Vascular types I and II transforming growth factor-beta receptor expression: differential dependency on tyrosine kinases during induction by TGF-β

Michael R. Ward*, Alex Agrotis, Garry Jennings, Alex Bobik

Cell Biology Laboratory, Baker Medical Research Institute and Alfred Hospital, Prahran, Vic., Australia

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Abstract Recent evidence indicates that the type II transforming growth factor-β (TGF-β) receptor (TβRII) is a serine-threonine-tyrosine kinase. However, the significance of its tyrosine kinase is unclear. We investigated in vascular smooth muscle cells the effects of tyrosine kinase inhibition on the expression of TGF-β receptor types I (ALK-5) and II (TβRII) mRNA, induced by TGF-β_1. TGF-β_1 elevated ALK-5 mRNA levels 5-fold; essentially similar TGF-β_1-dependent elevations were observed with growth factors, PDGF-BB and FGF-2. The tyrosine kinase inhibitor genistein abolished these TGF-β_1 and growth factor responses. TGF-β_1 also elevated TβRII mRNA levels which were not inhibited by genistein. We conclude that tyrosine kinases participate in defining how cells respond to TGF-β.

Key words: Transforming growth factor-β; Tyrosine kinase inhibition; Transforming growth factor-β receptor gene expression; Vascular smooth muscle

1. Introduction

The importance of protein phosphorylation in signalling events associated with smooth muscle cell proliferation and blood vessel remodelling is well documented. Most mitogenic growth factors interact with transmembrane tyrosine kinases, initiating autophosphorylation and triggering a cascade of phosphorylation events critical for cell proliferation [1,2]. These latter events can involve tyrosine kinases, serine/threonine kinases as well as dual specific kinases such as mitogen-activated protein (MAP) kinase which activates its substrate by phosphorylation on both tyrosine and threonine residues [3,4]. There is also evidence that members of the transforming growth factor-beta (TGF-β) family which regulate cell growth, differentiation and gene responses [5–7], also interact with receptors which can initiate phosphorylation on tyrosine, serine and threonine residues [8,9]. Signal transduction by TGF-β is dependent on the physical interaction of its type I and type II receptors, namely ALK-5 or ALK-2 and TβRII, forming a heteromeric complex at the cell surface and resulting in phosphorylation of the type I receptor on serine and threonine [10]. Types I and II receptors also exist in the surface membrane as homomeric receptor complexes [11] and in the case of the type II receptor, its cytoplasmic domain is constitutively autophosphorylated [12]. Recently its phosphorylation has been shown to involve not only serine and threonine but also tyrosine, and replacement of tyrosine residues with phenylalanine strongly inhibits its kinase activity [9]. The in vivo biological significance of tyrosine phosphorylation in the TGF-β initiated signalling cascade is not known. It is possible that tyrosine phosphorylation influences the pattern of TGF-β-inducible cell responses. We tested this hypothesis in cultured smooth muscle cells by examining the ability of exogenous and endogenously produced TGF-β_1 to increase the expression of the TGF-β receptors, types I (ALK-5 and ALK-2) and type II (TβRII), in the absence and presence of tyrosine kinase inhibition. We demonstrate that tyrosine kinase activity is essential for the induction by TGF-β_1 of expression of its types I but not its type II receptor, suggesting differentiation of signalling through tyrosine phosphorylation mechanisms.

2. Materials and methods

2.1. Cell culture

Vascular smooth muscle cells (VSMC) were obtained from the media of rat left common carotid arteries as previously described [6,13]. VSMC were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and were used between the 2nd and 6th passage. At confluency the VSMC were serum-deprived for 24 h and then incubated with either PDGF-BB (200 ng/ml, Sigma, St Louis, MO, USA), FGF-2 (50 ng/ml, Bachem, Torrence, CA, USA), or TGF-β_1 (2 ng/ml, Celltrix, Palo Alto, CA, USA) in DMEM or with DMEM alone. The cells were harvested 24 h later and the RNA immediately extracted (see below). Some experiments (see Section 3) were performed in the presence of a TGF-β_1-neutralising antibody (Becton Dickinson, MA, USA, 2.5 μg/ml [0.72 μg neutralises 1 ng TGF-β_1]), or the tyrosine kinase inhibitor genistein (200 μM, RBI, Natick, NJ, USA) at concentrations previously been shown by us and others to specifically inhibit the actions of TGF-β_1 and tyrosine kinases, respectively [6,14].

