutes after addition of 5 ng/ml TGFβ1 (Strathmann Biotec AG, Hamburg, Germany).

Statistics

Data is shown as mean±s.d. Statistical significance in the differences between two groups was analyzed using Student's *t*-test and values of $P<0.05$ were considered significant.

Results

Re-epithelialization

In the present study, we have investigated the role of TGFβ in re-epithelialization of cutaneous wounds using a transgenic mouse model with an interrupted TGFβ signaling pathway specifically in keratinocytes (Amendt et al., 1998). We monitored the re-epithelialization of full thickness excisional wounds on the back of transgenic and non-transgenic mice by histological examination of wound sections from day 10 to day 13 post wounding (Table 1). The re-establishment of the epidermis was completed at day 11 in transgenic mice, whereas in non-transgenic mice the wounds still exhibited a gap in the epithelium (Fig. 1). In non-transgenic mice, the re-epithelialization was completed 1 day later at day 12. We did not detect any differences in granulation tissue formation in the transgenic mice compared with non-transgenic mice (Fig. 1).

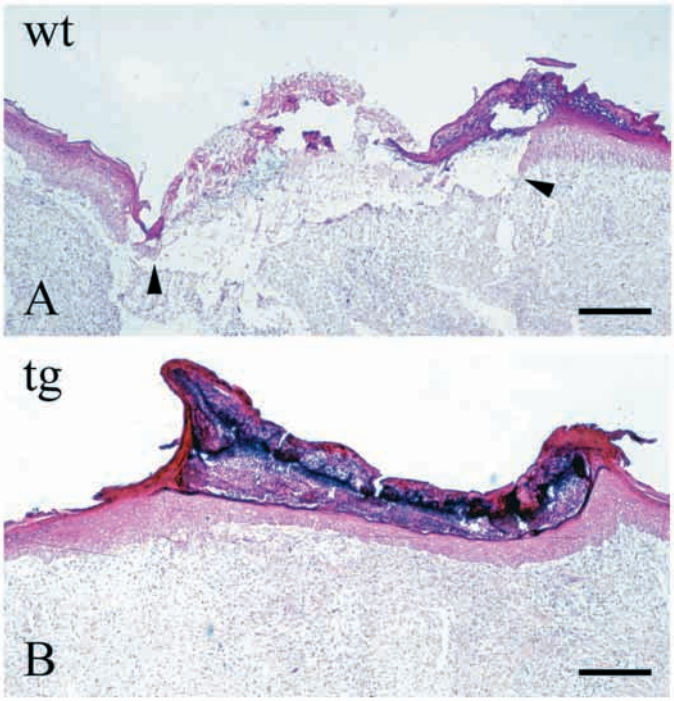

Fig. 1. Re-epithelialization of full thickness excisional wounds of wild-type and transgenic mice at day 11 after wounding. (A) Hematoxylin-Eosin stained cryosection through the mid-point of a wound from a wild-type animal showing an incomplete re-epithelialization. The wound edges were marked by arrow heads. (B) Section through the mid-point of a wound from a transgenic animal where the re-epithelialization process is already completed. Bars, 100 μm.

Remodeling

The accelerated re-epithelialization process in the transgenic mice correlated with an earlier loss of hematopoietic cells from the wound site (Fig. 2). Sections of wound specimens from at least four different animals were Giemsa stained, photographed and the number of hematopoietic cells were quantified in a 64 mm^2 field (Fig. 2). Ten days after wounding, no significant difference in the numbers of hematopoietic cells was evident (Fig. 2E). But at day 14, the number of hematopoietic cells in the wound was markedly decreased in the transgenic animals compared with the control animals (Fig. 2E; 1.6-fold reduction, $P<0.02$). To further clarify which blood-derived cells were retained in the wounds of non-transgenic animals, we performed immunohistochemical staining for macrophages, using the macrophage-specific marker Mac1, and for mast cells and neutrophils, using naphthol-ASD-chloroacetate-esterase staining, in two transgenic and non-transgenic animals (Fig. 3). In 14-day old wounds of transgenic animals we found fewer macrophages and mast cells or neutrophils (Fig. 3A,C) when compared with non-transgenic animals (Fig. 3B,D). The earlier disappearance of the hematopoietic cells in the wound site of the transgenic animals correlates with the accelerated wound closure.

