Original Article

Transforming Growth Factor-β Signaling Enhances Transdifferentiation of Macrophages into Smooth Muscle–Like Cells

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Hemopoietic cells or bone marrow–derived cells contribute to tissue formation, possibly by transdifferentiation into smooth muscle cells (SMCs) or myofibroblasts. In this study our goal is to examine the effects of transforming growth factor-β1 (TGF-β1) on the transdifferentiation of the monocyte/macrophage lineage into SMC-like cells. Using rat peritoneal exudate macrophages, we investigated the expression of smooth muscle–specific differentiation markers, such as α-smooth muscle actin, embryonic smooth muscle myosin heavy chain, and calponin. The treatment of macrophages with TGF-β1 enhanced the expression of SMC-specific markers at day 4; after 7 days in culture, a higher level of expression (approximately 3- to 5-fold) was detected on Western blots. In contrast, TGF-β1 decreased the expression of CD11b, which is a macrophage marker. Furthermore, we examined the effect of the TGF-β type 1 receptor inhibitor SB-431542 and a replication-defective adenovirus construct expressing Smad7 (Adeno-Smad7), which inhibits TGF-β signaling by interfering with the activation of other Smad proteins. Both SB-431542 and Adeno-Smad7 suppressed the expression of SMC-specific markers. These results indicated that TGF-β signaling is essential for the transdifferentiation of macrophages into SMC-like cells. Elucidating the mechanism by which macrophages transdifferentiate into SMC-like cells may reveal new therapeutic targets for preventing vascular diseases. (*Hypertens Res* 2006; 29: 269–276)

***Key Words*: transforming growth factor-β, macrophages, smooth muscle cell, transdifferentiation**

Introduction

Inflammatory cells play a pivotal role in vascular diseases (*1–3*). Infiltration into the vascular wall and the activation of monocytes/macrophages correlate with remodeling and arteriosclerosis. Recently, hemopoietic cells or bone marrow–derived cells have been shown to contribute to tissue formation, possibly by transdifferentiation into smooth muscle cells (SMCs) or myofibroblasts (*4–7*). Bone marrow–derived vascular progenitor cells circulating in adult peripheral blood have been shown to give rise to both vascular endothelial cells and SMCs (*8–11*). Thus, the potential contribution of bone marrow–derived cells to the formation of neointima is a new concept that may help in understanding the pathology of vascular diseases.

Overproduction of transforming growth factor (TGF)-β has been suggested to be involved in the various mechanisms of hypertension and organ damages (*12–14*). TGF-β is a multifunctional polypeptide signaling factor that can regulate cell proliferation, differentiation, apoptosis, and the expression of extracellular matrix proteins, resulting in vascular remodeling

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Received November 14, 2005; Accepted in revised form January 13, 2006.

