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Transforming Growth Factors β1, β2, and β3 and Their Receptors Are Differentially Regulated during Normal and Impaired Wound Healing*

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A series of studies has shown that application of transforming growth factor β (TGF-β) to a wound has a beneficial effect, especially in animals with wound healing disorders. In this study we have investigated the regulation of TGF-β1, β2, and β3 and their receptors during the repair process. We found a large induction of all three TGF-β isoforms and also of TGF-β types I and II receptors, although the time course of induction and the absolute expression levels were different for these genes. Furthermore, each TGF-β isoform had distinct sites of expression in the wound. Systemic treatment with glucocorticoids significantly altered the expression levels of TGF-βs and TGF-β receptors. Whereas expression of TGF-β1, TGF-β2, and TGF-β type II receptor was suppressed by glucocorticoids in normal and wounded skin, expression of TGF-β3 and TGF-β receptor type I mRNA was stimulated. These findings provide an explanation for the beneficial effect of exogenous TGF-β in the treatment of impaired wound healing in glucocorticoid-treated animals. Furthermore, they suggest that a disturbed balance between the levels of the three TGF-β isoforms and their receptors might underlie the wound healing defect seen in glucocorticoid-treated animals.

Wound healing is a highly ordered and well coordinated process that involves inflammation, cell proliferation, matrix deposition, and tissue remodeling (1). A series of studies indicates that peptide growth factors and their receptors are key modulators of this process. One of the most important modulators of wound repair is transforming growth factor β (TGF-β),[1] which is found in large amounts in platelets (2). Furthermore, it is produced by several cell types that are present in a wound, including activated macrophages, neutrophils, fibroblasts, and also keratinocytes (3–9). Three TGF-β isoforms (TGF-β1, β2, and β3) are present in mammals that share a 64–85% amino acid sequence homology (10). *In vitro*, TGF-βs have potent effects on many cell types. Thus, they are mitogenic for various types of fibroblasts, but they inhibit proliferation of most other cells, including endothelial cells and epithelial cells. Furthermore, TGF-βs modulate differentiation processes, and they are very potent stimulators of the expression of extracellular matrix proteins and integrins (7, 10). Therefore, they have the properties expected of wound cytokines. Indeed, all three types of mammalian TGF-β are expressed during wound repair, whereby each member of the TGF-β family has a unique distribution in pig wounds (8). The differential expression of TGF-βs in the wound and also in many embryonic mouse tissues (11, 12) suggested differential regulation of these genes but also different functions of their gene products. This was confirmed in *in vitro* studies where several differences in the potency and biological activity of TGF-β1, β2, and β3 have been demonstrated. These include the inhibitory effect on DNA synthesis in keratinocytes, where TGF-β3 is significantly more potent than TGF-β1 and TGF-β2 (13). Recently Shah *et al.* (14) were able to demonstrate differences in the biological effects of TGF-βs during wound repair. Their studies suggest that TGF-β1 and TGF-β2 induce cutaneous scarring, whereas TGF-β3 seems to inhibit this effect.

Whereas increased expression of TGF-β1 and TGF-β2 might have deleterious effects on the repair process by increasing scar formation, reduced expression of growth factors in a wound might result in a severe delay in wound healing. This has recently been demonstrated for keratinocyte growth factor, a member of the fibroblast growth factor family, which is expressed at significantly reduced levels during wound repair in genetically diabetic *db*/*db* mice (15) and glucocorticoid-treated mice (16). The wound healing defect seen in these animals can be reversed by topical application of exogenous growth factors. Thus, treatment of poorly healing wounds in glucocorticoid-treated animals with fibroblast growth factors or TGF-β had favorable effects on the repair process (17–20). Therefore, we speculated that expression of endogenous TGF-βs might also be impaired in these animals. Here we demonstrate severe effects of glucocorticoids on the expression of TGF-βs in normal and wounded skin. These data suggest that aberrant expression of these genes in glucocorticoid-treated mice might be associated with the wound healing defect seen in these animals.

MATERIALS AND METHODS

Glucocorticoid Treatment of Mice—Female BALB/c mice (3 months old) were injected subcutaneously at 9 a.m. daily with 1 mg of dexamethasone/kg of body weight for 7 days. Control mice were injected with phosphate-buffered saline. Three dexamethasone-treated mice and three control mice were subsequently injected with dexamethasone or phosphate-buffered saline for another 3 days but left unwounded. The other mice were wounded as described below. During the wound healing period, we continued the daily injection of dexamethasone or phosphate-buffered saline.

