Essential role of Smad3 in the inhibition of inflammation-induced PPAR β / δ expression

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Wound healing proceeds by the concerted action of a variety of signals that have been well identified. However, the mechanisms integrating them and coordinating their effects are poorly known. Herein, we reveal how PPAR β / δ (PPAR: peroxisome proliferator-activated receptor) follows a balanced pattern of expression controlled by a crosstalk between inflammatory cytokines and TGF- β 1. Whereas conditions that mimic the initial inflammatory events stimulate PPAR β / δ expression, TGF- β 1/Smad3 suppresses this inflammation-induced PPAR β / δ transcription, as seen in the late re-epithelialization/remodeling events. This TGF- β 1/Smad3 action involves an inhibitory effect on AP-1 activity and DNA binding that results in an inhibition of the AP-1-driven induction of the PPAR β / δ promoter. As expected from these observations, wound biopsies from Smad3-null mice showed sustained PPAR β expression as compared to those of their wild-type littermates. Together, these findings suggest a mechanism for setting the necessary balance between inflammatory signals, which trigger PPAR β / δ expression, and TGF- β 1/Smad3 that governs the timely decrease of this expression as wound healing proceeds to completion.

The EMBO Journal (2004) 23, 4211–4221. doi:10.1038/sj.emboj.7600437; Published online 7 October 2004

Subject Categories: signal transduction; molecular biology of disease

Keywords: PPAR; Smad3; TGF- β 1; wound healing

Introduction

Healing of cutaneous wounds, which is crucial for survival after an injury, proceeds via a well-tuned pattern of events including inflammation, re-epithelialization and finally matrix and tissue remodeling in the scar. The multitude of complex biological processes, which occurs during the different stages of wound repair, is regulated spatiotemporally by the concerted actions of several growth factors and cytokines.

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Received: 27 May 2004; accepted: 15 September 2004; published online: 7 October 2004

The variety of these signals and their effects on wound repair have been well studied (Werner and Grose, 2003). However, the mechanisms integrating these various signals, which translate early inflammation into gene expression changes and coordinate beneficial healing responses, are still unclear.

Among the genes that are initially activated by early inflammation signals are those encoding the peroxisome proliferator-activated receptors (PPARs). The PPARs are members of the nuclear receptor family. Three closely related PPAR isotypes have been identified, PPAR α (NR1C1), PPAR β / δ (NR1C2; called PPAR β below) and PPAR γ (NR1C3), which are encoded by separate genes and fulfill a variety of functions (Desvergne and Wahli, 1999; Kersten *et al*, 2000). PPAR β plays a critical role in the keratinocyte response to a skin injury (Michalik *et al*, 2001). Inflammatory stimuli present at a wound site, like TNF- α , increase the expression of PPAR β in keratinocytes via the stress-associated kinase/AP-1 signaling cascade (Tan *et al*, 2001). The enhanced PPAR β activity leads to the stimulation of a major cellular antiapoptotic survival pathway (Akt1 pathway), via the transcriptional upregulation of integrin-linked kinase (ILK) and 3-phosphoinositide-dependent kinase-1 (PDK1). The activities of ILK and PDK1 are stimulated by direct interaction with PIP₃ present at increased levels due to a PPAR β -dependent downregulation of PTEN (Di Poi *et al*, 2002). This protection against apoptosis via Akt1 is thought to be necessary to maintain a sufficient number of viable keratinocytes at the wound edge for subsequent re-epithelialization.

Of the numerous cytokines produced at the wound site, TGF- β 1 has undoubtedly the broadest effects, which comprise regulation of the inflammatory response, extracellular matrix deposition, cellular adhesion, migration and proliferation. TGF- β 1 signals through a complex of two membrane-bound receptor serine/threonine kinases that recruit and phosphorylate Smad2 and Smad3. Once phosphorylated, Smad2 and Smad3 oligomerize with Smad4 and translocate to the nucleus where they participate in regulating transcriptional events (Massague and Wotton, 2000). *In vivo*, analyses of the effects of TGF- β 1 on wound repair have provided conflicting results, depending on the animal models analyzed (Ashcroft and Roberts, 2000; Crowe *et al*, 2000; Koch *et al*, 2000).

The extensive temporal overlap in TGF- β 1 and PPAR β functions during wound healing suggests a crosstalk between these two signaling pathways, which fine-tunes wound healing. In this work, we demonstrate that the ability of TGF- β 1 to inhibit inflammation-mediated induction of PPAR β is Smad3-dependent and involves an inhibitory effect on AP-1 activity and DNA binding.

Results

TGF- β 1 inhibits inflammation-induced PPAR β expression

To determine whether a temporally overlapping action of PPAR β and TGF- β 1 in keratinocytes implicates a crosstalk

Figure 1 TGF- β 1 represses inflammation-induced PPAR β expression. (A) RNase protection assay (RPA) of PPAR β , keratin 5 (K5) and 6 (K6) after treatment of the primary keratinocytes with TNF- α (5 ng/ml), IFN- γ (5 ng/ml) or LPS (1 ng/ml) in the presence of increasing amounts of TGF- β 1 (1 and 5 ng/ml). (B) Regulation of PPAR β expression by TNF- α (5 ng/ml) and/or TGF- β 1 (5 ng/ml) in the presence (+) or absence (-) of cycloheximide (CH; 5 μ g/ml) as analyzed by RPA. (C) Control of CH efficiency by Western blot analysis of PPAR β , JunB and PAI-1, from keratinocytes treated as in (B). One representative RPA result out of four independent experiments is shown. RPA values were normalized using the ribosomal protein L27 mRNA levels. Values from unstimulated keratinocytes were arbitrarily assigned a value of 1. Values of PPAR β , JunB and PAI-1 were normalized using tubulin expression. The figures obtained in the absence of TGF- β 1, TNF- α and CH were given a value of 1. The other values indicated represent relative fold increase (decrease) as compared to unstimulated keratinocytes.