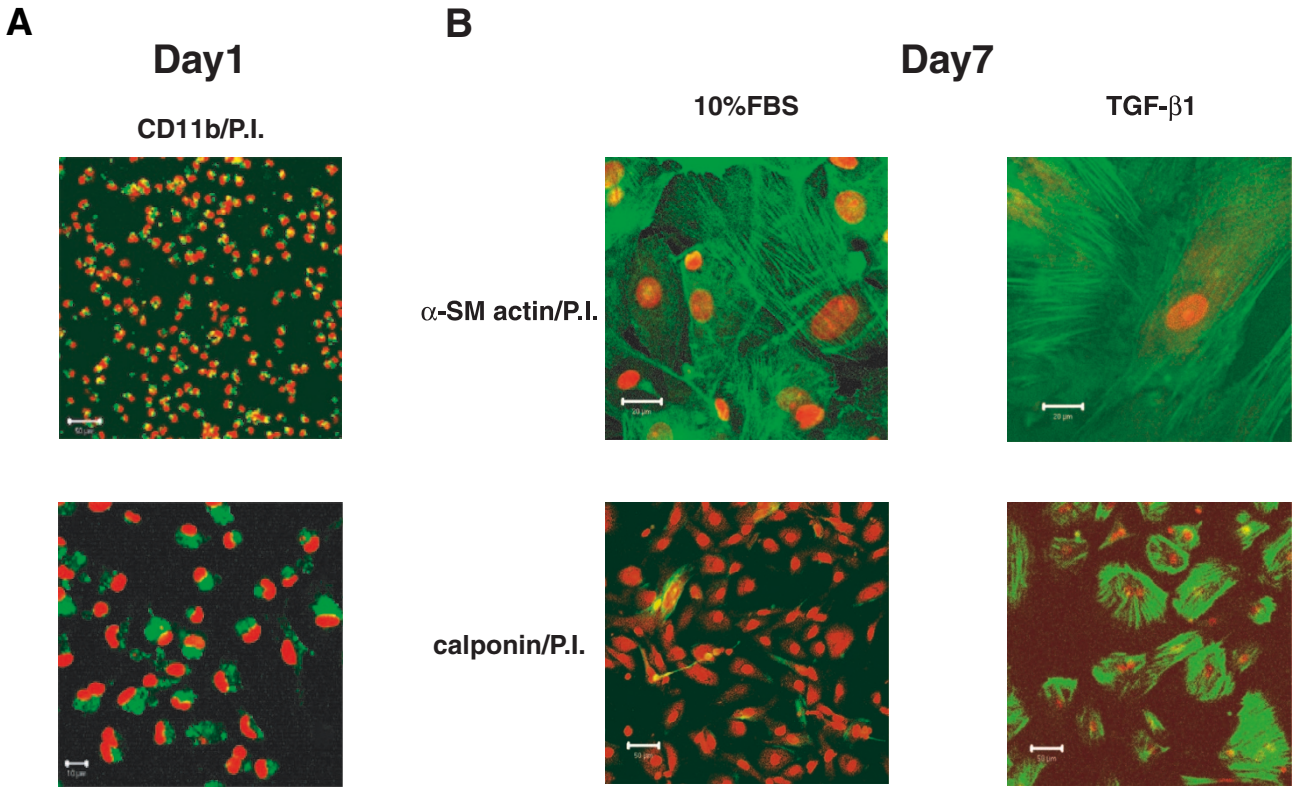

Fig. 1. *Effect of TGF-β1 on cell morphology. A: Immunofluorescence staining for CD11b on day 1. Green fluorescent signals show CD11b. Red fluorescent signals show the cell nuclei stained with propium iodide (P.I.). Upper panel, scale bar: 100 μm; lower panel, scale bar: 10 μm. B: Immunofluorescence staining for α-SM actin and calponin. Rat peritoneal macrophages were cultured in the presence of 10% FBS alone (left panel) or with 1 ng/ml TGF-β1 (right panel) for 7 days. Green fluorescent signals show α-SM actin (upper panel) or calponin (lower panel). The results represent two experiments. Upper panel, scale bar: 20 μm; lower panel, scale bar: 50 μm.*

(*15–18*). Two distinct serine/threonine kinase receptors, referred to as type I (TGF-βRI) and type II (TGF-βRII) TGF-β receptors, are necessary and sufficient for transducing most TGF-β–induced signals across the plasma membrane of a target cell (*19*). Upon TGF-β stimulation, TGF-βRII phosphorylates TGF-βRI, and activated TGF-βRI phosphorylates Smad2 and Smad3 (*20*). The Smad proteins constitute a novel class of signaling proteins that function as downstream effectors of TGF-β signaling by transmitting the TGF-β signal from the plasma membrane to the nucleus of a target cell (*21*, *22*). Smad2 and Smad3 are components of the TGF-β and activin signaling pathways, and Smad4 is a common factor required for TGF-β, activin, and bone morphogenetic protein signaling. In addition, Smad6 and Smad7 inhibit TGF-β signaling (*23*, *24*). Phosphorylated Smad2 and Smad3 then associate with each other as well as with Smad4 and subsequently translocate from the plasma membrane to the nucleus (*25*), where they activate the transcription of TGF-β–responsive genes.