Wounding and Preparation of Wound Tissues—To assess TGF-β and TGF-β receptor expression during wound healing, six full-thickness wounds were created on each animal, and skin biopsy specimens from four animals were obtained 1, 3, 5, 7, and 13 days after injury. To study the effect of dexamethasone on TGF-β expression, wounds from four dexamethasone-treated mice and four control mice were analyzed at day 2, 3, or 5 after injury. Mice were anesthetized with a single intraperitoneal injection of Avertin. The hair on the back of these mice was

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[1] The abbreviation used is: TGF-β, transforming growth factor β.

cut with fine scissors, and the back was subsequently wiped with 70% ethanol. Six full-thickness wounds (6-mm diameter, 3–4 mm apart) were made on the backs of these mice by excising the skin and panniculus carnosus. The wounds were allowed to dry to form a scab. An area of 7–8 mm in diameter which included the scab and the complete epithelial margins was excised at each time point. A similar amount of skin from the backs of three nonwounded animals was used as a control. In every experiment, the wounds from four animals (24 wounds) and the nonwounded back skin from three animals, respectively were combined, immediately frozen in liquid nitrogen, and stored at −70 °C until used for RNA isolation. In one experiment, four additional mice (two control mice and two glucocorticoid-treated mice) were wounded. The wounds of these mice were analyzed histologically at day 5 after injury to study the effects of glucocorticoid treatment on wound healing. All animal experiments were carried out with permission from the local government of Bavaria (permission number 211-2531-16/93).

Histology—Glucocorticoid-treated mice and control mice were wounded as described above. Animals were sacrificed at day 5 after injury. Complete wounds were isolated from the middle of the back, bisected, fixed overnight in 4% paraformaldehyde, and paraffin embedded. Six-μm sections from the middle of the wound were stained with hematoxylin and eosin.

Preparation of Tissue Lysate and Enzyme-linked Immunosorbent Assay—Normal and wounded back skin was frozen in liquid nitrogen. Eight-cm^2 of normal and wounded back skin (10 wounds) were homogenized in 3 ml of 2 × lysis buffer (1 × lysis buffer: 1% Triton X-100, 20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin (0.15 unit/ml), 15 μg/ml leupeptin, 10 μg/ml pepstatin). The tissue extract was cleared by centrifugation and the supernatant diluted 1:1 with water. One ml of each diluted sample was cleared by centrifugation. The amount of protein in the lysate was determined using the Bio-Rad protein assay (Bradford method). One μg of total protein from skin and wound lysates was treated with 1 N HCl to activate latent TGF-β. 65 and 130 ng of HCl-treated protein lysate were subsequently analyzed for the presence of immunoreactive TGF-β1 by enzyme-linked immunosorbent assay using the Predicta TGF-β1 kit (Genzyme) as described by the manufacturer.

Immunohistochemistry—Mice were wounded as described above. Animals were sacrificed at day 5 after injury. Complete wounds were isolated from the middle of the back, bisected, and frozen in OCT. Six-μm frozen sections were fixed with acetone and treated for 10 min at room temperature with 1% H_2O_2 in phosphate-buffered saline to block endogenous peroxidase activity. They were subsequently incubated for 60 min at room temperature with polyclonal antisera for TGF-β1, β2, or β3 (Santa Cruz, Biotechnologies) (1:250 diluted in phosphate-buffered saline, 0.1% bovine serum albumin). These antibodies have been shown to be specific for the different TGF-β isoforms as assessed by Western blot analysis and immunohistochemical staining. The slides were subsequently stained with the avidin-biotin-peroxidase complex system (Vector Laboratories, Burlingame, CA) using 3-amino-9-ethylcarbazole as a chromogenic substrate. After development, they were rinsed with water, counterstained with hematoxylin, and mounted.

RNA Isolation and RNase Protection Assay—RNA isolation was performed as described (21). Fifty μg of total RNA from wounded or nonwounded skin was used for RNase protection assays. RNase protection assays were carried out as described recently (22). Briefly, DNA probes were cloned into the transcription vector pBluescript KSII (+) (Stratagene) and linearized. An antisense transcript was synthesized *in vitro* using T3 or T7 RNA polymerase and [^{32}P]UTP (800 Ci/mmol). RNA samples were hybridized at 42 °C overnight with 100,000 cpm of the labeled antisense transcript. Hybrids were digested with RNases A and T1 for 1 h at 30 °C. Under these conditions, every single mismatch is recognized by the RNases. Thus, cross-reaction of the individual probes with other TGF-β isoforms can be excluded. Protected fragments were separated on 5% acrylamide, 8 M urea gels and analyzed by autoradiography. All protection assays were carried out with at least two different sets of RNA from independent wound healing experiments.