between them, the expression of PPAR β was examined in primary mouse keratinocytes challenged with inflammatory stimuli in the presence or absence of TGF- β 1. The PPAR β levels were low in unstimulated keratinocytes, but increased upon exposure of these cells to inflammatory stimuli such as those produced at a wound site (TNF- α , IFN- γ), or to LPS as occurs during an infection (Figure 1A). In response, these activated keratinocytes expressed high levels of keratin 6 (K6), a keratinocyte hyperproliferative and inflammatory marker, with a simultaneous decrease in keratin 5 (K5), a basal keratinocyte protein. Interestingly, TGF- β 1 treatment of activated keratinocytes abrogated the inflammation-induced expression of PPAR β and K6 in a dose-dependent manner (Figure 1A). PPAR β downregulation was via a direct transcriptional regulatory mechanism, since this repression was unaffected by blocking protein *de novo* synthesis with cyclo-

heximide (Figure 1B). The concentration of cycloheximide used was not toxic to the cells, as reflected by the absence of effect on basal levels of PPAR β and L27 expression (Figure 1B), but sufficient to inhibit protein synthesis (Figure 1C). In the absence of cycloheximide, the expression of PPAR β protein was induced by TNF- α . The expression of plasminogen activator inhibitor 1 (PAI-1), a known target gene for both TNF- α and TGF- β 1, was induced as well by these two cytokines. The expression of JunB was only weakly induced by TGF- β 1 in keratinocytes, consistent with its cell-type-specific regulation (Mauviel *et al*, 1996; Verrecchia *et al*, 2001a) (Figure 1C). In each case, cycloheximide was able to prevent an increase of these proteins during TNF- α treatment.

TGF- β 1 modulates PPAR β expression via Smad3

To gain further insight into the inhibitory mechanism of TGF- β 1 on inflammation-induced PPAR β , we performed transient transfections using various PPAR β promoter constructs. We found that the AP-1(-414) site in the promoter was the only site critical for both TNF- α responsiveness and TGF- β 1-mediated inhibition (Figure 2A). Overexpression of TAM-67, a dominant-negative form of c-JUN, confirmed this result by provoking a similar diminution of the activity of the PPAR β promoter constructs (PPAR β (-445) and PPAR β (Δ 308/ Δ 197)) harboring an intact AP(-414)-binding site (Figure 2B). Conversely, the overexpression of c-JUN resulted in an increase of basal PPAR β promoter activity and overcame the inhibitory effect of TGF- β 1 (Figure 2B). Thus, the two different signaling pathways triggered by TNF- α and TGF- β 1 culminate at the AP-1(-414) site on the PPAR β promoter with opposite consequences: transcriptional stimulation by TNF- α and repression of the stimulated transcription by TGF- β 1.

Next, we examined the mechanism by which TGF- β 1 was mediating this inhibition. Overexpression of Smad3 and to some extent Smad4 reproduced the inhibitory effect of TGF- β 1, whereas the expression of a dominant-negative form of Smad3 (Smad Δ 3), and to a lesser extent of Smad4 (Smad Δ 4), abolished this repression (Figure 2C). Controls included Western blot analyses of the expression levels of the various Smad constructs and the use of a reporter gene containing three copies of the Smad-binding element (3SBE-luc), as positive control for transcriptional stimulation (Figure 2C). To further determine the role of Smad3 in mediating the inhibitory effect of TGF- β 1, we performed loss-of-function studies. Smad3^{+/+} and Smad3^{-/-} primary keratinocytes were examined for PPAR β expression in response to TNF- α in the presence or absence of TGF- β 1. These cells were also tested for their ability to transactivate the PPAR β (-445) promoter construct containing the AP-1 site. TGF- β 1 inhibited both TNF- α -mediated induction of endogenous PPAR β expression and activity of the exogenous PPAR β (-445) promoter in Smad3^{+/+} cells. However, this inhibitory effect of TGF- β 1 was completely abolished in Smad3^{-/-} keratinocytes, except when they were cotransfected with increasing amounts of a Smad3 expression vector (Figure 2D). These data indicate a major role for Smad3 in the TGF- β 1 inhibition of TNF- α -induced PPAR β promoter activity.

Differential regulation of PPAR β expression in an *in vitro* model mimicking different stages of wound healing

Biological signals produced during different phases of wound repair can be mimicked, at least in part, by conditioned media