The purpose of this study was to examine the role of TGF-β in controlling the expression of SMC markers in macrophages during transdifferentiation into SMC-like cells.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin, fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Life Technologies, Inc. (Rockville, USA). TGF-β1 was purchased from PeproTech EC, Ltd. (London, UK). SB-431542 was purchased from Tocris Cookson Inc. (Ellisville, USA).

Isolation of Peritoneal Macrophages Using Thioglycolate and Culture Conditions

All experiments were performed in accordance with the Guidelines for Animal Experimentation at Kobe University Graduate School of Medicine. Male Sprague-Dawley rats weighing 200–250 g were purchased from Japan SLC (Shizuoka, Japan). Thioglycolate medium (Nissui, Japan) was

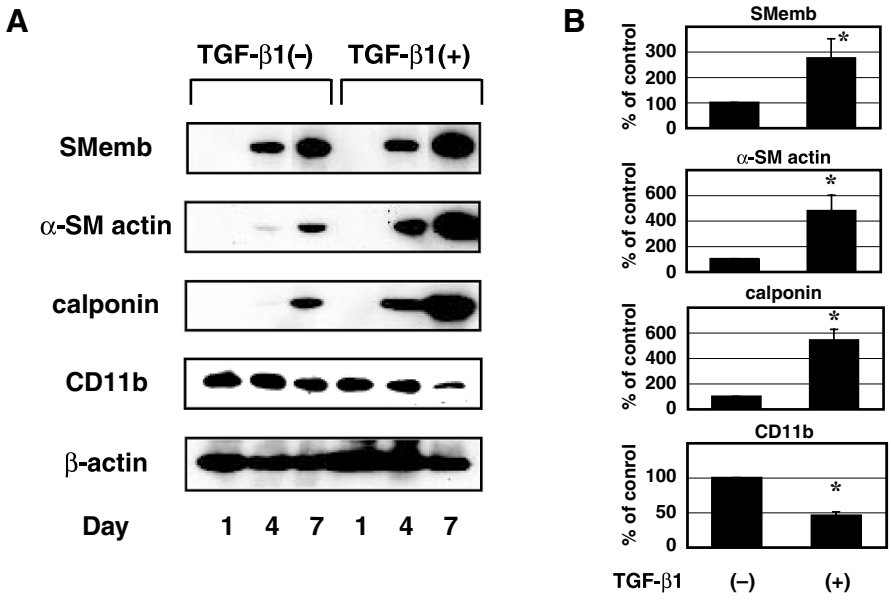

Fig. 2. *TGF-β1 enhances the expression of SMC markers. A: Rat peritoneal macrophages were cultured in the presence of 10% FBS with or without 1 ng/ml TGF-β1 for the indicated number of days. The expression of α-SM actin, SMemb, calponin, CD11b, and β-actin was examined by Western blotting. The data in the left panel represent three independent experiments. B: The bands in the right panel were quantified by an image analyzer. The degree of expression of each protein was determined as the value relative to that of 10% FBS–treated cells on day 7. The values (the mean ±SEM in three independent experiments) are expressed as percentages relative to the control.* *p < *0.01* vs. *the control by unpaired Student's* t-*test.*

dissolved in distilled water (4%, w/v) and autoclaved at 121°C for 20 min. Thioglycolate medium (7 ml) was injected into the peritoneum of rats. After 4 days, the rats were sacrificed by cervical dislocation. The peritoneal wall was soaked with 70% ethanol and then lifted away from the cavity and injected with approximately 10 ml of cold PBS supplemented with 2 mmol/l EDTA and 0.5% bovine serum albumin, using a 10-ml syringe with an 18-gauge needle. The peritoneum was massaged, and the fluid was drawn back into the syringe. The cells were washed three times by centrifugation at 300 × *g* for 10 min at 4°C and were resuspended in DMEM containing 10% FBS. Then the cells were cultured in basal medium (DMEM supplemented with 10% FBS) with or without TGF-β1 for 7 days. The medium was changed every day. In some cases, SB-431542 was added.