2.2. RNA extraction and standardised reverse transcriptase-polymerase chain reaction (sRT-PCR)

Total RNA was extracted from cells using the method of Chomczynski and Sacchi [15], then treated with DNase (Stratagene). The RNA was dissolved in sterile water to a concentration of 266 ng/μl. Two hundred nanograms were then used for each reverse transcriptase-polymerase chain reaction (RT-PCR). A standardised (s)RT-PCR was performed in a manner which ensured that the respective PCR products reflected the levels of mRNA encoding the different TGF-β receptors in VSMC, as previously described (for details see [6,16]). This was performed using the following oligonucleotide primers and cycles: ALK-5 sense, base pairs (bp) 70–102 and antisense, bp 484–513, 34 cycles; ALK-2 sense, bp 128–152 and antisense, bp 435–459, for 34 cycles; TβRII sense, bp 1214–1237 and antisense, bp 1525–1552. The amount of PCR product for each target mRNA was expressed relative to the amount of PCR product for L7, a ribosomal protein which is encoded by a non-inducible cell cycle-independent gene [17] using the following primers and cycle number: sense, bp 143–162 and antisense, bp 405–428 for 23 cycles. PCR products

*Corresponding author. Cell Biology Laboratory, Baker Medical Research Institute, Commercial Rd, Prahran, Vic. 3181, Australia. Fax: (61) (3) 9521 1362. E-mail: mward@baker.edu.au

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were quantitated using laser densitometry estimation of intensities from photograph negatives of 2% agarose electrophoresis gels [6].

3. Results

3.1. Effects of growth factors on expression of TGF-β receptor mRNAs in VSMC

Initially we examined how exposure of VSMC to TGF-β_1 affected mRNA levels encoding the type I TGF-β receptors, ALK-5 and ALK-2, and the type II receptor (TβRII). Twenty four hours exposure of the cells to TGF-β_1 (2 ng/ml) elevated ALK-5 mRNA levels 8.3-fold; ALK-2 was also elevated at this time by 2.5-fold (Fig. 1). TGF-β_1 was also a potent stimulator of TβRII gene expression in these cells elevating mRNA levels during the 24-h period by 5.2-fold. Since TGF-β_1 is known to be induced in VSMC by PDGF-BB and FGF-2 we next investigated the extent to which these growth factors increased mRNA encoding the type I and type II TGF-β receptors. Twenty four hours exposure of these cells to PDGF-BB (200 ng/ml) resulted in elevated ALK-5 mRNA levels by 5.8-fold, an increase in ALK-2 mRNA by 2.5-fold and TβRII mRNA by 3.9-fold (Fig. 1); FGF-2 (50 ng/ml) also elevated ALK-5 mRNA (by 6.6-fold), ALK-2 mRNA by 2.0-fold and TβRII by 4.8-fold (Fig. 1).