Table 1. Re-epithelialization

	10 days post wounding		11 days post wounding		12 days post wounding		13 days post wounding	
	Re-epithelializaton completed							
	Yes	No	Yes	No	Yes	No	Yes	No
Animal transgenic	1	5	4	1	4	0	4	0
Wild type	0	5	1	4	4	0	4	0

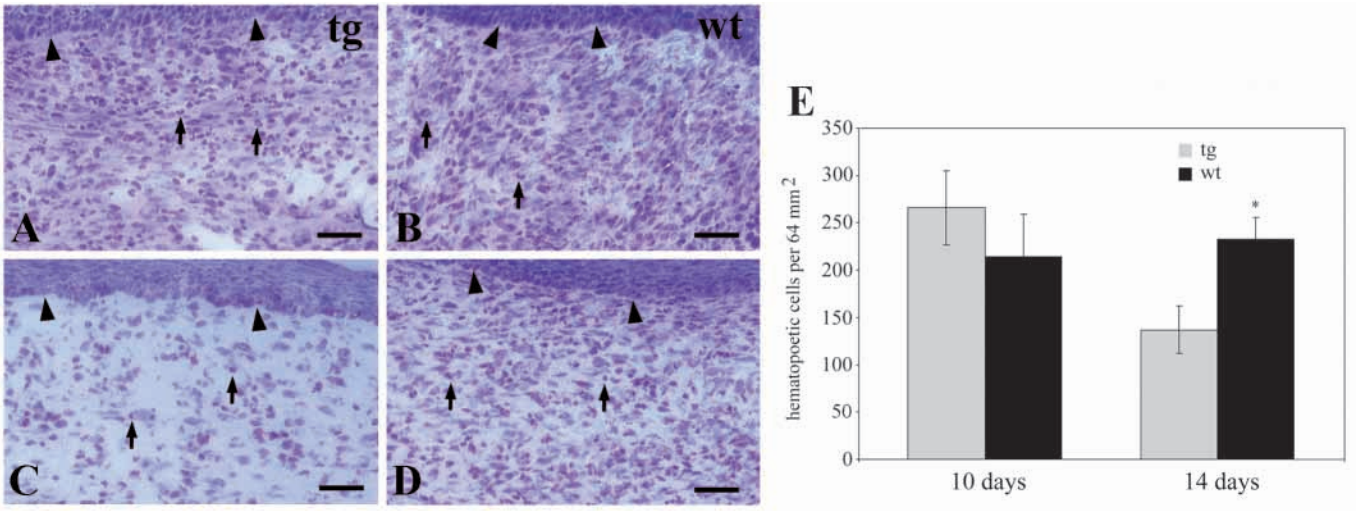

Fig. 2. Hematopoietic cells in the granulation tissue. Giemsa stained cryosections of 10-day-old wounds (A,B) and 14-day-old wounds (C,D). At day 10, the number of giemsa stained cells is similar in both transgenic (A) and non-transgenic (B) animals, whereas at day 14, the number of giemsa stained cells is clearly reduced in transgenic animals (C) in comparison with non-transgenic animals (D). Arrowheads indicate newly formed epidermis; arrows indicate hematopoietic cells. Bars, 50 μm. (E) Quantification of hematopoietic cells. Counting of giemsa stained cells in 64 mm^2 fields of a photograph from wounds of at least four different animals revealed a significantly reduced number in transgenic animals compared with non-transgenic animals at day 14 after wounding (P<0.02). Error bars represent the standard deviation.

Keratinocyte proliferation and apoptosis

To test if the interrupted TGFβ signaling pathway in keratinocytes leads to a relaxed cell cycle control and thus to a higher proliferation rate in the wound edge of transgenic mice, we performed BrdU-labeling experiments with at least three different animals per group (Fig. 4). At days 3, 5 and 7, mice of the transgenic line 114 showed significantly increased numbers (P<0.05, Student's t-test) of labeled nuclei in the wound edge compared with non-transgenic animals (Table 2). Similarly, transgenic mice of line 54, which shows a lower level of transgene expression than line 114, also displayed elevated BrdU labeling indices in comparison with controls, albeit these differences were less pronounced than in line 114 (Table 2). This finding correlates well with the different levels of transgene expression in these two lines (Amendt et al., 1998). These results demonstrate that the accelerated re-epithelialization in transgenic mice could be attributed at least in part to an increased proliferation rate in keratinocytes at the wound edge.