Adenovirus Vector Construction and Infection

The replication-defective adenovirus construct expressing Smad7 (Adeno-Smad7) was provided by Kohei Miyazono (Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo) (*26*). The construct expressing β-galactosidase (Adeno-β-gal) was previously described (*27*). All constructs were amplified in 293 cells and purified by ultracentrifugation. The viral titer was determined as plaque forming units (pfu). For infection, macrophages were typically incubated with adenovirus at a multiplicity of infection (M.O.I.) of 50 or 150 pfu in medium for 12 h. The virus was removed when the medium was replaced with fresh medium. Under these conditions, the transfection efficiency was >90%.

Western Immunoblotting

Western blotting was performed as previously described (*27*). The following antibodies were used in the present study: anti–α-smooth muscle actin, anti-β actin, and anti-Flag M2 antibodies from Sigma Chemical (St. Louis, USA); anti-embryonic smooth muscle (SM) myosin heavy chain (SMemb) antibody from the Yamasa Corporation (Chiba, Japan); anti-calponin antibody from DAKO Corporation (Carpinteria, USA); anti–phospho-Smad2 antibody from Upstate Biotechnology (Lake Placid, USA); anti-Smad2 antibody from Zymed Laboratories, Inc. (San Francisco, USA); and anti-CD11b antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Equal amounts of protein were electrophoresed on 5% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Tokyo, Japan). The same membrane was reprobed for the detection of different antibodies after treatment of the membrane with Restore Western blot stripping buffer (Pierce, Rockford, USA). In some instances, band intensities were scanned for quantitation using an image analyzer.

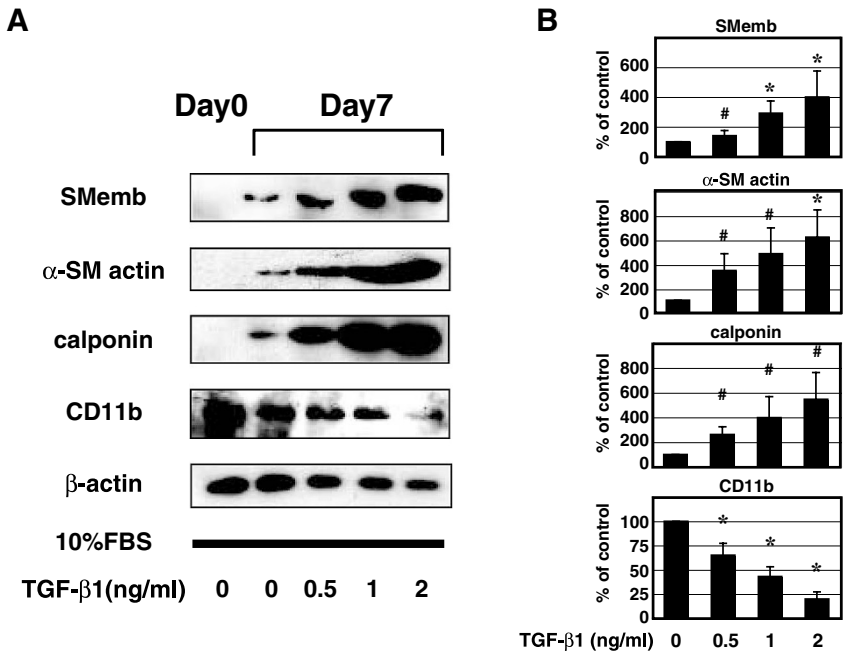

Fig. 3. *Dose effect of TGF-β1 on the expression of SMC markers. A: Macrophages were cultured in the presence of 10% FBS with TGF-β1 at the indicated doses for 7 days. The data in the left panel represent three independent experiments. B: In the right panel, the degree of expression of each protein marker was determined as the value relative to that of 10% FBS–treated cells. The values (the mean±SEM in three independent experiments) are expressed as percentages relative to the control.* $^{*}p < 0.01$ *and* $^{\#}p < 0.05$ vs. *the control by ANOVA with Bonferroni/Dunn test.*