Probe DNAs—Human and murine cDNA probes for TGF-β1, β2, and β3 were cloned by polymerase chain reaction using 5′-GA(A/G)TGG(C/T)TI(A/T)(C/G)ITT(C/T)GA(C/T)GT-3′ as a 5′-primer and 5′-GG(C/T)TC(A/G)TG(A/C/G/T)A(C/T)CCA(C/T)TTCCA-3′ as a 3′-primer. These primers correspond to regions that are highly conserved among the different forms of TGF-β; therefore, they could be used for the amplification of the three TGF-β cDNAs. The murine TGF-β type I receptor (*Alk-5*) cDNA was cloned by polymerase chain reaction using 5′-ATCCATGAAGACTATCAGTTGCCT-3′ as a 5′-primer and 5′-CATTTTGATGCCTTCCTGTTGGCT-3′ as a 3′-primer. The amplified fragment corresponds to nucleotides 1258–1509 of the published sequence (23, 24). The murine TGF-β type II receptor cDNA probe was cloned by polymerase chain reaction using degenerated primers corresponding to the human cDNA (25) (5′-primer: 5′-GGNGARACNTTYTTYATGTG-3′; 3′-primer: 5′-TTDATRTTRTTNGCRCANGT-3′).

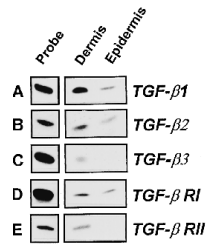

FIG. 1. **Expression of TGF-βs and TGF-β receptors in the dermis and the epidermis of mouse tails.** Mouse tail skin was incubated for 30 min at 37 °C in a solution of 2 M NaBr. The epidermis was subsequently separated from the underlying dermis, immediately frozen in liquid nitrogen, and used for RNA isolation. Fifty μg of RNA from dermis and epidermis was subsequently analyzed by RNase protection assay for the expression of TGF-βs and TGF-β receptors. One thousand cpm of the hybridization probes was used as a size marker. The same batch of RNAs was used for all protection assays. *RI* and *RII*, types I and II receptor, respectively.

RESULTS

Expression of TGF-βs in the Dermis and the Epidermis—To determine potential sites of action of TGF-βs in normal skin, we first investigated the endogenous expression of TGF-β1, TGF-β2, and TGF-β3 as well as their receptors in the dermis and epidermis of mouse skin. Tail skin was used for this purpose, since this is the only place where the dermis can be separated from the epidermis. For detection of the individual transcripts, RNase protection assays were performed under conditions where a single base mismatch can be detected. Thus, cross-reaction of the probes with other TGF-β isoforms could be excluded. This was further proven by hybridization of the probes with TGF-β1, β2, and β3 sense transcripts (data not shown). We found expression of TGF-β1 and TGF-β2 in the dermis and at lower levels in the epidermis (Fig. 1, *A* and *B*). By contrast, TGF-β3 mRNA could only be detected in the dermis, although at much lower levels compared with the other types of TGF-β (Fig. 1*C*). TGF-β signals by contacting two distantly related transmembrane serine/threonine kinases called receptors I and II (25–27). As shown in Fig. 1, *D* and *E*, both receptors were expressed in mouse tail skin. mRNA encoding the type I receptor (*TGF-βRI*) was found at similar levels in the dermis and the epidermis (Fig. 1*D*). The type II receptor (*TGF-βRII*) was found at high levels in the dermis, whereas epidermal expression was low and could only be detected after longer exposure times (Fig. 1*E* and data not shown).

Expression of TGF-β1, TGF-β2, and TGF-β3 Is Differentially Regulated during Wound Repair—To investigate the mRNA expression of TGF-βs in normal and wounded skin, we isolated RNA from full-thickness wounds at different intervals after injury and performed RNase protection assays. For each time point, 24 wounds from four mice were excised, combined, and used for RNA isolation. Normal skin from the back of nonwounded mice was used as a control. As shown in Fig. 2, *A–C*, TGF-β1, β2, and β3 were expressed in normal back skin. Upon injury, a strikingly increased expression of all TGF-β isoforms was observed, although the kinetics of induction was different for the three variants. Expression of TGF-β1 mRNA increased 9-fold within 24 h after wounding and remained high for several days after injury (Fig. 2, *A* and *D*). Expression of TGF-β3 mRNA was low during the first 3 days after injury. However, TGF-β3 mRNA levels subsequently increased, and a maximal