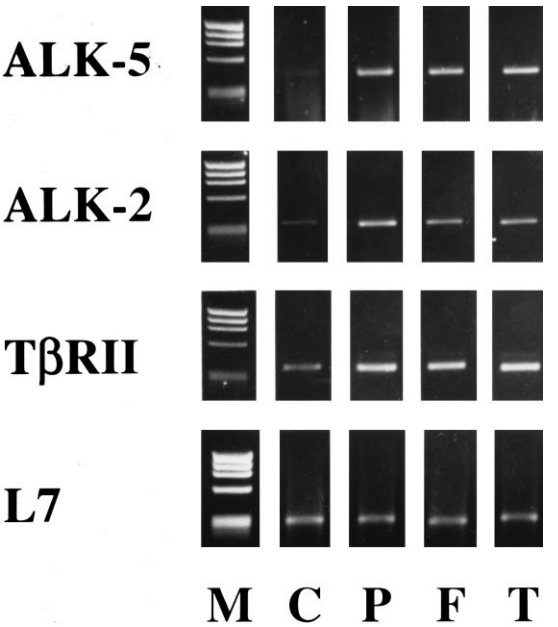

Fig. 1. Agarose gels depicting RT-PCR analysis of type I (ALK-5 and ALK-2) and type II (TβRII) TGF-β receptor and L7 mRNA levels in cultured vascular smooth muscle cells after 24 h exposure to PDGF-BB (P), FGF-2 (F) or TGF-β_1 (T) or culture medium only (C). M represents molecular weight markers from ΦX174 DNA digested with *Hae*III (Promega, WI, USA). Results are typical of 6 experiments.

3.2. Inhibition of tyrosine kinase activity alters the profile of TGF-β receptor mRNAs induced by TGF-β_1

Since the type II TGF-β receptor is known to undergo tyrosine phosphorylation in vitro, we next investigated the extent to which tyrosine kinase activities might regulate induction of types I and type II receptor mRNA by TGF-β_1. Simultaneous exposure of the VSMC to TGF-β_1 (2 ng/ml) and 200 μM genistein, a concentration which maximally and specifically inhibits tyrosine kinases in vascular smooth muscle [6] without affecting serine/threonine kinases, Ca^{2+}-dependent calmodulin and related kinases [14], completely prevented the cytokine-induced elevations in ALK-2 and ALK-5 mRNA levels (Figs. 2 and 3). In contrast genistein did not affect the ability of TGF-β_1 to elevate mRNA encoding TβRII (Figs. 2 and 3), providing evidence that distinct tyrosine kinase-dependent and -independent pathways are involved in the regulation of mRNAs of the two classes of receptor in response to TGF-β_1. Genistein (200 μM) also inhibited PDGF-BB and FGF-2 induced elevations in ALK-2, ALK-5 and TβRII mRNA in the cultures (Fig. 3), consistent with its high efficacy for inhibition of tyrosine kinase activities in vascular smooth muscle [6]. Thus, tyrosine phosphorylation appears necessary for the control of specific responses to TGF-β_1 in VSMC.

3.3. Dependency of induction of types I and II TGF-β receptors in VSMC on autocrine actions of TGF-β_1

Since exogenously added TGF-β_1 markedly elevated mRNA levels encoding the type I receptors ALK-5 and ALK-2 and also TβRII, it was of interest to determine whether increased secretion of this cytokine by VSMC could also cause similar elevations in mRNA levels. Accordingly, we next examined, using TGF-β_1-specific neutralising antibodies, the extent to which endogenously secreted, biologically active TGF-β_1 is responsible for the ability of RTKGFs to increase mRNA levels encoding the different TGF-β receptor types. The TGF-β_1 neutralising antibody (2.5 μg/ml) completely prevented the ability of TGF-β_1 (2 ng/ml) to elevate the mRNAs encoding ALK-5, ALK-2 and TβRII (Fig. 4). However this

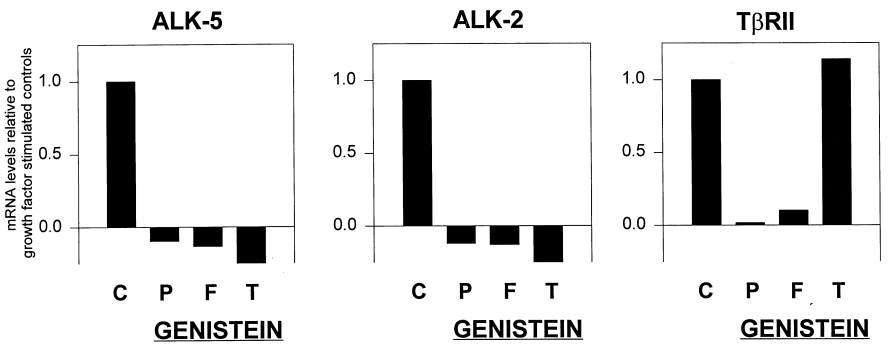

Fig. 2. Dependency of elevations in TGF-β receptor mRNAs on tyrosine phosphorylation in cultured vascular smooth muscle cells during 24 hours incubation with PDGF (P), FGF (F) and TGF-β_1 (T) in the presence of the tyrosine kinase inhibitor genistein. C represents control (growth factor stimulated in the absence of genistein). Results are expressed relative to mRNA levels of L7 and are the mean of 3 experiments.

