TGF-β₁ and Smad4 overexpression induce a less invasive phenotype in highly invasive spindle carcinoma cells

Juan Francisco Santibáñezᵃ,ᵇ*, Miguel Quinterillaᵇ, Jorge Martinezᵃ

ᵃLaboratorio de Biología Celular, INTA, Universidad de Chile, Casilla 138, Santiago 11, Chile
ᵇInstituto de Investigaciones Biomédicas Alberto Sols, CSIC-UAM, 28029 Madrid, Spain

First published online 8 May 2002

Edited by Veli-Pekka Lehto

Abstract We have examined the effect of transforming growth factor β₁ (TGF-β₁) and overexpression of the Smad4 gene on the phenotype of Car C, a ras mutated highly malignant spindle carcinoma cell line. TGF-β₁-treated Car C cells overexpressing Smad4 spread with a flattened morphology with membrane ruffles abundant in vinculin and show a reduction in their invasive abilities. TGF-β₁ treatment and overexpression of Smad4 also enhanced the production of PAI-1 measured by the activation of the p3TP-lux reporter gene containing a PAI-1-related promoter. This activation was abolished with a dominant-negative Smad4 construct. These results lead us to conclude that both TGF-β₁ and Smad4 overexpression reduce the invasive potential of Car C cells, probably via the Smad pathway. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: TGF-β₁; Smad4; Spindle; Cat C

1. Introduction

Transforming growth factors β (TGF-β) are a family of growth factors which express a wide range of biological activities including effects on cell proliferation, extracellular matrix deposition, fibrosis, angiogenesis, morphogenesis, and immune regulation [1,2]. Epithelial cells are particularly sensitive to TGF-β-induced growth arrest while most cells of mesenchymal origin show little or no growth inhibition [1]. During epithelial carcinogenesis, TGF-β₁ acts as a tumor suppressor at early stages of tumorigenesis by inhibiting cell proliferation and inducing terminal differentiation [3–5]. However, growth arrest is lost at later stages of carcinogenesis [6,7]. Thus, carcinoma cells respond to TGF-β₁ by eliciting an epithelial–mesenchymal transition (EMT) associated with the development of highly invasive and metastatic spindle cell carcinoma [8]. This role of TGF-β₁ in promoting malignancy has been demonstrated in the model of mouse skin carcinogenesis [9–11], as well as in human carcinoma cell lines [12–14]. Our previous work has demonstrated that in transformed keratinocytes TGF-β₁-dependent EMT proceeds through the activation of the Ras-Erk pathway or the blockage of Smad4 and includes the enhancement of urokinase-type plasminogen activator (uPA) production and invasiveness [15,16].

Smad4 is a candidate tumor suppressor gene whose protein product acts as a cofactor that binds TGF-β₁ receptor-acti- 
Smad4 gene were analyzed for expression of Smad4 and Flag tag with antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and M2 (Sigma, St. Louis, MO, USA) respectively.

2.3. Transient transfection and reporter gene measurements

For the invasive assay, Car C cells (10^6) were transiently transfected using the above-mentioned methods with 5 µg of C-terminal truncated dominant-negative Smad4 (1-514) pCMV construct or 5 µg of pcDNA3 EV along with 1 µg of SV40 lacZ vector as an internal control. To measure the PAI-1 promoter activity Car C cells (3 × 10^4) were transiently co-transfected as above with 0.5 µg of full-length pCMV or 1-514 truncated Smad4, pCDNA3 as a control vector and 0.5 µg of 3TPLux-specific luciferase construct along with 200 ng of SV40 lacZ vector as an internal control for transfection efficiency. After 18 h, the medium was changed and TGF-β1 (10 ng/ml) was added for an additional 24 h, cells were lysed and luciferase activity was determined [25].

2.4. Invasion and uPA activity assays

The capacity of the cells to migrate through Matrigel-coated filters was measured by using Transwell chambers (Costar, Cambridge, MA, USA) as described [25]. uPA secreted activity, radial caseinolytic and zymographic assays were also measured as described [25].