Figure 2 TGF- β 1 modulates PPAR β expression via Smad3. (A) The activity of the full-length (PPAR β (-1880)) PPAR β promoter carrying various deletions (PPAR β (-587), (-445) and (-277)) and site-directed mutations was quantified in transactivation assays in primary keratinocytes. The PPAR β (-445) promoter construct contains three putative AP-1 sites (solid bar). The mutations of the AP-1 sites and their position relative to the transcription start site are indicated by the hatched bars and associated italic numbers. The PPAR β (Δ 308/ Δ 197) promoter construct is similar to PPAR β (-445), but has two putative AP-1-binding sites (at positions -308 and -197) mutated, while the -414 was left intact. The PPAR β (Δ 414) promoter construct contains a mutated AP-1 at -414. The AP-1(-414) site is the only site critical for both TNF- α (5 ng/ml) responsiveness and TGF- β 1-mediated (5 ng/ml) repression. The means of at least six independent experiments are shown. (B) A dominant-negative c-JUN (TAM-67) or wild-type c-JUN was cotransfected in increasing amounts with either PPAR β (-445), PPAR β (Δ 308/ Δ 197) or PPAR β (Δ 414) promoter constructs. TNF- α -induced PPAR β promoter activity was repressed upon overexpression of TAM-67 (left panel). Overexpression of c-JUN overcomes TGF- β 1-mediated repression (right panel). The means of at least six independent experiments are shown. (C) Cotransfections of PPAR β (-445) reporter construct with Smad1, 2, 3 or 4 (left panel) or with dominant-negative Smad 3(Δ 3) or Smad 4(Δ 4) (middle panel). As positive control, cotransfections were performed using Smad3, 4, Smad Δ 3 and Δ 4 with a luciferase reporter promoter construct containing three copies of the Smad-binding elements (3SBE-luc) (right panel). The means of at least three independent experiments are shown. Smad3 and Smad4 as well as dominant-negative Smad 3(Δ 3) or Smad 4(Δ 4) are equally expressed and protein integrity equally well preserved when transfected in primary keratinocytes. All Smad expression constructs contain an N-terminal FLAG tag. Integrity and expression levels of Smad3, 4, Smad Δ 3 and Δ 4 were verified by Western blot analysis using anti-FLAG antibody (see the right of the third panel). As a control of the FLAG antibody, the unrelated Flag-PPAR γ 2 protein was *in vitro* transcribed and translated. (D) Smad3 $^{+/+}$ and Smad3 $^{-/-}$ keratinocytes were transfected with the PPAR β (-445) construct and stimulated with TNF- α in the presence or absence of TGF- β 1. Smad3 deficiency blocked TGF- β 1 repression of TNF- α -induced PPAR β promoter activity. Cotransfection with increasing Smad3, in Smad3 $^{-/-}$ keratinocytes, restored responsiveness to TGF- β 1 inhibition. The means of at least six independent experiments are shown. V: empty expression vector.

from mixed leukocyte reactions (MLRs) (Banchereau and Steinman, 1998; Gallucci *et al*, 1999). Necrosis-derived conditioned medium (NM), initiated using minced skin (MS) or freeze-thawed (FT) fibroblasts, mimics early inflammation. Conversely, apoptotic-derived conditioned medium (AM), initiated using UV- or dexamethasone (Dex)-induced apoptotic fibroblasts, mimics the late re-epithelialization/remodeling stages of wound healing, when removal of apoptotic cells predominates (Greenhalgh, 1998).

In primary keratinocytes exposed to NM, there was a dose-dependent upregulation of PPAR β expression, which

was repressed by TGF- β 1 treatment (Figure 3A). Inflammatory stimuli, like TNF- α and LPS, increased the expression of PPAR β as well (Figure 3B). Exposure of primary keratinocytes to AM reduced the TNF- α -mediated stimulation of PPAR β expression. To identify the repressing activity, we preincubated AM with anti-TGF- β antibody. This treatment, but not the treatment with a control antibody, abolished the inhibitory effect of AM, which pointed to TGF- β 1 as the repressing cytokine (Figure 3B).

To analyze the complex bound to the AP-1 site, which is critical for both TNF- α - and TGF- β 1-mediated effects on

Figure 3 Differential regulation of PPAR β expression of *in vitro* model mimicking different stages of wound repair. (A, B) RPA analyses of PPAR β , keratin 5 (K5) and 6 (K6) after exposure of the keratinocytes to various treatments, as indicated. NM: necrosis-derived conditioned medium mimicking early inflammation, initiated by using either minced skin (MS) or freeze/thawed fibroblasts (FT); AM: apoptotic-derived conditioned medium mimicking the context of late remodeling stages of wound healing, initiated by using either UV (UV)- or dexamethasone (Dex)-treated fibroblasts. The importance of TGF- β 1 in AM was assessed by preincubation of AM with anti-TGF- β 1 antibody (50 μ g/ml). TGF- β 1 (5 ng/ml), TNF- α (5 ng/ml) and LPS (1 ng/ml) were used. In all RPA analyses, data were normalized using the ribosomal protein L27 mRNA levels. Values represent the mean of at least three independent experiments. (C) ChIP performed on primary keratinocytes treated as in (B) with AM or NM in the presence of TNF- α or TGF- β 1, as indicated. The results show a PCR amplification of the AP-1(-414) site (right panel) or control sequence (left panel) after ChIP with anti-c-JUN antibody. Preimmune serum serves as a control for ChIP. (D) JunB is not binding to the AP-1 site of the PPAR β promoter. ChIP assays were performed with the anti-JunB antibody. Keratinocytes exposed to NM, in the absence or presence of TGF- β 1, were used for ChIP. The AP-1(-414) site of the PPAR β promoter could not be amplified, indicating the lack of JunB binding to this site (top panel). As a positive control, the AP-1 site at position -783 of the mouse p16^{INK4a} gene was amplified using senescent (passages 6 and 12) primary fibroblasts (bottom panel). For (C) and (D), the figure shows a representative result out of four independent experiments.

PPAR β expression, we performed chromatin immunoprecipitation (chIP) on keratinocytes in various conditions. c-JUN occupancy of the AP-1(–414) site on the PPAR β promoter was triggered by exposure of keratinocytes to TNF- α or NM (Figure 3C). Interestingly, this induced c-JUN occupancy was reduced in a dose-dependent manner upon additional treatment with TGF- β 1 or AM. Preincubation of AM with anti-TGF- β antibody removed this inhibitory activity of NM. Also, the expression of JunB was not robustly increased upon TGF- β 1 treatment in keratinocytes (Figure 1C) and was not present on the AP-1(–414) site of the PPAR β promoter. (Figure 3D). However, JunB was detected on the AP-1(–783) site of the mouse p16^{INK4a} gene (Passegue and Wagner, 2000) in senescent primary fibroblasts used as positive control. Hence, TGF- β 1 inhibits the inflammation-induced PPAR β expression by preventing AP-1 DNA binding to the PPAR β promoter in keratinocytes.