Immunocytofluorescence

Macrophages were cultured on collagen type 1–coated culture slides (Becton Dickinson Labware, Bedford, USA). Cells were fixed with 4% paraformaldehyde. The first antibodies used in staining were mouse monoclonal Alexsa Fluor 488-conjugated anti-CD11b (Serotec Ltd., Oxford, UK), anti–α-SM actin (Sigma) and anti-calponin antibody (DAKO). Anti-CD11b antibody was used at 1:100 in a buffer consisting of 3% BSA in PBS. Anti–α-SM actin was used at 1:400. Anti-calponin antibody was used at 1:100. Biotinylated anti-mouse immunoglobulin (DAKO) was applied as a secondary antibody. Then Alexsa Fluor 647–conjugated, or fluorescein-conjugated streptavidin solution (DAKO) was applied to detect the signal. The cellular nucleus was stained with Propium Iodide (Molecular Probe, Leiden, Netherlands). The samples were examined by a high-resolution laser scanning confocal imaging system (LSM5 PASCAL; Carl Zeiss, Japan).

Statistical Analysis

Values are expressed as the mean±SEM. Comparisons between groups were performed using either Student's *t*-test or by analysis of variance (ANOVA) with Bonferroni/Dunn test. Values of $p<0.05$ were considered statistically significant. Analyses were performed with Stat View J 5.0 software (SAS Institute Inc., Cary, USA).

Results

Effect of TGF-β1 on the Expression of SMC Markers

Initially, we examined the surface antigens of monocytes/macrophages by immunohistochemical analyses. On day 1, almost all cells expressed CD11b antigen, which is known as a macrophage marker (Fig. 1A). After 7 days of incubation with TGF-β1 (1 ng/ml), the expression levels of both α-SM actin and calponin, which are known as SMC/myofibroblast markers, increased compared with those in control monocytes/macrophages that were maintained in 10% FBS (Fig. 1B).

Time Course of the Expression of SMC Markers in Response to TGF-β1

Next, we examined the effects of TGF-β1 (1 ng/ml) on cultures of monocytes/macrophages by Western blots. Incubation with TGF-β1 led to an increase in the level of SMemb, α-SM actin, and calponin (Fig. 2). We examined the expression of SMC markers on days 1, 4, and 7 in the presence and absence of TGF-β1. The expression of SMemb and α-SM