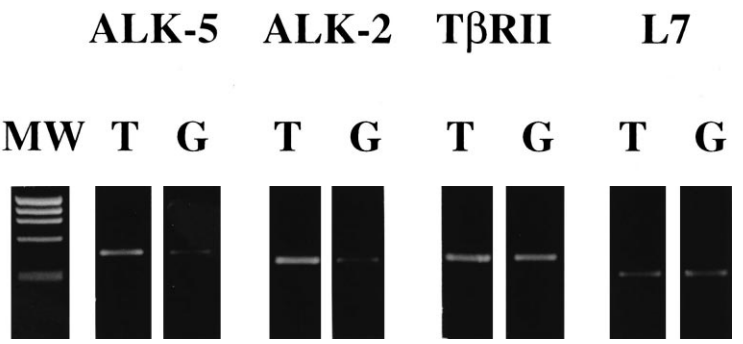

Fig. 3. Agarose gel illustrating the sRT-PCR products of mRNA obtained after 24 h stimulation of VSMC with TGF-β_1 in the absence (T) and presence (G) of 200 μM genistein. M represents molecular weight markers from ΦX174 DNA digested with *Hae*III (Promega, WI, USA). Results are typical of 3 similar experiments.

concentration of antibody, which we have previously shown prevents TGF-β_1-dependent increases in integrin α_v induced by PDGF-BB in these cells [6], had only a minimal effect on PDGF-BB- and FGF-2-induced elevations in these type I receptors; it had no effect on PDGF-BB-induced elevations in ALK-5 mRNA and only reduced the ALK-2 mRNA by 17%. While the antibody attenuated FGF-2-induced increases in ALK-5 mRNA by approximately 9% it had more potent effects on ALK-2 mRNA levels, which were reduced by 55% (Fig. 4). The antibody did not affect the levels of mRNA encoding these receptor types in the absence of growth factor stimulation (not shown). Thus, PDGF-BB and FGF-2, at concentrations which induce near-maximal effects on VSMC proliferation [7], exert effects on ALK-5 mRNA levels that are relatively independent of any autocrine action of bioactive TGF-β_1, while the increases they induce in ALK-2 mRNA levels are partially dependent on TGF-β_1. In contrast to the apparent lack of dependency of the elevation in ALK-5 mRNA on TGF-β_1, the PDGF-BB- and FGF-2-induced elevations in TβRII mRNA levels were nearly completely dependent on TGF-β_1 (Fig. 4). Alone, the TGF-β_1-specific neutralising antibody did not affect basal TβRII mRNA levels (not shown); however the increases induced by 24 h exposure to PDGF-BB and FGF-2 were reduced by 75% and 98%, respectively (Fig. 4). Thus, elevations in mRNAs encoding the predominant type I receptor required for TGF-β action, ALK-5, and its type II receptor induced by PDGF-BB and FGF-2 exhibit a differential dependency on the production of bioactive TGF-β_1.

4. Discussion

The initiation of TGF-β-induced cell signalling involves the heteromeric association of type I and type II receptor serine/threonine kinases, followed by transphosphorylation of the type I receptor [10]. The large array of cellular responses initiated by this ligand-receptor complex suggest that the initiated intracellular signals are likely to vary depending on the requirements of the cells. Our study indicates that part of the diversity in cell signalling by TGF-β in VSMC is mediated through alterations in tyrosine phosphorylation. The precise sites in the signalling cascade in cells where tyrosine phosphorylation creates diversity of cell responses are yet to be fully defined but likely involves tyrosine phosphorylation of the type II TGF-β receptor since type II receptors of the TGF-β superfamily have recently been reported to also possess tyrosine kinase activity [8,9].