Table 2. Epidermal BrdU labeling indices

Animal	3 days post wounding	5 days post wounding	7 days post wounding
Line 114	43±9	32±1	33±4
Line 54	34±7	30±7	34±4
Wild type	23±4	22±6	23±4

It had been shown that the decrease of cellularity in the

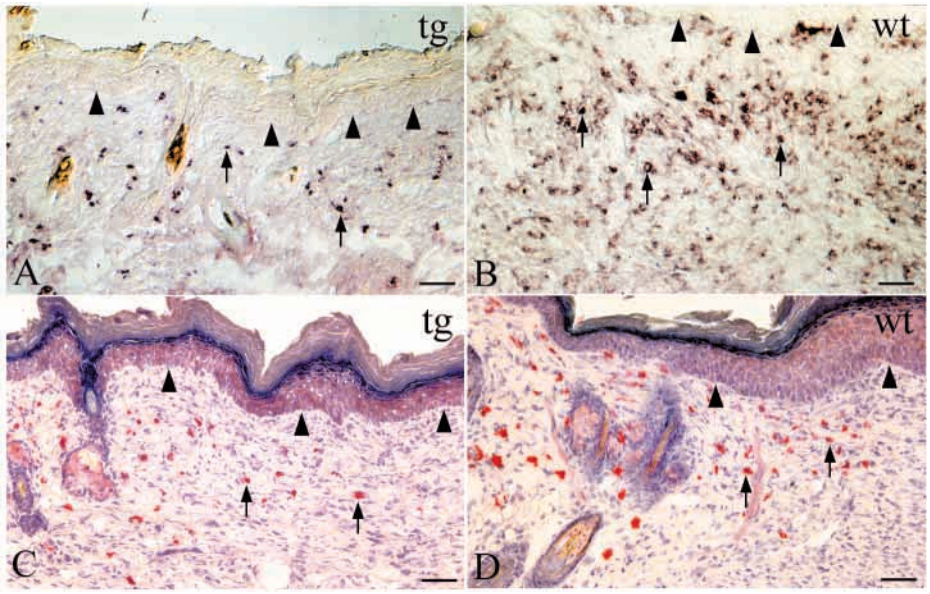

Fig. 3. Characterization of hematopoietic cells in the granulation tissue. Immunohistochemistry using Mac1 (CD11b)-antibody for detection of macrophages (A,B), and naphthol-ASD-chloroacetate-esterase histochemistry for detection of neutrophils and mast cells (C,D). Transgenic mice (A,C) exhibited significantly fewer numbers of macrophages (A), as well as neutrophils and mast cells (C) in cryosections of 14-day-old wounds when compared with the non-transgenic controls (B,D, respectively). Arrows indicate macrophages (A,B), and mast cells or neutrophils (C,D); arrowheads indicate the epidermal/dermal junction (A-D). Bars, 50 μm.