2.5. Immunofluorescence analysis

Indirect immunofluorescence staining of vinculin and vimentin was performed in cells grown on glass coverslips and fixed in methanol using mouse monoclonal antibodies (Sigma) at a 1:100 dilution and a FITC-labeled secondary antibody. Double immunofluorescence staining of uPA and PAI-1 was performed in non-permeabilized cells fixed in paraformaldehyde (4% in phosphate-buffered saline (PBS)) using an anti-uPA mouse monoclonal antibody [25] at 10 µg/ml and a goat anti-PAI-1 polyclonal antibody (Santa Cruz Biotechnology) at 1:100 dilution. Secondary antibodies were FITC-labeled anti-goat IgG and TRITC anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA).

2.6. Western blot analysis

For p-ERK-1,2 (Santa Cruz Biotechnology) and p-Smad2 (Upstate, Lake Placid, NY, USA) determinations, cells were lysed in PBS buffer.
containing 150 mM NaCl, 1% Triton X-100, pH 7.4 and 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, 1 mM orthovanadate and 1 mM NaF. For Smad4 analysis, cells were lysed in the same buffer mentioned above and in the absence of phosphatase inhibitors. Proteins (50 μg/lane) were resolved by 10% SDS-PAGE and transferred to Immobilon P membranes (Millipore, Bedford, MA, USA). PAI-1 was detected in the conditioned media of both intact and Smad4wt transfected cells untreated or treated for 48 h with TGF-β1, in serum-free medium. Five-fold concentrated conditioned media were processed by Western blot analysis as above using a goat anti PAI-1 polyclonal antibody (Santa Cruz Biotechnology) at 1:500 dilution and developed using the enhanced chemiluminescent system (ECL, Amersham, Arlington Heights, FL, USA).

3. Results

3.1. TGF-β1 and overexpression of wtSmad4 stimulate spreading and induce morphological changes in Car C cells

Car C are spindle-shaped fibroblastic cells that grow in culture forming a densely packed tubular-like network (Fig. 1). Treatment with TGF-β1 or transfection with the wild type Smad4 gene disrupted these structures and cells dissociated from each other adopting a flat and well spread morphology. Immunofluorescence analysis also revealed a poor organization of vinculin-containing adhesion plaques in untreated Car C cells while cells treated with TGF-β1 or overexpressing the Smad4 gene (Smad4wt clone 2) displayed a well developed adhesion plaque system suggesting that both treatments promoted increased adhesion to the substratum and reinforced focal adhesion (Fig. 1A, middle row). Morphological changes induced by TGF-β1 or overexpression of the Smad4 gene also included significant alterations of the microfilament cytoskeleton, generating a branched network as is shown in cells assayed by immunofluorescence to vimentin (Fig. 1A, lower row). The expression level of Smad4 protein and Flag tag Smad4 protein of control cells and cells transfected with EV and two different clones of Smad4wt+ cells is also shown (Fig. 1B).

3.2. TGF-β1 and overexpression of Smad4 gene decrease invasiveness of Car C cells and stimulate PAI-1 expression and secretion

We examined the effect of TGF-β1 on the invasiveness of Car C cells, which normally show extremely high invasive abilities through the reconstituted basement membrane Matrigel. We studied the effects of TGF-β1 and the stable transfection with wild type Smad4 gene and a transiently transfected dominant-negative (Smad4dn) version of Smad4 on the invasiveness of Car C spindle cells. As shown in Fig. 2A, TGF-β1 significantly reduced invasiveness of the intact spindle Car C cells (C). A similar inhibition was observed in cells treated with 2 μg/ml PAI-1 (P). Cells transfected with EV behave as the controls parental cells. However, wtSmad4 cells show a strong inhibition of the invasiveness in the presence or the absence of TGF-β1. In contrast, transfection with the dominant-negative version of Smad4 does not affect the invasiveness of Car C cells and block the TGF-β1-induced inhibition of invasiveness.