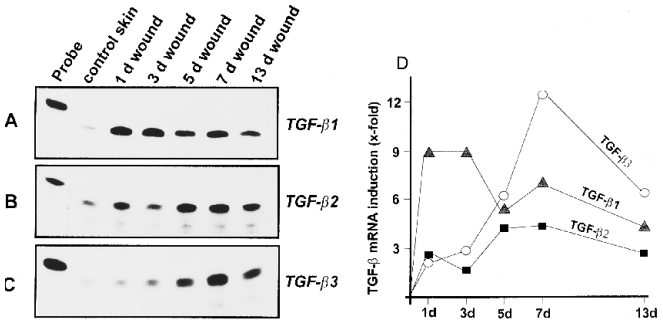

FIG. 2. **Differential expression of TGF-β1, TGF-β2, and TGF-β3 mRNA during wound repair.** Total cellular RNA (50 μg) from normal and wounded back skin was analyzed by RNase protection assay with RNA hybridization probes complementary to mRNA encoding TGF-β1 (*panel A*), TGF-β2 (*panel B*), and TGF-β3 (*panel C*). The same RNA preparations were used for all hybridizations of this figure and Fig. 5. The time after injury is indicated at the *top* of each *lane*. One thousand cpm of the hybridization probes was used as the size marker. The degree of TGF-β1, β2, and β3 mRNA induction as assessed by laser scanning densitometry of the autoradiograms is shown schematically in *panel D*.

induction (12.5-fold) was observed at day 7 after wounding (Fig. 2, *C* and *D*). Thus, TGF-β3 expression levels were highest during the period of maximal granulation tissue formation and reepithelialization. Similar kinetics were observed for TGF-β2, although induction of this gene was only 4.5-fold (Fig. 2, *B* and *D*). At day 13 after injury all wounds were completely reepithelialized, but the granulation tissue still revealed a high cellularity (data not shown). At that stage of the repair process, expression of all TGF-β variants had declined, although the expression levels were still significantly higher compared with the basal level (Fig. 2*D*). This suggests that TGF-βs are also involved in the late stage of the repair process which is characterized by significant tissue remodeling. This time course of TGF-β1, β2, and β3 expression was seen in three different RNase protection assays with RNAs from independent wound healing experiments.

To determine the absolute levels of the individual TGF-β transcripts, defined amounts of the corresponding sense transcripts were used as positive controls and compared with the signals obtained with 20 μg of wound RNA. All sense transcripts had the same length and were derived from homologous regions of the TGF-β cDNAs. These results revealed a similar expression of TGF-β1 and β3 at later stages of the repair process (5–7 days after injury), whereas TGF-β2 mRNA levels were 90% lower (data not shown). Since TGF-β3 expression is significantly lower during the early phase of wound healing (days 1–3 after injury) (Fig. 2), TGF-β1 seems to be the predominant isoform during this period.

Induction of TGF-β mRNA Expression after Injury Correlates with Induction of Immunoreactive TGF-β Protein—To determine whether the observed induction of TGF-β expression after injury correlates with induction of TGF-β protein, we determined the amounts of immunoreactive TGF-β1 protein in normal and wounded skin by enzyme-linked immunosorbent assay. For this purpose, tissue lysates were prepared from normal and wounded skin (2 days after injury), and 65 or 130 ng of total protein was analyzed for the presence of TGF-β1. Lysate from normal skin contained 0.1 ng/ml immunoreactive TGF-β1, whereas the levels of this factor were 4-fold higher (0.43 ng/ml) in the lysate of wounded skin. This result demonstrates that induction of TGF-β1 mRNA correlates with increased production of TGF-β1 protein.

To determine the localization of the different types of TGF-β in normal and wounded skin, serial sections from 5-day full-thickness mouse wounds were stained with monospecific antibodies against TGF-β1, β2, and β3. At that time point after injury, all types of TGF-β are expressed at high levels (Figs. 2 and 3). As shown in Fig. 3, *A–C*, TGF-β1, β2, and β3 proteins were found at distinct places within the wound. A remarkably high expression of all three isoforms was seen in a population of cells in the dermis at the wound edge, which might either represent migrating fibroblasts or macrophages. Furthermore, all three isoforms were found in the granulation tissue, whereas TGF-β2 protein was particularly abundant below the hyperproliferative epithelium (Fig. 3*B*). TGF-β3 protein was found at high levels in the hyperproliferative epithelium at the wound edge (Fig. 3*C*) but not in normal epidermis (data not