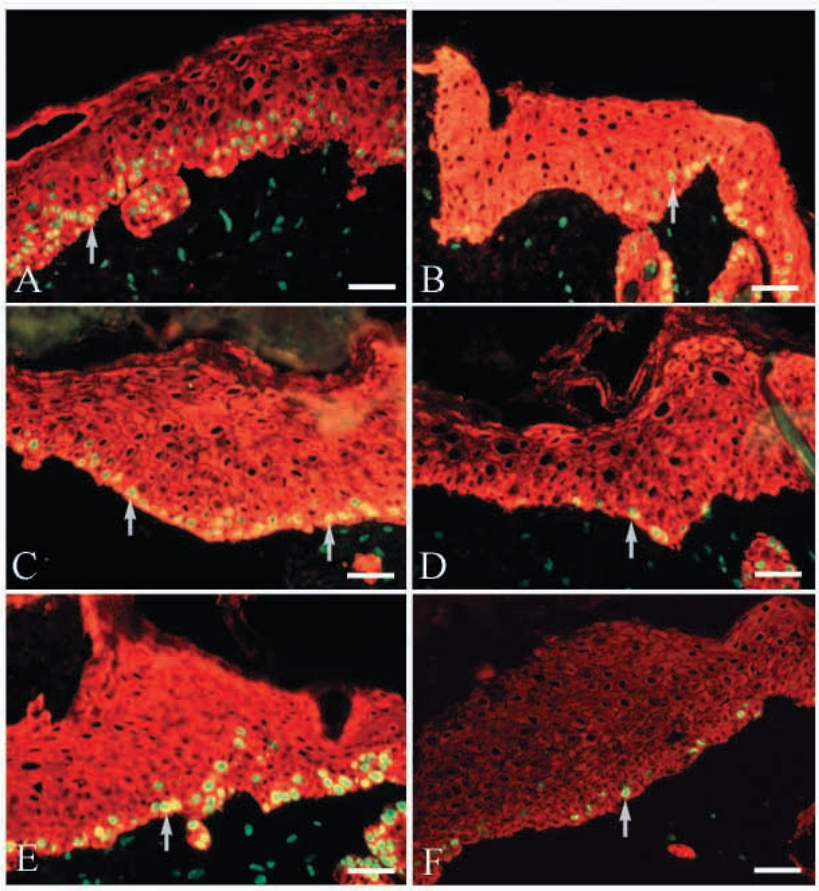

Fig. 4. Keratinocyte proliferation during wound healing. (A-F) At least three mice of every group were injected intraperitoneally with BrdU and sacrificed after a labeling period of 1 hour. Skin sections were stained for detection of BrdU-labeled S-phase nuclei (green, arrows) and for keratin 14 marking the epidermis (red). Transgenic animals (A,C,E) exhibit a higher number of labeled nuclei at the wound edge at 3, 5 and 7 days post wounding compared with wild-type animals (B,D,F). 3 days post wounding (A,B); 5 days post wounding (C,D); and 7 days post wounding (E,F). Bars, 50 μm.

remodeling phase during wound healing involves apoptosis (Desmouliere et al., 1995). As TGFβ induces programmed cell death in various cell types (Lomo et al., 1995; Nass et al., 1996), we tested if apoptosis was modulated in keratinocytes during the late phase of wound healing. Apoptotic cells in 13-day-old wounds were marked by TUNEL labeling and counted versus total number of basal cells in at least four different transgenic and non-transgenic animals (Fig. 5). In transgenic animals, a significantly lower number of apoptotic cells were found in comparison with wild-type animals. Only 0.42±0.16% basal epidermal cells in transgenic animals undergo apoptosis, whereas in wild-type animals 0.77±0.10% of the basal epidermal cells were apoptotic (P<0.005, Student's t-test). RNAse protection assays using RNA from