To investigate if the diminished invasive capacity of TGF-β1-treated and Smad4wt Car C was due to an alteration in the balance of the cellular proteolytic potential, we analyzed the expression of PAI-1, a specific inhibitor of uPA, in both experimental conditions. Fig. 2B shows that Smad4wt-2 cells express a higher amount of PAI-1 than control cells and that TGF-β1 was able to increase the production of PAI-1 in both control and Smad4wt cells. In a separate experiment, we transiently co-transfected Car C cells with Smad4, Smad4dn and pcDNA3 EV with the p3TP-lux reporter gene which contains a TGF-β1-responsive PAI-1-related promoter. As Fig. 2C shows, TGF-β1 stimulates PAI-1 promoter activities in cells harboring EV. Interestingly, Smad4dn Car C cells express a lower promoter activity (compared with control cells) and PAI-1 is not stimulated by the addition of TGF-β1. The overexpression of Smad4wt in Car C cells results in a cellular phenotype with a potent promoter activity that retains the potential to be stimulated by TGF-β1.
3.3. TGF-β1 treatment and Smad4wt transfection do not affect uPA production. PAI-1 and uPA co-localize at the cell surface

Measurements were also made to investigate if TGF-β1 treatment and stable Smad4wt gene transfection affect the production of uPA. As Fig. 3A shows, neither TGF-β1 treatment nor Smad4wt transfection affects the net secreted activity of uPA analyzed by zymography (Z). However, when enzymatic activity was assayed by radial diffusion (RD), inhibition of uPA activity was observed in parental control cells (C) treated with TGF-β1. Smad4wt cells secreted a very low uPA activity in the presence or absence of TGF-β1.

The interaction of uPA and PAI-1 was also analyzed by double immunofluorescence experiments in non-permeabilized cells. Fig. 3B shows that uPA was concentrated at plasma membrane extensions in untreated, TGF-β1-treated and Smad4wt cells while no sign of PAI-1 was observed in untreated cells. Upon stimulation with TGF-β1, PAI-1 was produced and co-localized with uPA in the same regions of the plasma membrane.

3.4. TGF-β1 treatment and Smad4wt transfection activate Smad signaling in Car C cells

To assess if the phenotypic changes provoked by TGF-β1 treatment or by transfection with the Smad4 gene in Car C cells occur by activation of the Smad pathway, we measured the kinetics of phosphorylation of Smad2 in parental as well as in Smad4wt-2 Car C cells with different kinetics. We also studied the possible activation by TGF-β1 of the Ras-MAPK signaling route in both cell types. As Fig. 4 shows, the same experimental conditions that induce the activation of Smad2 do not modify ERK-1,2 activity.

4. Discussion

In the present study, we show that TGF-β1 treatment and overexpression of Smad4 gene in Car C cells induce a set of
phenotypic changes that generate a less invasive phenotype. These results clearly show that these changes are not dependent on the activation of the Ras-ERK pathway but rather on Smad activation.

Smad4 plays a central role in TGF-β signaling by serving as a common partner of other Smad proteins and accumulates in the nucleus in a highly regulated process [26]. The potential tumor suppressive activity of Smad4 has also been evaluated in Smad4-defective colon cancer cells by stable re-expression of Smad4, a phenomenon associated with reduced uPA expression [27]. In mouse cells, the Smad4 gene is located on chromosome 18q [28]. Car C cells possess an intact pair of chromosome 18 and express a non-mutated version of Smad4 (Quintanilla et al., unpublished results). In our data, clones transfected with Smad4wt express three to four times the amount of Smad4 protein of control cells and also express the Flag-tagged protein confirming the exogenous origin of the gene (Fig. 1B).

TGF-β1-treated and Smad4wt Car C cells show changes in morphology illustrated in the expression of a network of vimentin and an increased peripheral localization of vinculin, an essential component of focal contact. This cellular shape is suggestive of a high cellular contact with the extracellular matrix and a less motile phenotype as has been previously demonstrated [29]. The vimentin network collaborates to maintain cellular mechanical stability, as has been demonstrated in other cellular phenotypes [30].