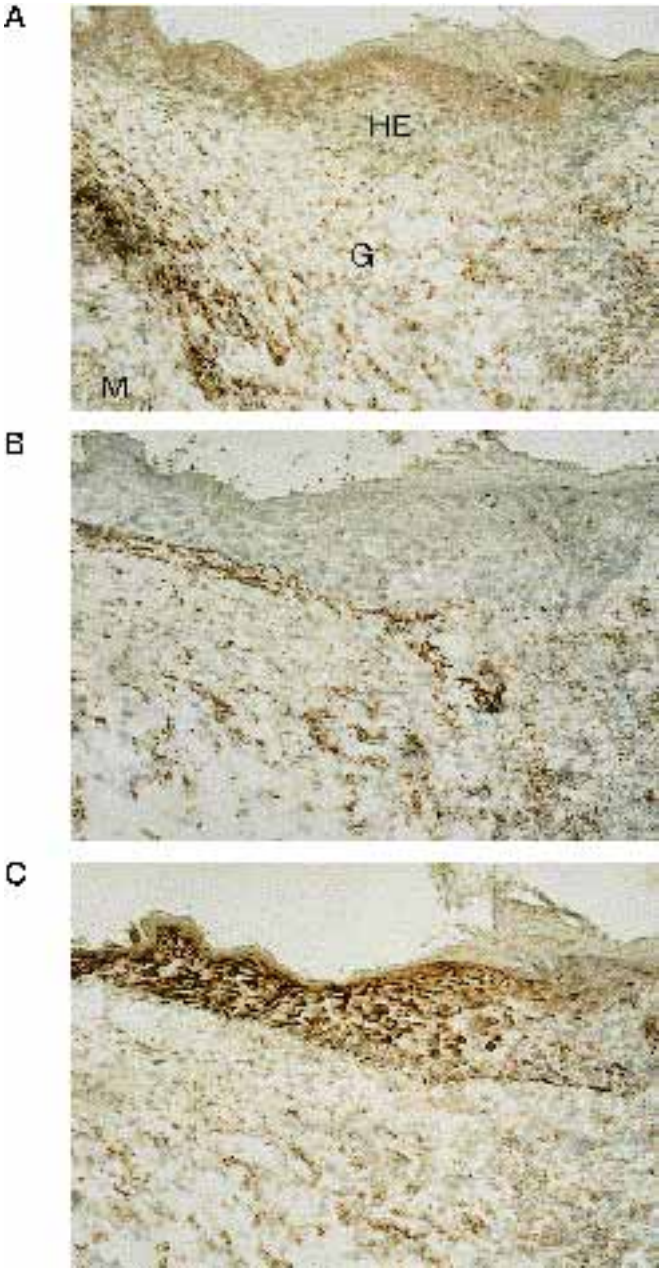

FIG. 3. **Differential expression of TGF-β1, β2, and β3 proteins in 5-day mouse wounds.** Frozen serial sections from a 5-day mouse wound were incubated with monospecific antibodies directed against TGF-β1 (*panel A*), TGF-β2 (*panel B*), or TGF-β3 (*panel C*) and stained with the avidin-biotin-peroxidase complex system using 3-amino-9-ethylcarbazole as a chromogenic substrate. Nuclei were counterstained with hematoxylin. An overview of the complete wound is shown in *panels A–C*. *G*, granulation tissue; *HE*, hyperproliferative epithelium; *M*, muscle layer.

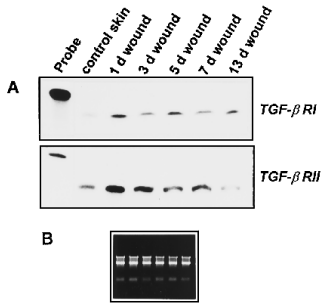

FIG. 4. **TGF-β type I and type II receptors are expressed at high levels in normal and wounded skin.** *Panel A*, 50 μg of total cellular RNA from normal and wounded back skin was analyzed by RNase protection assay for expression of TGF-β type I (*TGF-βRI*) and type II (*TGF-βRII*) receptors. The time after injury is indicated at the *top* of each *lane*. One thousand cpm of the hybridization probes was used as a size marker. One μg of the same batch of RNA is shown in *panel B*. The same RNA preparations were used for the protection assays shown in Figs. 2 and 5.

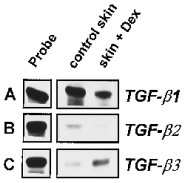

FIG. 5. **Glucocorticoids have differential effects on TGF-β1, β2, and β3 expression in normal skin.** BALB/c mice were treated with glucocorticoids as described under "Materials and Methods." Nonwounded back skin from dexamethasone-treated mice (*skin + Dex*) and control mice (*control skin*) was isolated and used for RNA isolation. Fifty μg of total cellular RNA was analyzed by RNase protection assay for expression of TGF-β1, β2, and β3 mRNA. One thousand cpm of the hybridization probes was used as a size marker. The same set of RNAs was used for the three protection assays shown in this figure.