Smad3 inhibits the binding of the c-JUN–p300 complex on the PPAR β promoter

The observations made so far (Figures 2C and 3C) suggested that in response to TGF- β 1, Smad3 may interfere with c-JUN-mediated transactivation. Various Smad3/c-JUN interaction studies have already shown that the N-terminal domain of Smad3 and the basic DNA-binding domain and leucine zipper of c-JUN mediate this physical interaction (Zhang *et al*, 1998). Furthermore, substitution of four critical lysine residues with alanine, at positions 40, 41, 43 and 44, within the N-terminal domain of Smad3 (construction Smad3(4A)) was shown to block this interaction significantly (Qing *et al*, 2000). We verified both the occurrence of a similar interaction between Smad3 and c-JUN in primary keratinocytes and the specificity of the anti-Smad3 antibody via co-immunoprecipitations (Supplementary Figure S1A and B). The impact of such an interaction in the inhibition of AP-1-mediated PPAR β expression was confirmed by the poor inhibition resulting from the overexpression of the mutant FLAG-Smad3(4A), compared to that observed with the functional FLAG-Smad3 (Figure 4A, left panel). It could be argued that the mutation of the four N-terminal lysines of Smad3(4A) prevented its nuclear translocation and thus its ability to interact with nuclear c-JUN. Therefore, we performed transient transfections with Smad3-NLS, which comprises the N-terminal region of Smad3 that interacts with c-JUN (Zhang *et al*, 1998) and the SV40 T-antigen nuclear localization signal (NLS) (Werner *et al*, 2000). In line with the results reported above, the overexpression of Smad3-NLS inhibited TNF- α -induced PPAR β promoter activity (Figure 4A, middle panel). Using chIP with anti-c-JUN, followed by Western blot with anti-Smad3 antibody, we examined the occupancy of the AP-1(–414) site by c-JUN on the one hand and the formation of a c-JUN–Smad3 complex on the other hand (Figure 4B). In the presence of NM or TNF- α , c-JUN bound to AP-1(–414), and did not interact with Smad3. In contrast, the activation of Smad3 in response to AM or TGF- β 1 resulted in the formation of an ‘off AP-1(–414)’ c-JUN–Smad3 complex (Figure 4B, top panel). Consistent with the role of Smad4 as a common Smad mediator, Western blot analysis with anti-Smad4 antibody showed that Smad4 is associated with the c-JUN–Smad3 complex. The presence of Smad4 in this complex is likely via its interaction with Smad3 as it does not make contacts with c-JUN (Zhang *et al*, 1998). Taken together, these data

indicate that in response to AM or TGF- β 1, the increased interaction between c-JUN and Smad3 can mediate the inhibitory action of TGF- β 1/Smad3 on PPAR β expression by interfering with AP-1(–414) binding activity.

The recruitment of coactivator like p300 is required for the activation of gene transcription by DNA-bound AP-1 (Kamei *et al*, 1996; Feng *et al*, 1998). We therefore explored the possibility that the recruitment of p300 to the AP-1(–414) site could also be inhibited. We tested whether the overexpression of myc-tagged Smad3 Δ C, an N-terminal truncated Smad3 that constitutively binds to p300 but lacks the c-JUN interacting region (Shen *et al*, 1998), could also affect TNF- α -induced PPAR β (–445) promoter activity. As shown in Figure 4A (right panel), cotransfection of Smad3 Δ C with the PPAR β (–445) reporter construct caused a decrease in TNF- α -induced transcriptional activity. This transcriptional inhibition, which is independent of Smad3–c-JUN interaction, may be the result of p300 sequestration, although we cannot rule out the possibility that titration of endogenous Smad4 may also play a role in this process.

Consistent with the requirement of p300 for optimal AP-1-mediated transactivation, chIP conducted with anti-p300 on keratinocytes exposed to NM or TNF- α resulted in AP-1(–414) site occupancy, whereas Smad3 was not part of this complex as shown by Western blot (Figure 4B, middle panel). The formation of a c-JUN–p300 complex on the AP-1(–414) site was further confirmed by a chIP(anti-c-JUN)/re-chIP(anti-p300) assay (Figure 4B, bottom panel). Conversely, upon coexposure of the cells to AM or TGF- β 1, the anti-p300 chIP did not detect any AP-1(–414) binding, whereas Western blotting revealed a co-immunoprecipitation of Smad3 with p300. Smad4 was also detected, which is consistent with its role in this complex. Since the anti-Smad3 antibodies were not suitable for immunoprecipitation of endogenous Smad3, chIP does not allow us to clearly distinguish between two ‘off AP-1(–414)’ complexes, Smad3–c-JUN and p300–Smad3, or a single ternary complex of Smad3–c-JUN–p300.