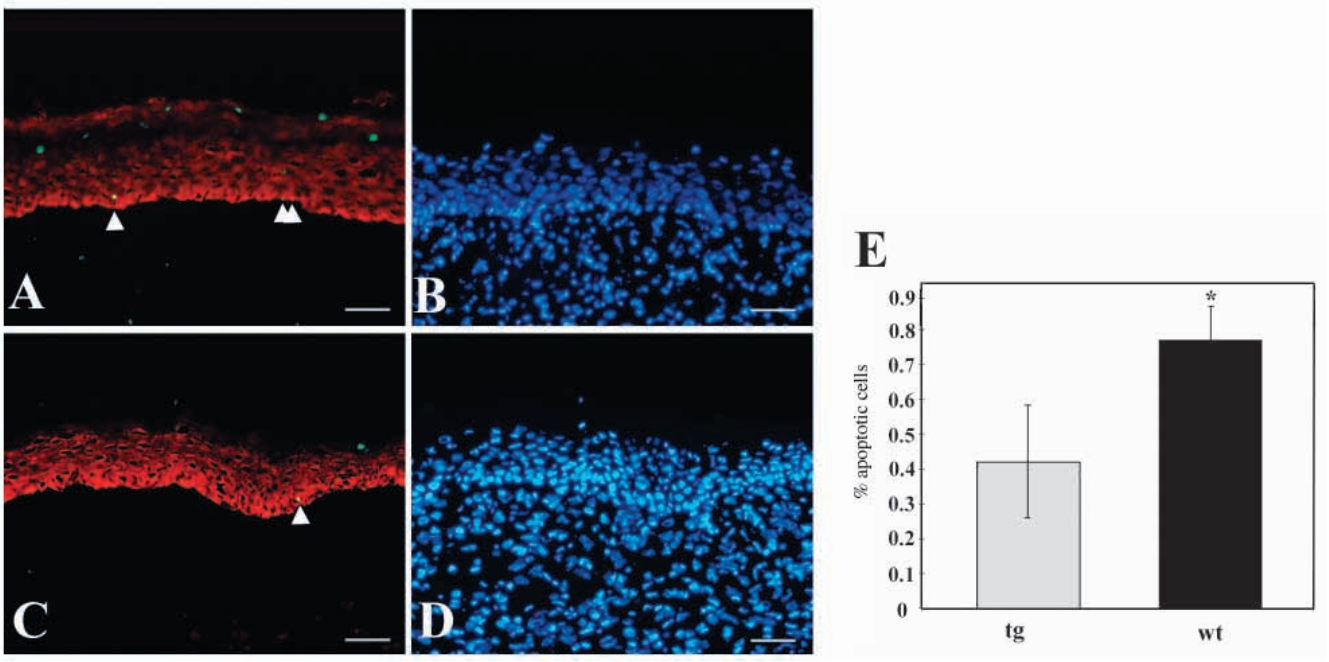

Fig. 5. Apoptosis in the epidermis of 13-day-old wounds. (A,C) Apoptotic cells in the newly formed epidermis, which is stained by an anti-keratin 14 antibody (red), were marked by TUNEL-labeling (green; arrow heads). (B,D) All cell nuclei were stained with DAPI (blue). In non-transgenic animals (A,B), a significantly higher number of apoptotic cells were detected compared with transgenic animals (C,D). (E) The percentage of apoptotic cells. Only 0.42±0.16% basal epidermal cells in transgenic animals undergo apoptosis, whereas in wild-type animals 0.77±0.10% basal apoptotic cells are found. Non-transgenic animals, n=5; transgenic animals, n=4; *P<0.005. Bars, 50 μm.

pooled wounds obtained from three different animals at 3 days and 5 days after wounding showed that the Bcl2 family members Bcl2, Bcl-X, Bax, Bak, Bad and A1 were not differentially regulated on the RNA level between transgenic and wild-type animals (data not shown).

Target genes

To find TGFβ target genes in keratinocytes during wound healing, we performed a cDNA array hybridization. cDNA from pooled wounds of at least three transgenic and non-transgenic animals collected at day 7 post wounding were used as probes. We found that the gene encoding early growth response factor 1, a transcription factor, was markedly downregulated (sevenfold) in transgenic animals compared with non-transgenic animals (Fig. 6A). We confirmed the finding that Egr1 is upregulated in controls even at this late phase of the wound healing process using northern blot analysis of 13-day-old wounds. For this analysis pooled RNA from three different animals were used. In unwounded skin, the expression of Egr1 mRNA was low in both transgenic and non transgenic animals. Whereas in 13-day-old wounds, the expression in non-transgenic animals was 4.8-fold (mean of three independent experiments) higher compared with unwounded skin. By contrast, in 13-day-old wounds of transgenic animals, only a 1.5-fold upregulation (P<0.05, Student's t-test) of Egr1 expression was detected (Fig. 6B,C show one representative experiment).

The cells mainly accounting for Egr1 expression in response to wounding are keratinocytes, as demonstrated by Egr1 immunostaining in 13-day-old wounds (Fig. 7). Keratinocytes of the newly re-epithelialized epidermis showed strong Egr1 staining, mainly in the cytoplasm (Fig. 7A). By contrast, in unwounded areas, Egr1 expression in keratinocytes was not detectable (Fig. 7B).

For further confirmation of an impaired upregulation of Egr1 in keratinocytes by an interrupted TGFβ signaling pathway, we stably transfected the human keratinocyte line HaCaT with an expression vector containing the dominant negative type II TGFβ receptor (Fig. 8). The expression of the dominant negative type II TGFβ receptor in two transfected HaCaT clones was analyzed by Northern Blot analysis (Fig. 8A). Clone 1 expressed the dominant negative type II TGFβ receptor at high levels, in contrast to clone 2, which is used as vector control (neo). The induction of Egr1 mRNA in response