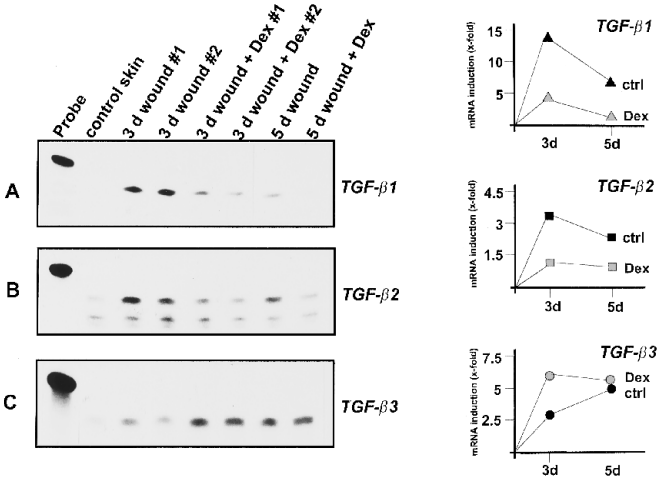

FIG. 6. **TGF-β1, TGF-β2, and TGF-β3 are differentially regulated by glucocorticoids during wound healing.** BALB/c mice were treated with glucocorticoids as described under "Materials and Methods." Mice injected with phosphate-buffered saline were used as a control. RNA was isolated from nonwounded skin of control mice (*control skin*) and from wounded skin (3 and 5 days after wounding) of control mice and dexamethasone-treated mice (*+Dex*). Fifty μg of total cellular RNA from two independent experiments (experiments 1 and 2) was analyzed by RNase protection assay for TGF-β1 (*panel A*), TGF-β2 (*panel B*), and TGF-β3 (*panel C*) expression. One thousand cpm of the hybridization probes was used as a size marker. The degree of TGF-β1, β2, or β3 mRNA induction as assessed by laser scanning densitometry of the autoradiograms is shown schematically on the *right side* of the figure. The same set of RNAs was used for the three protection assays shown in this figure.

shown). By contrast, only a weak expression of TGF-β1 protein was seen in differentiating keratinocytes of the hyperthickened epidermis (Fig. 3*A*), and TGF-β2 protein was restricted to differentiated keratinocytes of the uppermost layers of the epithelium (Fig. 3*B*). These results demonstrate a differential expression of all three TGF-β isoforms in the wound tissue.

Expression of TGF-βs during Wound Healing Correlates with Expression of TGF-β Type I and Type II receptors—To determine whether expression of TGF-βs correlates with expression of the corresponding receptors, we analyzed the mRNA levels of both types of TGF-β receptor at different stages of the repair process. As shown in Fig. 4, high levels of TGF-β type II receptor mRNA but significantly lower levels of the type I receptor mRNA were found in normal murine back skin. A transient increase in expression of both receptors was seen after injury. Expression levels were maximal at day 1 after injury and subsequently declined. After completion of the proliferative phase of wound repair (13 days after injury) expression levels were still elevated compared with control skin. The presence of both types of receptors in normal and wounded skin and their up-regulation after injury demonstrate a strong correlation between expression of the ligands and their receptors.

Glucocorticoids Have Differential Effects on TGF-β1, β2, and β3 Expression in Normal Skin and during Wound Healing—Inhibition of wound healing by glucocorticoids is a well established phenomenon. To determine a possible role of TGF-β in impaired wound healing of glucocorticoid-treated mice, dexamethasone was injected daily at 9 a.m. over a period of 7 days before wounding and 2–5 days after wounding. To prove the deleterious effect of dexamethasone treatment on wound repair, wounds were analyzed histologically at day 5 after injury. A significant amount of granulation tissue had formed in control mice at this time point, whereas in dexamethasone-treated mice granulation tissue formation was hardly detectable. Furthermore, reepithelialization was dramatically delayed in glucocorticoid-treated mice (data not shown). RNA was isolated from nonwounded skin and from wounded skin of glucocorticoid-treated mice and control mice at day 2, 3, or 5 after injury and analyzed for TGF-β mRNA expression.

In nonwounded skin, expression levels of TGF-β1 and TGF-β2 were reduced by dexamethasone (Fig. 5, *A* and *B*), whereas TGF-β3 mRNA levels increased upon glucocorticoid treatment (Fig. 5*C*). These differences were also seen after injury, and expression levels of TGF-β1 and TGF-β2 were 65% lower in glucocorticoid-treated mice compared with control mice at day 3 after wounding (Fig. 6, *A* and *B*). A similar reduction was seen at day 2 and 5 after injury (Fig. 6, *A* and *B*, and data not shown). In contrast to TGF-β1 and TGF-β2, expression of TGF-β3 mRNA was stimulated by dexamethasone, although this difference was only seen in the early phase of wound repair when TGF-β3 mRNA expression is normally very low (Fig. 6*C*). Thus, systemic treatment with glucocorticoids resulted in a premature onset of TGF-β3 expression. These *in vivo* data demonstrate that glucocorticoids modulate the normal induction of TGF-β1, β2, and β3 expression after cutaneous injury.