Although TGF- β 1/Smad3 can repress inflammation-induced PPAR β expression by preventing the binding of c-JUN–p300 complex on the AP-1(–414) site of the PPAR β promoter, it is conceivable that such a complex may be tethered to promoters that are activated in response to TGF- β 1 via Smad3. To assess this possibility, we turned to the characterized human PAI (hPAI) promoter, which contains both AP-1 and Smad-binding elements (SBEs) in the same promoter region (–782 to –503) and is stimulated by either TNF- α or TGF- β 1 (Figure 1C). Interestingly and in contrast to the PPAR β promoter, coinubation of TNF- α or NM with AM or TGF- β 1, respectively, resulted in increased p300–c-JUN occupancy on the hPAI AP-1 sites (Figure 4C, top panel). Due to the lack of anti-Smad3 antibodies for chIP, chIP(anti-FLAG)/re-chIP(anti-p300) was performed on hPAI with transfected FLAG-Smad3. The results showed that the ‘p300–Smad3’ complex was only recruited to the SBEs of the hPAI promoter in response to TGF- β 1 or AM (Figure 4C, bottom panel), and suggested that both AP-1 and Smad3 can recruit the p300 coactivator to stimulate hPAI expression in response to TNF- α /NM and TGF- β 1/AM, respectively.

Taken together, these data indicated that TGF- β 1 is a major player in AM, which mimics late re-epithelialization/remodeling phase of wound repair and inhibits NM- or

Figure 4 Effects of Smad3–c-JUN interaction are promoter context dependent. (A) Cotransfection of the PPAR β (–445) promoter construct with increasing amounts of Smad3, Smad3(4A) (left panel), Smad3-NLS (middle panel) and Smad3 Δ C (right panel). Similar expression levels of Smad3 and Smad3(4A) were verified by immunoblotting with anti-FLAG antibody, whereas Smad3-NLS and Smad3 Δ C were verified by anti-myc antibody (see top of each panel); V: empty expression vector. The means of at least six independent experiments are shown. (B) Smad3–c-JUN interaction inhibits PPAR β expression. Protein–protein crosslink and chIP with anti-c-JUN (top panel) and anti-p300 (middle panel) antibodies, followed by Western blot (WB) with anti-Smad3, anti-Smad4 antibody or PCR amplification of the AP-1(–414) site on the PPAR β promoter are shown. In re-chIP experiment (bottom panel), first chIP was performed with anti-c-JUN antibody. After dissociation from the anti-c-JUN antibody, a second chIP (or re-chIP) was performed with anti-p300 antibody. Prior chIP, equal input was verified by immunoblotting with anti-tubulin antibody. A representative result out of three independent experiments is shown. (C) p300–c-Jun and p300–Smad3 complexes stimulate human PAI expression. ChIPs were performed on keratinocytes transfected with a 800 bp hPAI luciferase promoter construct and coincubated with TNF- α , AM, NM or TGF- β 1 as indicated. ChIP assays with anti-c-JUN (top panel), chIP/re-chIP with anti-c-JUN/anti-p300 (middle panel) and anti-FLAG/anti-p300 (bottom panel) antibodies are shown. Our results also suggest that both transcription factors can recruit p300 coactivator to the hPAI promoter in response to either TNF- α /NM or TGF- β 1/AM. One representative result out of three independent experiments is shown.

TNF- α -induced PPAR β expression. Importantly, the effect of TGF- β 1/AM on TNF- α /NM-mediated gene expression is highly dependent on promoter context, inhibiting PPAR β while increasing PAI expression.

Overexpression of p300 or c-JUN rescues TGF- β 1/Smad3 inhibition of PPAR β

As shown above, the inhibitory action of TGF- β 1 on the TNF- α -induced expression of PPAR β is due to increased ‘off-DNA’

c-JUN/Smad3 complex formation that prevented p300-dependent AP-1-mediated transactivation. We reasoned that the overexpression of either c-JUN or p300 should overcome the inhibitory effect of Smad3 by shifting the equilibrium such that more p300–c-JUN complex is formed. Indeed, overexpression of c-JUN (Figure 2B) or p300 (Figure 5A) in transfected keratinocytes was able to overcome the inhibitory effect of TGF- β 1/Smad3 on TNF- α -mediated PPAR β (–445) transactivation. Consistently, chIP/re-chIP experiments on keratinocytes overexpressing c-JUN (Figure 5B, top panel) or p300 (Figure 5B, middle panel) showed that, under these conditions, the p300–c-JUN complex binds to the PPAR β AP-1(–414) site even in the presence of TGF- β 1. Thus, the highly increased availability of p300 or c-JUN allowed the transfected cells to escape the TGF- β 1-dependent inhibition of the AP-1-mediated expression of PPAR β .

Smad3 does not bind to the AP-1(–414) site of PPAR β promoter

Analysis of the PPAR β promoter sequence did not reveal any potential SBEs. To further assess the inhibitory effect of Smad3 on the PPAR β promoter, keratinocytes were transfected with a FLAG-Smad3-expressing construct together with increasing amounts of p300, to avoid titration of this coactivator (Figure 5B, bottom panel). Regardless of the conditions, chIP experiments with anti-FLAG antibody showed that Smad3 did not occupy the AP-1(–414) site. Importantly, immunoblotting confirmed that Smad3 can clearly inhibit the binding of p300–c-JUN complex on the AP-1(–414) site of the PPAR β promoter (Figure 5B, bottom panel).

Altogether, these results indicated that the inhibitory effect of TGF- β 1/Smad3 on inflammation-induced PPAR β expression involves the formation of an ‘off AP-1(–414)’ Smad3/c-JUN complex that reduces the AP-1-dependent transactivation of the PPAR β promoter.