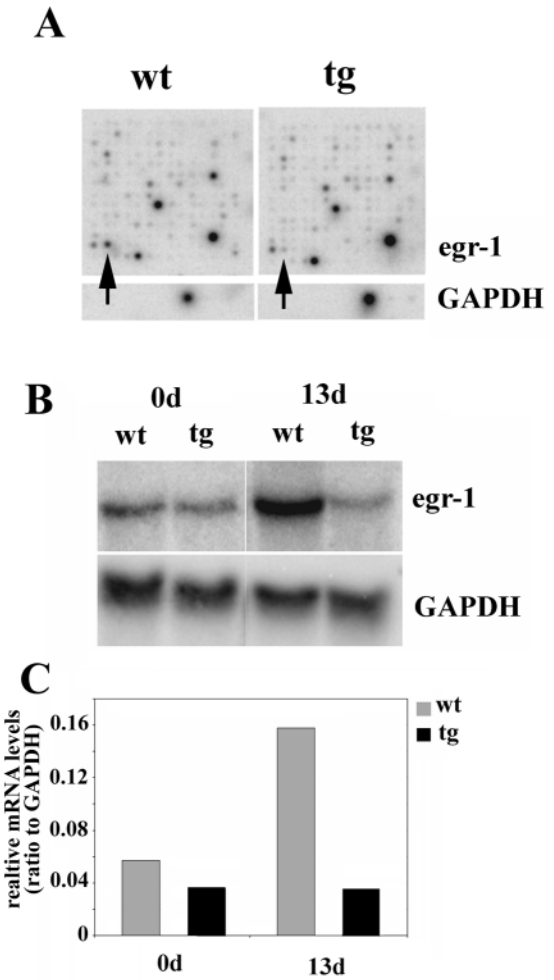

Fig. 6. Expression of Egr1 in wounds. (A) A cDNA-array was probed with cDNA derived from 7-day-old wounds of transgenic and non-transgenic animals. Egr1 expression (arrows) was reduced 7 times in transgenic animals compared with non-transgenic animals. (B) Northern blot analysis for Egr1 using RNA form 13-day-old wounds. In normal skin, Egr1 expression (3.2 kb) is low, whereas in 13-day-old wounds Egr1 expression is elevated in comparison with unwounded skin only in non-transgenic animals. GAPDH expression (1.3 kb) was used as loading control. (C) Quantification of the northern blot analysis by PhosphorImager system. In 13-day-old wounds, the expression level of Egr1 is threefold higher in non-transgenic animals in comparison with unwounded skin. By contrast, no upregulation is found in transgenic animals.

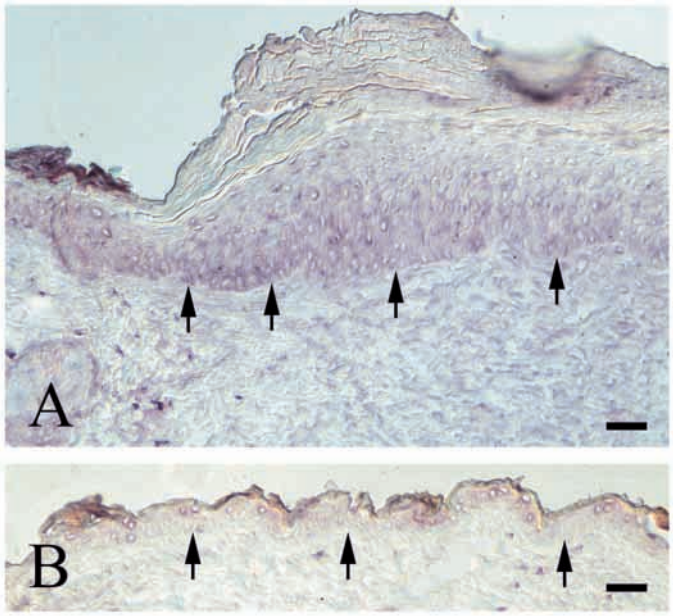

Fig. 7. Immunodetection of Egr1 in wounds. Cryosections of 13-day-old wounds from wild-type animals were stained with an antibody against Egr1. Keratinocytes in the newly formed epithelium at the wound margin exhibit strong induction of Egr1 (A), whereas distal to the wound margin, hardly any upregulation is seen (B). Arrowheads indicates the epidermal/dermal junction. Bars, 50 μm.