Differential Modulation of TGF-β Type I and II Receptors by Glucocorticoids—In addition to its effects on TGF-β expression, dexamethasone also modulated the expression levels of both TGF-β receptors. In nonwounded skin, glucocorticoids had no effect on TGF-β type I receptor expression (Fig. 7*A*), whereas TGF-β type II receptor expression was significantly reduced upon dexamethasone treatment (Fig. 7*B*). During wound healing, glucocorticoid treatment resulted in increased expression of the type I receptor (Fig. 7*A*), whereas type II receptor mRNA levels were slightly reduced in the wounds of dexamethasone-treated mice (Fig. 7*B*). This finding demonstrates a differential regulation of the two TGF-β receptors, suggesting that the

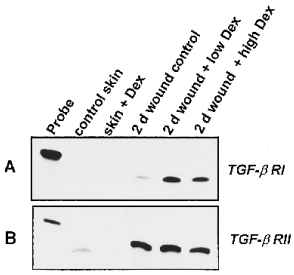

FIG. 7. **Modulation of TGF-β receptor expression by glucocorticoids in nonwounded and wounded skin.** BALB/c mice were treated with glucocorticoids as described under "Materials and Methods." RNA was isolated from nonwounded back skin of control mice (*control skin*), from 2-day wounds of control mice (*2d wound control*) and from mice treated with two different concentrations of dexamethasone (0.2 or 1 mg/kg body weight) (*2d wound + low Dex, 2d wound + high Dex*). Fifty μg of total cellular RNA was analyzed by RNase protection assay for expression of TGF-β type I and II receptors (*TGF-βRI* and *TGF-βRII*, respectively). One thousand cpm of the hybridization probes was used as a size marker.

responsiveness to TGF-β might be altered upon glucocorticoid treatment.

DISCUSSION

Wound healing is a highly organized process that is regulated by a wide variety of growth factors and cytokines. Thus, the regulation of the temporal and spatial expression of these factors is of major significance for normal repair. One of the key players in the repair process is TGF-β. Expression of different TGF-β isoforms in rabbit, porcine, and human wounds has been demonstrated by immunohistochemical staining, yet with variable results (8, 9, 28–30). Although these studies provide important information on the spatial distribution of these factors in the wound, little is known about the time course of TGF-β expression during the repair process. In this study we demonstrate a strong up-regulation of TGF-β1, β2, and β3 expression after injury. These findings correlate with results from other authors who demonstrated increased TGF-β-like activity in wound fluid from rats (31).

The kinetics of expression was different for the three TGF-β variants. Particularly remarkable was the strong induction of TGF-β3 expression at later stages of the repair process. Since TGF-β3 has been shown to reduce connective tissue deposition and subsequent scarring during wound healing in normal rats (14), this finding suggests that up-regulation of this factor after completion of the proliferative phase of wound healing might be important for the limitation of the fibrotic process. However, studies from other authors have yielded contradictory results concerning the effect of TGF-β3 on connective tissue deposition. They demonstrated an increase in new dermal matrix by exogenous application of TGF-β3 to wounds in age-impaired animal models (32). However, the effect of TGF-β3 on scarring was not determined in this study, and increased production of dermal matrix during early wound repair might not necessarily lead to increased scar formation. Besides the effects on the mesenchyme, TGF-βs are likely to be important in the epidermis. TGF-β3 is the most abundant TGF-β isoform in the hyperproliferative epithelium and might therefore play an important role in keratinocyte differentiation. Thus, increased expression of TGF-β3 during the early phase of wound healing in glucocorticoid-treated mice could lead to a premature onset of keratinocyte differentiation and inhibition of epithelial cell proliferation. This could provide an explanation for the severe delay in reepithelialization seen in these animals.

The distribution of TGF-β1, β2, and β3 in normal and wounded mouse skin showed several differences compared with the expression pattern of these factors in porcine and human skin, particularly in the epidermal compartment (8, 29). Thus, TGF-β2 was found at high levels in all layers of the hyperproliferative epithelium of pig wounds (8), whereas in mouse wounds only the outermost layers expressed this protein (this study). In normal murine epidermis, TGF-β3 mRNA and protein were not detectable, whereas expression of this factor was found at high levels in porcine and human skin (8, 29). This difference may be related to the different rate of epidermal cell turnover in murine and human skin. In contrast to TGF-β2 and TGF-β3, the expression pattern of TGF-β1 in the epidermis was similar in all species, and TGF-β1 protein was found at highest levels in the upper layers of the epidermis.