In vitro and in vivo downregulation of PPAR β target genes by TGF- β 1/Smad3

To examine downstream effects of Smad3 in the inhibitory action of TGF- β 1, the expression of PPAR β and of two of its target genes was examined using Smad3^{+/+} and Smad3^{–/–} primary keratinocytes. Smad3^{+/+} primary keratinocytes

exposed to TNF- α showed increased PPAR β expression that was inhibited by coexposure to TGF- β 1. However, the inhibitory effect of TGF- β 1 was completely abolished in Smad3^{–/–} keratinocytes (Ashcroft *et al*, 1999) (Figure 6A). The *in vivo* relevance of this crosstalk was further examined using wound fluid collected from mice day 1 (WFD1) and 7 (WFD7) postinjury, corresponding to the early inflammation and re-epithelialization phases of wound repair, respectively. Primary keratinocytes exposed to WFD1 showed a dose-

Figure 5 Smad3 inhibits AP-1-mediated transactivation of PPAR β expression. (A) Primary keratinocytes were transfected with the PPAR β (–445) reporter construct and promoter activity was measured upon induction by TNF- α (5 ng/ml). Inhibition of TNF- α -induced PPAR β promoter activities by TGF- β 1 was obtained either by additional treatment with TGF- β 1 (5 ng/ml) (left panel) or cotransfection with a Smad3 expression plasmid (right panel). This repression was partially overcome by overexpression of increasing amounts of p300. V: empty expression vector. Values represent the mean of four independent experiments. Top panel: Control by Western blot of the amounts of p300 expression in the corresponding keratinocyte whole-cell extracts, using baculovirus-produced His-tagged p300 as positive control. (B) ChIP and re-chIP of the AP-1(–414) site with indicated antibodies on keratinocytes treated with TNF- α and/or TGF- β 1 as indicated, and transfected with increasing amounts of the c-JUN expression plasmid (top panel) or p300 expression plasmid (middle panel). TGF- β 1 signaling was mimicked by overexpressing Smad3 (FLAG-Smad3) and ChIP performed with an anti-FLAG antibody, since the commercially available anti-Smad3 antibodies were not suitable for ChIP of endogenous Smad3 (bottom panel). A representative result out of three independent experiments is shown.

dependent increase in the expression of PPAR β and its target genes, ILK and PDK1, which was inhibited by the addition of WFD7 (Figure 6B). Preincubation of WFD7 with excess of TGF- β neutralizing antibody abolished its inhibitory effect, suggesting that TGF- β 1 is a major player in the downregulation of inflammation-induced PPAR β expression *in vivo* during wound healing (Figure 6B, see below). Consistent with the above results, chIP analysis using anti-c-JUN antibody

showed reduced c-JUN occupancy on the AP-1(-414) site of the PPAR β promoter in keratinocytes coexposed to WFD7. Similar observations were also made in PPAR $\beta^{+/+}$ keratinocytes coexposed to inflammatory signals (NM or TNF- α) and TGF- β 1 (or AM) (Supplementary Figure S1C and D).

To examine the biological outcome of this crosstalk, the expression of PPAR β was examined in wound biopsies from Smad3 $^{+/+}$ and Smad3 $^{-/-}$ mice. As shown in Figure 6C,

unwounded skin from both wild-type and Smad3^{-/-} mice showed similar levels of PPAR β expression. In wild-type mice, the expression of PPAR β was increased 24 h postinjury and returned to just above the basal level after 7 days. In contrast and consistent with the above results, the loss of the suppressive effect of Smad3 on c-JUN in the Smad3^{-/-} mice allowed for enhanced and sustained expression of PPAR β . The expression of ILK and PDK1 followed the same general pattern, as expected for PPAR β target genes. Presumably, this prolonged increase in PPAR β , ILK and PDK1 expression contributed to the accelerated wound closure rate that was observed in Smad3^{-/-} mice, as compared to their wild-type littermates (Ashcroft *et al*, 1999).

Discussion

Skin injury is accompanied by an early inflammation that is eventually resolved as wound repair proceeds into the re-epithelialization and remodeling phases. A variety of growth factors and cytokines released during wound healing regulate and synchronize a multitude of cellular processes, such as proliferation and apoptosis, that are essential for efficient wound repair. We showed herein that TGF- β 1 can inhibit inflammation-induced PPAR β expression via Smad3. Our results underscore that a spatiotemporal interplay/integration of different signaling pathways involving inflammation, TGF- β 1, Smad3 and PPAR β is necessary for proper wound healing.

Effects of Smad3–c-JUN interaction are promoter context specific

We reported herein that TGF- β 1, via Smad3, abrogates the stimulation of PPAR β expression induced by inflammatory cytokines. The activation of Smad3 by TGF- β 1 results in the formation of an 'off-DNA' c-JUN–Smad3 complex, which inhibits the binding of c-JUN to the PPAR β promoter. In this specific context, Smad4 is also part of the complex, in contrast to reports on Smad4-independent action of Smad3 in keratinocytes (Kretschmer *et al*, 2003). The interaction between Smad3 and c-JUN, which requires the N-terminal region of Smad3 and DNA-binding/leucine zipper of c-JUN, is likely to inhibit the DNA-binding property of this complex. In

line with this proposal, EMSA experiments showed that the interaction between Smad3 and AP-1 inhibited binding of the latter to AP-1 elements (Verrecchia *et al*, 2001b). In concordance, the inhibitory action of TGF- β 1/Smad3 also prevented p300-dependent AP-1 transactivation on the AP-1(–414) site of the PPAR β promoter. It is unlikely that PPAR β is the only AP-1-mediated target gene that is inhibited by TGF- β 1. Similar mechanisms have been proposed for the inhibition of the genes encoding human metalloelastase and inducible nitric oxide synthase (Feinberg *et al*, 2000). After completion of the work presented herein, the inhibitory action of Smad3 on AP-1-dependent promoter activity was also reported for MCP-1 in macrophages (Feinberg *et al*, 2004). Interestingly, MCP-1 expression was also found to be downregulated by ligand-activated PPAR β , a transrepression mechanism involving BCL-6 (Lee *et al*, 2003). Finally, TGF- β 1 was reported to suppress NF- κ B-dependent activation of MMP-1, via the sequestration of p300 by Smad3 (Yuan and Varga, 2001).