Diversity of TGF-β responses in cells have been attributed to serine and threonine dependent phosphorylation changing the properties of the types I or type II receptor. Thus, serine-172 and threonine-176 of the type I TGF-β (ALK-5) receptor are dispensable for extracellular matrix production by TGF-β but critical for its ability to inhibit cell proliferation [18]. Similarly, phosphorylation of the glycine/serine rich (GS) domain of ALK-5 is critical for antiproliferative and transcriptional responses to TGF-β [19]. Phosphorylation of serine-409 in the type II receptor is also critical for TβRII kinase signalling and the ability of TGF-β to induce growth inhibition, whilst phosphorylation of serine-416 inhibits type II TGF-β receptor

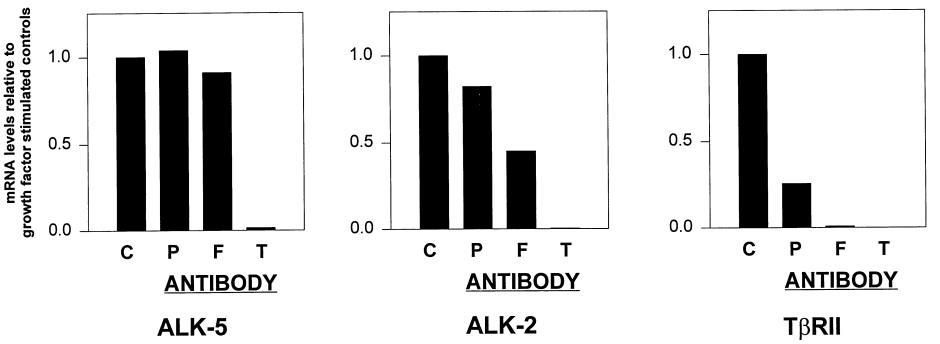

Fig. 4. Dependency of elevations in TGF-β receptor mRNAs on endogenously produced TGF-β_1 in cultured vascular smooth muscle cells during 24 h incubation with PDGF (P) and FGF (F) in the presence of the TGF-β neutralising antibody (ANTIBODY). C represents control (growth factor stimulated in the absence of antibody). T indicates TGF-β_1 stimulated mRNA levels in the presence of the antibody. Results are the mean of 3 similar experiments.

function [12]. Very recent studies indicate that the type II TGF-β receptor is also subject to autophosphorylation on tyrosine residues at positions 259, 336 and 424; replacement of these residues with phenylalanine greatly attenuated the kinase activity of the receptor in vitro but the significance of this finding in intact cells was not defined [9]. Our findings in VSMC indicate that tyrosine phosphorylation may participate in defining transcriptional responses to TGF-β_1, such as elevations in TGF-β receptor types.

In the present study PDGF-BB and FGF-2 were also shown to induce elevations in TGF-β receptor subtypes. However, only TβRII mRNA elevations exhibited dependency on endogenously produced TGF-β_1, while ALK-2 mRNA elevations exhibited partial dependency and ALK-5 no dependency. These different dependencies on endogenous TGF-β_1 when the VSMC are stimulated by these growth factors which are known to elevate TGF-β secretion [7], most likely reflect differences in the characteristics of the promotor regions of the receptor genes. The TβRII promotor region contains both AP-1 and SP-1 binding sites while the ALK-5 promotor has predominantly SP-1 binding sites [20,21]. TGF-β_1 exerts many of its effects via AP-1 binding sites [22], and has recently been reported to affect activity of promotors containing SP-1 binding sites [23].

Both common and apparently independent mechanisms appear to be important in regulating the expression of types I and II TGF-β receptors in blood vessels. In injured carotid arteries up-regulation of both receptor types has been reported [6,24] but in other vessels type I receptor serine-threonine kinases are preferentially expressed [25,26]. Also in osteoblasts [27], during kidney development [28] and in malignancy [29] independent changes in type I and type II receptors for TGF-β have been reported. The present results indicate the differentiation of TGF-β signalling, altering the profile of TGF-β responses in cells, may occur by mechanisms dependent on tyrosine kinases and probably involving tyrosine phosphorylation on TβRII. Further studies will be necessary to understand the mechanisms by which tyrosine kinases alter cell signals involved in the expression of TGF-β receptor types.

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