TGF-β1-treated and Smad4wt Car C cells display a diminished invasive capacity as measured in a Matrigel assay, probably associated with the changes in cellular shape and the induction of the expression and secretion of PAI-1. The invasive capacity of spindle Car C was unaffected by transient transfection with Smad4dn or by treatment of these transformed cells with TGF-β1 (see Fig. 2A). In contrast, cells transfected by the wild type version of Smad4 show inhibition of invasiveness even in the absence of added factor. These results permit us to suggest that the overexpression of Smad4 has as a consequence the recruitment of Smad2/3 and the onset of a cellular response similar to those of stimulated parental TGF-β1-treated cells. The inhibition of the invasive capacity by TGF-β1 treatment or Smad4wt transfection in Car C cells can also be attributable to the stimulated expression of PAI-1. This hypothesis was confirmed in experiments where invasion was inhibited directly by the addition of PAI-1 (see Fig. 2A). Induction of PAI-1 appears to require Smad signaling, since Smad4wt cells show similar PAI-1 secretion to parental TGF-β1-treated cells (Fig. 2B) and the transcriptional activation of the p3TP-lux reporter gene by TGF-β1 was blocked by a dominant-negative construct (Fig. 2C). That the augmented production of PAI-1 inhibits the uPA-mediated proteolytic potential was suggested by the specific uPA-PAI-1 interaction observed on the surface of parental TGF-β1-treated and Smad4wt cells (Fig. 3B). Also, it is important to note that TGF-β1 induced the expression and secretion of PAI-1 in Car C cells without affecting the net uPA secreted activity. In a radial assay, where uPA and PAI-1 activities co-exist, a diminished uPA activity was observed in parental TGF-β1-treated cells. In the same assay, Smad4wt showed a strong inhibition of uPA activity (Fig. 3A) probably attributable to the formation of a PAI-1-uPA complex. Interestingly, the exogenous addition of PAI-1 exerts similar effects to TGF-β1 treatment or Smad4wt transfection on cell invasiveness but does not induce phenotypic changes (data not shown).

Results in Fig. 4 permit us to confirm that in intact Car C cells TGF-β1 stimulates the Smad2 phosphorylation in a time-dependent manner while, in the same conditions, activation of ERK-1,2 is not observed. These results are in agreement with the assumption that Car C cells exhibit a Ras-ERK pathway permanently activated by the expression of a mutated version of H-Ras and the loss of the normal allele [23,24]. A similar result was obtained when Smad4wt cells were used, although, interestingly, in this case a basal activation of the pathway was observed. This finding can be compared with results of PAI-1 expression, where in the absence of added factor, Smad4wt cells express a two-fold increased stimulation in the TGF-β1-responsive PAI-1 promoter activity which is increased further with the addition of the factor. It is important to note that other authors have reported that components of the JNK pathway can also regulate the TGF-β-dependent PAI-1 promoter activity but this possibility was not tested by us [31].

In conclusion, our results suggest that spindle Car C cells respond to TGF-β1 stimulation oppositely than transformed keratinocytes because the expression of a different signaling behavior. In fact, while spindle Car C shows a very low response through its permanently active Ras-Erk pathway, transformed keratinocytes mount a robust EMT that depends specifically on this signaling pathway [15]. Our results also suggest that Car C cells conserve an intact Smad signaling pathway and that the loss of the TGF-β1 antiproliferative response cannot be linked to inactivation of Smad signaling.

Acknowledgements: We thank Dr. J. Massagué for kindly providing Smad4 plasmids and Dr. Carmelo Bernabeu for the 3TP-lux reporter. This work was supported by Fondecyt 3000045 and post-graduate fellowship of Fundación Andes (to J.F.S.), 1010703 (to J.M.) and by the Comisión Interministerial de Ciencia y Tecnología and Comunidad Autónoma de Madrid of Spain (Grants SAF98-0085-C03-02 and 8.1/22/97, to M.Q.).

References