Although this novel 'off-DNA' c-JUN–Smad3 complex suggests a mechanism for the observed antagonism between AP-1 and TGF- β 1/Smad3 on AP-1-dependent genes, such as the PPAR β and MCP-1 genes, it can only partially account for the more complex pattern of regulation observed for genes containing composite response elements, as studied most extensively in the case of the PAI promoter (Keeton *et al*, 1991; Dennler *et al*, 1998). On this promoter, Smad3 and AP-1 can act cooperatively or antagonistically, depending on the identity of the AP-1 complex (c-JUN, c-Fos or JunB) bound to the composite element (Mauviel *et al*, 1996; Verrecchia *et al*, 2001b). The outcome, either gene activation or repression, of Smad3–AP-1 interaction depends on many other factors, including cell type (Uria *et al*, 1998; Verrecchia *et al*, 2001a), cell context (Zhang *et al*, 1998; Feinberg *et al*, 2004), induction time (Verrecchia *et al*, 2001a) and promoter specificity (Verrecchia *et al*, 2001b). It is worth noting that in most reports, the inhibitory action of Smad3 was revealed in the presence of inflammatory cytokines/stimuli (Werner *et al*, 2000; Feinberg *et al*, 2004). It is thus conceivable that exposure of cells to inflammatory signals leads to different TGF- β 1 responses, which can be exacerbated by AP-1/Smad3 overexpression.

Figure 6 Smad3 is essential for inhibition of inflammation-induced PPAR β expression and downregulation of its target genes. **(A)** RPA analysis of PPAR β mRNA expression after treatment of Smad3^{+/+} and Smad3^{-/-} primary keratinocytes with TNF- α (5 ng/ml) in the presence or absence of TGF- β 1 (5 ng/ml). Ribosomal protein (L27) mRNA was used as internal control. Vehicle (PBS)-treated keratinocytes served as control. **(B)** RPA analyses of PPAR β , ILK and PDK1 mRNA expression in keratinocytes exposed to day 1 wound fluid (WFD1; 0.1 and 0.5% v/v) in the presence or absence of day 7 wound fluid (WFD7; 0.1 and 0.5% v/v). The importance of TGF- β 1 in WFD7 was assessed by preincubation of WFD7 with anti-TGF- β 1 antibody (10 and 50 μ g/ml). ChIP analysis with anti-c-JUN antibody confirmed reduced c-JUN binding to AP-1(–414) of the PPAR β promoter in the presence of untreated or control Ig-treated (10 and 50 μ g/ml) WFD7. The means of at least four independent experiments are shown. **(C)** RPA analyses of expression levels of PPAR β , ILK and PDK1 in day 1 (inflammation phase) and 7 (re-epithelialization phase) wound biopsies from Smad3^{+/+} and Smad3^{-/-} mice, normalized against ribosomal protein (L27) mRNA levels. The experimental value, from unwounded wild-type skin, used as normalization unit was arbitrarily assigned a value of 1. The other values indicate relative fold increase (decrease) as compared to unwounded wild-type skin. Values represent the mean of three independent experiments. **(D)** Model for the role of Smad3 in PPAR β regulation. Upon injury, early inflammation signals, like TNF- α , predominate at the wound site. TNF- α stimulates PPAR β expression and activity via the SAPK signaling cascade resulting in the activation of AP-1 complex that binds at the AP-1(–414) site and the production of PPAR β ligands, respectively (Tan *et al*, 2001). Activated PPAR β upregulates the expression of ILK and PDK1, which activate Akt1 by phosphorylation (Di Poi *et al*, 2002). Phosphorylated Akt1 interacts with Smad3, resulting in a decrease in Smad3-mediated transcription (Conery *et al*, 2004; Remy *et al*, 2004). Hence, during early phases of wound repair, the growth–survival Akt1 pathway is dominant. As wound repair proceeds into the re-epithelialization/remodeling phase, TGF- β 1 produced by both the infiltrating immune cells and the wound fibroblasts leads to strong activation of the TGF- β 1/Smad3 pathway. Phosphorylation of Smad3 prevents the formation of Akt1–Smad3 complex, forms a complex with Smad4, which translocates to the nucleus and regulates gene expression. Smad3 either interacts with c-JUN or sequesters the p300 coactivator, which inhibits inflammation-induced PPAR β expression. Reduced Akt1 phosphorylation amplifies the effect of Smad3. Importantly, this mechanism allows for a shift in the control of PPAR β and downstream effects, from the prevalence of growth–survival pathway to the prevalence of TGF- β 1-mediated growth arrest.

Biological significance of the crosstalk between TGF- β 1/Smad3 and PPAR β signaling

During wound repair, a conundrum of growth factors and cytokines are produced that simultaneously activate a network of signaling cascades that often have opposing effects. For example, keratinocyte growth factor (KGF), TGF- β 1 and glucocorticoid are produced during wound repair. However, the former stimulates keratinocyte proliferation, whereas the latter two cause cell-cycle arrest. It is still unclear how some of these opposing signaling pathways culminate to a specific cellular response. In the context of wound healing, we showed an opposite regulation of PPAR β expression by inflammatory cytokines and TGF- β 1, which is reflected on the PPAR β target genes, ILK and PDK1, and then on the Akt1 signaling pathway.