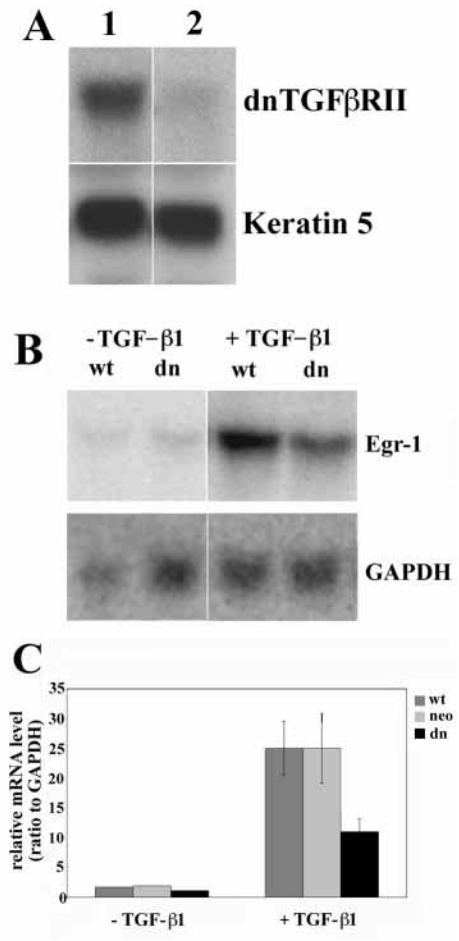

Fig. 8. Expression of Egr1 in wild-type HaCaT cells and HaCaT cells transfected with an expression vector for the dominant-negative type II TGFβ receptor. (A) Expression of the dominant negative type II TGFβ receptor in two transfected HaCaT clones. The northern blot was probed with cDNA of the dnTGFβRII and with keratin 5 cDNA. Lane 1 shows HaCaT clone dn, which has a high expression level of the dnTGFβRII (2.4 kb). Lane 2 shows the very low expressing clone neo. Keratin 5 expression (2.6 kb) was used as loading control. (B) Northern blot analysis of RNA from HaCaT cells transfected with the dominant-negative type II TGFβ receptor (wt) and transfectants (dn). Without addition of TGFβ1, the expression of Egr1 in wild-type and transfectant cells is very low. Forty-five minutes after addition of 5 ng/ml TGFβ1, the expression of Egr1 (3.2 kb) is strongly induced in untransfected HaCaT cells only. GAPDH expression (1.3 kb) was used as loading control. (C) Quantification of the northern blot analysis by PhosphorImager system. The expression level of Egr1 after addition of 5 ng/ml TGFβ1 (+TGFβ1) is elevated 24-fold in HaCaT cells and neo controls. By contrast, in HaCaT cells expressing the dominant-negative type II receptor at high levels (dn), only a ninefold increase is found.

to TGFβ was measured by northern blot analysis and subsequent quantitative evaluation was performed using a PhosphorImager system (Fig. 8B,C). In wild-type HaCaT cells and in the transfected control cells (neo), the expression of Egr1 mRNA was strongly induced by TGFβ1 (25-fold). By contrast, HaCaT cells, which express the dominant negative type II TGFβ receptor at high levels showed a markedly reduced upregulation of Egr1 mRNA (ninefold, n=3, P=0.0005, Student's t-test). This confirms the assumption that the dominant negative type II TGFβ receptor is able to abolish or reduce induction of Egr1 expression in keratinocytes.

Discussion

Owing to its multifunctionality, TGFβ is involved in nearly every aspect of wound healing (O'Kane and Ferguson, 1997; Roberts and Sporn, 1996). It stimulates chemotaxis and proliferation, and modulates gene expression that is dependent upon cell type and differentiation. One important step during cutaneous wound healing is the re-establishment of the epithelial barrier. The role of TGFβ in the re-epithelialization process appears to be contradictory. On the one hand, TGFβ, which is upregulated in keratinocytes after wounding, is a strong inhibitor of keratinocyte proliferation (Coffey et al., 1988; Sellheyer et al., 1993). This could interfere with the supply of keratinocytes from the wound edge needed to cover the wound bed. On the other hand, TGFβ induces the expression of integrins necessary for keratinocyte migration over the provisional fibronectin matrix (Gailit et al., 1994; Zambruno et al., 1995). In most studies investigating the role of TGFβ in re-epithelialization, it was not possible to distinguish between direct and indirect effects of TGFβ. The interruption of the TGFβ pathway in keratinocytes of the epidermis in our transgenic mice enabled us to address TGFβ involvement in a cell type-specific manner. In the present study, we have shown that the expression of the dominant negative type II TGFβ receptor in keratinocytes of transgenic mice resulted in increased keratinocyte proliferation at the wound edges of full thickness excisional wounds, which in turn led to accelerated re-epithelialization. This result is in agreement with a wound healing study using mice that have a partly disrupted TGFβ signaling pathway, owing to lack of Smad3 (Ashcroft et al., 1999). Similarly, keratinocyte proliferation at the wound edge was elevated in both models accompanied by an increased rate of re-epithelialization. Together with our results, this provides strong evidence that TGFβ indeed impairs re-epithelialization at least in part by repressing keratinocyte proliferation. The less pronounced effect on re-epithelialization in our model compared with the *Smad3* null mice could be attributed to the restricted expression of the dominant negative type II receptor only in keratinocytes. Thus, beneficial effects of a loss of TGFβ signaling on other cell types must exist (e.g. granulation tissue) that also lead to an accelerated wound healing (Ashcroft et al., 1999). As a consequence of accelerated wound closure in transgenics, we also observed an accelerated clearance of the dermis, in particular from macrophages and mast cells or neutrophils.