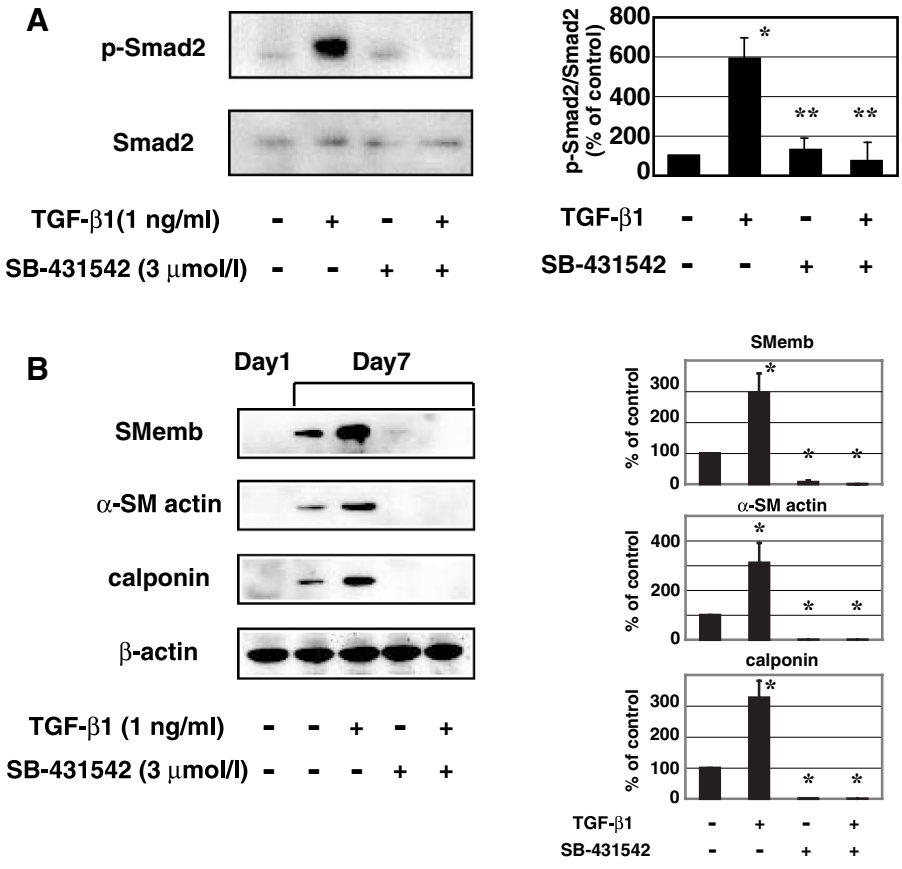

Fig. 4. *SB-431542 suppresses the expression of SMC markers. A: Rat peritoneal macrophages on day 2 were pretreated with 3 μmol/l SB-431542 for 1 h and then stimulated with TGF-β1 (1 ng/ml) for 30 min. Western blot analysis was performed with the anti–phospho-Smad2 antibody, and the same membrane was reprobed with anti-Smad2 antibody. Representative results are shown in the left panel. In the right panel, a quantitative analysis of the level of phosphorylated Smad2 relative to the level of total Smad2 is shown. Band intensities for phosphorylated Smad2 and total Smad2 were quantified by an image analyzer. The values (the mean±SEM, in three independent experiments) are expressed as percentages relative to those of the untreated cells.* *p < *0.01* vs. *the control;* **p < *0.01* vs. *TGF-β only, by ANOVA with Bonferroni/Dunn test. B: Rat peritoneal macrophages were cultured in the presence of 10% FBS with 3 μmol/l SB-431542 in the presence or absence of TGF-β1 (1 ng/ml) for 7 days. The expression of SMemb, α-SM actin, calponin, and β-actin was examined by Western blotting. Representative results are shown in the left panel. In the right panel, the values (the mean±SEM, in three independent experiments) are expressed as percentages relative to those of 10% FBS–stimulated cells.* *p < *0.01* vs. *the control by ANOVA with Bonferroni/Dunn test.*

actin was observed on day 4 in the absence of TGF-β1 (Fig. 2A). Cells incubated with TGF-β1 showed increased SMemb, α-SM actin, and calponin expression on day 7 (Fig. 2A). To determine the increases in the expression of these SMC markers relative to the expression in control cells (10% FBS), three independent experiments were performed, and the band intensities were quantified (Fig. 2B). The level of expression of the SMC markers began to increase at day 4, and a higher level of expression (approximately 3- to 5-fold) was detected at day 7 when TGF-β1 was included in the medium. In contrast, TGF-β1 decreased the expression of CD11b (Fig. 2).