After completion of this work, two reports showed that Akt can also modulate TGF- β 1 signaling via an interaction with Smad3 (Conery *et al*, 2004; Remy *et al*, 2004). Importantly, this interaction does not inhibit Akt kinase activity, but inhibits Smad3-mediated gene regulation. This mechanism, combined to those reported herein, reveals a novel regulatory crosstalk between TGF- β 1/Smad3 and PPAR β /Akt signaling at different stages of wound repair (Figure 6D). Altogether, they provide novel insights into the temporal articulation of the sequential dominance of intricate signaling cascades, such as the antiapoptotic Akt signaling early after injury and the proapoptotic TGF- β 1 pathway at later stages of wound healing. We speculate that this articulation is not unique to skin repair, but may well be part of a more general healing pattern that can be activated after injury in different parts of the body.

Materials and methods

Reagents

Antibody providers are as follows: anti-JunB and anti-PPAR β / δ (Affinity Bioreagents); anti-PAI (BD Biosciences); anti-ILK, anti-PDK1 and agarose-conjugated anti-c-JUN (Santa Cruz Biotechnology); anti-FLAG (Sigma); anti-p300 (Upstate Biotechnology), anti-tubulin (Pharmingen); neutralizing TGF- β 1 antibody (Shah *et al*, 1995) (R&D System, AB-101-NA) and corresponding control antibody (Upstate Biotechnology, 12-332); anti-c-JUN and Akt Kinase Assay Kit (Cell Signaling); anti-Smad3 (Zymed; Western blot analyses) and anti-Smad3 (Santa Cruz Biotechnology). Cytokines were from PeproTech EC.

Cell culture and transfection

Primary mouse keratinocytes were cultured and transfected with full-length (PPAR β (-1880)) and truncated (PPAR β (-587), (-445) and (-277)) mouse PPAR β promoter reporter constructs as previously described (Tan *et al*, 2001). Plasmids are named according to the length of the promoter region relative to the transcription start site, subcloned upstream of a promoterless luciferase reporter construct (pGL-luc) (Tan *et al*, 2001). The PPAR β (-445) promoter construct contained a 445 bp truncated mouse PPAR β promoter with its three putative AP-1 sites. The human Smad expression constructs contained a FLAG tag fused to the N-terminus of Smad3. Smad Δ 3 is a dominant-negative mutant with three C-terminal serine phosphorylation sites changed to alanines. Smad3 Δ C (amino acids 199–424) is an N-terminal

truncated Smad3 that interacts with p300, but not with c-JUN (Shen *et al*, 1998).

Smad3(4A) is a mutant, in which the four lysines (position 40, 41, 43 and 44) are changed to alanines, and which is not capable of interacting with c-JUN. Smad Δ 4 was created by deletion of the C-terminal 61 amino acids (Lagna *et al*, 1996). Smad Δ 3-NLS contains amino acid 1–144 of Smad3 subcloned between a myc tag and three copies of SV40 T-antigen NLS of pCMV-myc-nuc (Invitrogen). Mutations were performed by site-directed mutagenesis (QuikChange, Stratagene).

RNase protection assay

Direct RNase protection assay (RPA) was performed as previously described (Tan *et al*, 2001), for PPAR β , L27, ILK, PDK1, K5 and/or K6, using RNA isolated from primary mouse keratinocytes subjected to the indicated treatments. Their relative expression levels were normalized against ribosomal protein (L27).

In vivo protein–protein crosslink, chromatin immunoprecipitation and re-chIP

In vivo protein–protein crosslink and chIP were performed as previously described (Di Poi *et al*, 2002; Hall and Struhl, 2002), with some modifications. Briefly, proteins and chromatin were cross-linked by treating the cells with 0.5% formaldehyde for 15 min at 37°C before sonication in lysis buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1% SDS, protease inhibitor cocktail (Roche Biochemicals)) to achieve crosslinked DNA of 200–600 bp in length. After centrifugation, the supernatant was diluted 10-fold with dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl, pH 8.1). Nonspecific binding was eliminated via preincubation with preimmune serum and Sepharose–Protein A at 4°C for 2 h. Sepharose beads were removed by centrifugation and 10% of the supernatant was used as input. The supernatant was incubated with indicated antibodies overnight at 4°C. Beads were collected and sequentially washed twice with 1 ml of wash buffers (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl; same buffer with 500 mM NaCl; 0.25 M LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, 10 mM Tris-HCl, pH 8.1; TE). For chIP/re-chIP assay (Ijpenberg *et al*, 2004), the immunoprecipitates were reverse crosslinked for PCR or boiled for 10 min in 1 $\times$ SDS loading buffer for Western blot analysis.

Wounding experiment

Smad3^{+/-} mice were intercrossed to produce Smad3^{+/+}, Smad3^{+/-} and Smad3^{-/-} offsprings (Ashcroft *et al*, 1999). Wound fluid was derived from injured wild-type mice as previously described (Schaffer *et al*, 1996; Shah *et al*, 1999) with some modifications. Briefly, full-thickness excisional wounds were inflicted as described (Michalik *et al*, 2001), a 5 mm $\times$ 5 mm polyvinyl alcohol sponge was placed subcutaneously adjacent to the wound and then the wound was covered with transparent polyurethane dressing (3M). At indicated days, sponges were removed, wound fluids from five animals were pooled and quickly frozen in liquid nitrogen. Four independent groups of five animals were used. For protein/RNA analysis, the entire wound including a 5 mm margin was excised and snap-frozen in liquid nitrogen until further analysis. RNA/proteins were individually extracted from single wound biopsy for analysis.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank L Gelman for the baculovirus-produced FLAG-tagged PPAR γ and His-tagged p300 proteins as controls for Western blot analysis. NS Tan is a recipient of an A*STAR (Singapore) international fellowship. This work was supported by grants from the Swiss National Science Foundation to WW and BD and the Etat de Vaud.

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