Members of the immediate-early gene family, which are expressed instantly after injury, regulate and orchestrate the tissue repair process (Khachigian et al., 1996; Liu et al., 2000b; Wang and Deuel, 1992). Because of this, studies investigating the role of Egr1, a member of this immediate-early gene family, during injury have focused on monitoring the expression of Egr1 for only a short period after wounding (Bryant et al., 2000; Khachigian et al., 1996). We demonstrate that Egr1 upregulation in wounds is predominantly seen in keratinocytes. The cytoplasmic pattern of staining correlates with observations from kidney epithelial cells from Wilms tumors (Ghanem et al., 2000).

Using a cDNA array and northern blot analysis, we found an elevated expression level of Egr1 in late phase wounds of wild-type animals. This novel finding of late-stage Egr1 expression during wound healing is of significance in consideration of the observed functions of Egr1 in developmental and differentiation processes (Milbrandt, 1987; Sukhatme et al., 1988). Moreover, we could demonstrate that this late upregulation of Egr1 during wound healing is caused by TGFβ, which is upregulated during wound repair with peak levels as early as 1-2 days post-wounding (Frank et al., 1996). Transgenic animals with an interrupted TGFβ signaling cascade show no upregulation of Egr1 expression. This TGFβ-dependent Egr1 upregulation in keratinocytes is further confirmed by our finding that interruption of the TGFβ signal pathway in the human keratinocyte cell line HaCaT also leads to impaired upregulation of Egr1. Hence, there is the possibility that Egr1 expression is regulated by an autocrine loop. On the one side, Egr1 is induced by TGFβ in various cell lines such as the osteoblastic cell line MC3T3, the fibroblast cell line NIH 3T3 and the epithelial cell line NMuMG (Koskinen et al., 1991; Ohba et al., 1994). On the other side, Egr-1 induces TGFβ1 by binding to GC-rich binding sites in the promotor region of TGFβ1 (Kim et al., 1994; Liu et al., 1996).

Two recently published wound-healing studies have demonstrated the importance of Egr1 in early wound healing. The delivery of the cDNA for Egr1 by a gene gun in a full excisional wound model in mice leads to accelerated wound healing, owing to induction of cytokines such as vascular endothelial growth factor (VEGF), PDGF-A and TGFβ1 (Bryant et al., 2000). A DNA-based enzyme that degrades Egr1 mRNA resulted in impaired wound healing of arterial neointima injured by a balloon catheter (Santiago et al., 1999). In contrast to the function of Egr1 in the immediate response to injury, very little is known about the function of Egr1 expression in late phases of wound repair. But consistent with its role in developmental and differentiation processes, as well as in the control of cell growth, Egr1 is very probably an important factor for the remodeling and termination phase of the wound healing process (Dinkel et al., 1998; Krishnaraju et al., 2001; McMahon et al., 1990; Santiago et al., 1999). The finding that Egr1 is expressed more continuously in developmental and differentiation processes favors this hypothesis (Milbrandt, 1987; Sukhatme et al., 1988). Additional support for this assumption is the finding that in chronic wounds like atheriotic lesions, levels of Egr1 expression remain elevated. In these lesions, Egr1 seems to repress the transcription of the type II TGFβ receptor and thus contributes to acquired resistance of the lesional cells to effects of TGFβ (Du et al., 2000; McCaffrey et al., 2000). Furthermore, Egr1 is able to induce the expression of fibroncetin in cell lines derived from a glioblastoma and a fibrosarcoma and of metalloproteinases in endothelial cells (Haas et al., 1999; Liu et al., 1999; Liu et al., 2000a). This indicates that Egr1 is capable of transactivating genes relevant for later phases of wound repair.

Resolving the wound repair process is another crucial step in wound healing that involves apoptosis in order to decrease cellularity (Desmouliere et al., 1995). Egr1 and other members of the immediate-early gene family have been linked to apoptosis induction whereby Myc, Fos and Egr1 mediate the proapaptotic signal via p53 (Estus et al., 1994; Hermeking and Eick, 1994; Liu et al., 2001; Muthukkumar et al., 1995; Nair et al., 1997; Preston et al., 1996; Woronicz et al., 1994). The reduced Egr1 expression found in 13-day-old wounds in transgenic animals correlated with a reduced rate of apoptosis in the epidermis. This suggests that TGFβ-induced Egr1 also plays a role in the resolution phase of wound repair by inducing apoptosis in keratinocytes.

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