Dose Effect of TGF-β1 on the Expression of SMC Markers

We also examined the dose-dependent effect of TGF-β1 on the expression of SMC markers. The TGF-β1–mediated increases of SMC markers (SMemb, α-SM actin, and calponin) were substantially induced at 0.5 ng/ml TGF-β1, reached a maximum at 1 ng/ml, and remained at this level at higher doses (2 ng/ml). To determine the relative increases in the expression of the SMC markers, we conducted three independent experiments and quantified the intensities of the bands on Western blots. As shown in Fig. 3B, the TGF-β1–induced expression of SMC markers relative to the control (10% FBS)

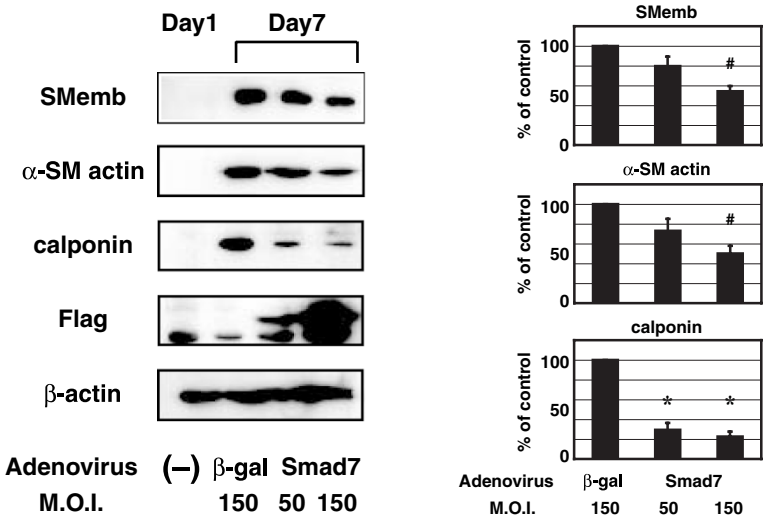

Fig. 5. *Adeno-Smad7 suppresses the expression of SMC markers. Macrophages were seeded on culture dishes; after 24 h, the cells were infected with the indicated M.O.I. of adenovirus carrying Adeno-β-gal or Flag-tagged Adeno-Smad7 for 12 h and were cultured with TGF-β1 (1 ng/ml) for 7 days. Representative results are shown in the left panel. Exogenous Smad7 expression was confirmed by staining with anti-Flag antibody. In the right panel, the values (the mean±SEM, in three independent experiments) are expressed as percentages relative to those of Adeno-β-gal–infected cells.* $^{*}p < 0.01$ *and* $^{\#}p < 0.05$ vs. *the control by ANOVA with Bonferroni/Dunn test.*

significantly increased by 4- to 6-fold with 2 ng/ml TGF-β1. On the other hand, the expression of CD11b decreased in response to TGF-β, and this effect was dose-dependent for 0.5–2 ng/ml TGF-β1 (Fig. 3).

Expression of SMC Markers Was Suppressed by SB-431542 and Adeno-Smad7

We examined the effect of SB-431542, a synthetic molecule that inhibits the kinase of the TGF-β receptor (*28–30*), on the expression of SMC markers in the presence and absence of TGF-β1. When 3 μmol/l of SB-431542 was added in the presence of TGF-β1, Smad2 phosphorylation decreased to approximately basal levels (Fig. 4A). Importantly, Smad2 phosphorylation was weakly detected in the absence of exogenous ligand (Fig. 4A), suggesting that endogenous TGF-β or activin acts on these cells. As we already showed, TGF-β1 (1 ng/ml) increases the expression of SMemb, α-SM actin, and calponin by 3- to 4-fold (Fig. 3B). The addition of 3 μmol/l SB-431542 completely reversed the effect of TGF-β1 (Fig. 4B).