Recently, two different TGF-β receptors have been cloned which mediate TGF-β signal transduction. However, expression of these receptors in a wound has not yet been demonstrated. Here we show that both types of TGF-β receptor are expressed at high levels in normal and wounded skin, suggesting that these receptors are indeed mediators of TGF-β action in the wound. Interestingly, glucocorticoids had differential effects on the expression levels of both receptors, and the ratio of TGF-β receptor type I to type II was increased significantly by steroid treatment. Recent studies had suggested that the ratio of type I to type II receptor could influence the biological effect of TGF-βs on proliferation and target gene expression (33, 34). Thus, increased expression of the type I receptor as seen during wound healing in glucocorticoid-treated mice might alter the cellular responses to the ligands during the repair process.

The strong up-regulation of TGF-βs during wound healing suggested that defects in the regulation of their expression might be associated with wound healing defects. This is supported by the beneficial effect of exogenous TGF-β in the treatment of impaired wound healing as seen, for example, in glucocorticoid-treated mice (17, 19, 20). Glucocorticoids are potent anti-inflammatory agents that both stimulate and inhibit the transcription of a variety of genes (35). The influence of glucocorticoids on wound healing is particularly remarkable, and the prolonged administration of anti-inflammatory steroids leads to a delay in wound repair and an increase in local wound complications (36). This wound healing defect may be due to the suppression of the inflammatory phase of healing by inhibiting leukocyte and macrophage infiltration (37–39) but also to the direct inhibition of genes that play a role in the repair process. Thus, a negative regulation by glucocorticoids has been shown for collagen type I and tenascin mRNA expression (40, 41). Furthermore, keratinocyte growth factor expression in fibroblasts is suppressed by dexamethasone (16).

The beneficial effect of exogenous TGF-β on wound healing in glucocorticoid-treated animals (17, 19, 20) suggested that endogenous TGF-β might also be limited during wound healing in these animals, and this hypothesis is supported by the results described in this study. We found a strong decrease in TGF-β1 and β2 mRNA expression by dexamethasone in normal and wounded skin. Since these factors have been shown to play an important role in granulation tissue formation (14, 42), our findings provide a likely explanation for the delay of this process in glucocorticoid-treated mice.

The molecular mechanisms that underlie the aberrant expression of TGF-βs during wound healing in glucocorticoid-treated mice are currently unknown. However, our experiments suggest that a combination of direct and indirect mechanisms might be responsible. Thus, inhibition of inflammatory cell infiltration and activation by glucocorticoids might indirectly reduce expression of TGF-β in the wound, since activated macrophages and neutrophils are an important source of TGF-βs, particularly of TGF-β1 (4, 5). However, a

direct effect of glucocorticoids on other TGF-β-producing cells in the wound cannot be excluded. Our finding that TGF-β1 and β2 expression is also reduced by dexamethasone treatment in nonwounded skin supports this hypothesis, since macrophages are present at low levels in normal skin. Furthermore, preliminary experiments from our laboratory suggest a negative regulation of TGF-β2 expression in keratinocytes.[2] Since these cells are an important source of TGF-βs in the wound (8, this study), inhibition of TGF-β2 expression in these cells by dexamethasone might also contribute to the reduced expression of this gene in glucocorticoid-treated mice. Furthermore, decreased expression of TGF-β1 and increased expression of TGF-β3 in the wounds of dexamethasone-treated mice might be due to a direct effect of glucocorticoids on mesenchymal cells. Thus, TGF-β1 mRNA and protein expression have been shown to be reduced by glucocorticoids in lung fibroblasts (43), and TGF-β3 was recently identified as a glucocorticoid-induced gene in the same cell type (44).

In summary, our data demonstrate a strong up-regulation of TGF-β and TGF-β receptor expression after injury which is modulated in a complex manner by glucocorticoids. Since these steroids are potent inhibitors of the wound healing process, our findings suggest that a correct regulation of TGF-β and TGF-β receptor expression is important for normal wound repair. Most importantly, these findings provide a molecular explanation for the beneficial effects of high concentrations of exogenous TGF-β on wound healing in glucocorticoid-treated animals.

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[2] M. Madlener, unpublished data.

Transforming Growth Factors 1, 2, and 3 and Their Receptors Are Differentially Regulated during Normal and Impaired Wound Healing

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