Next, we used adenovirus vectors expressing the FLAG-tagged Adeno-Smad7 construct because Smad7 acts as an "inhibitory" Smad (*24*). In this system, monocytes/macrophages are transduced at a frequency >90%, permitting biochemical analyses of transdifferentiating cultures under a variety of conditions. To assess the usefulness of these reagents in this system, the transduction-dependent alterations in the expression of SMC markers were examined in the presence of TGF-β1 (1 ng/ml). The inclusion of TGF-β1 (1 ng/ml) in the basal medium led to increased expression of SMC markers at day 7 in the control cells transduced with Adeno-β-gal (Fig. 5). We did not observe any detectable differences in the cell morphology between mock- and Adeno-β-gal–infected cultures (unpublished data). Transduction with FLAG-tagged Adeno-Smad7 reduced the enhanced expression of SMC markers induced by TGF-β1, and these inhibitory effects were dose-dependent (Fig. 5). These data suggest that TGF-β signaling plays an important role in the expression of SMC markers during transdifferentiation.

Discussion

In this study, we showed that TGF-β signaling plays an important role during the transdifferentiation of monocytes/macrophages into SMC-like cells. Western blots data confirmed that the addition of TGF-β1 to the basal medium potently induced the expression of α-SM actin, SMemb, and calponin. Previous studies have shown that synovial tissues are capable of differentiating *in vitro* into myofibroblast-like cells containing α-SM actin and that this differentiation can be triggered by TGF-β or interleukin-4 (*31*). In fibroblasts, TGF-β–mediated fibroblast–myofibroblast terminal differentiation is differentially regulated by Smad proteins (*32*). Our results agree with those reports that TGF-β up-regulates α-SM actin expression.

An inflammatory environment seems to play an important role in the transdifferentiation of macrophages into SMC-like

cells. Several reports support this assertion. Earlier studies have shown that cultured macrophages from the buffy coat or peritoneal exudates transdifferentiate into SMC-like cells (*33*). In this report, we presented data showing that TGF-β1 enhances transdifferentiation, but there may be other cytokines that also stimulate transdifferentiation (*4*). For example, we examined the effect of interferon-γ, but found that the effect was comparable to that of 10% FBS (data not shown). Within the limits of our investigation, TGF-β1 had the strongest effect on macrophage-SMC–like cell transdifferentiation.

There may be a concern that the thioglycolate injected into the rat peritoneum might have carried some non-macrophage cells. In this study, we cannot completely exclude the possibility that contaminating non-macrophage cells, such as hemopoietic stem cells, might have served as progenitors of SMC-like cells. However, we observed that almost all cells expressed CD11b antigen on day 1, suggesting that the cells we treated were macrophages.

In the present study, TGF-β signaling was essential for the transdifferentiation of macrophages into SMC-like cells. The TGF-β inhibitor SB-431542 strongly suppressed the expression of SMC markers, and this inhibitory effect was confirmed by Smad2 phosphorylation. Interestingly, SB-431542 also facilitated the proliferation of macrophages. As Smad2 phosphorylation was weakly detected in the absence of TGF-β1, endogenous TGF-β may act on these cells to inhibit cell proliferation. The expression of Smad7, an inhibitor of Smad-dependent signaling, in the cells decreased the expression of SMC markers in a dose-dependent manner, suggesting that the TGF-β–Smad pathway is essential for transdifferentiation. However, TGF-β stimulates not only Smad signaling but also Smad-independent TGF-β responses (*34*, *35*). Given that the inhibitory effect of SB-431542 was much stronger than that of Smad7, Smad must be only a partial contributor to the transdifferentiation process, and other Smad-independent pathways may be involved.

Smad7 has also been shown to play an important role after balloon injury in an *in vivo* model. The overexpression of vascular Smad7 attenuated remodeling and the contribution of adventitial fibroblasts to neointimal formation after balloon angioplasty (*36*). Although it remains unclear whether Smad7 affects the transdifferentiation of macrophages into SMC-like cells *in vivo*, Smad7 is a hopeful novel therapeutic target for reducing vascular remodeling.

In the studies presented here, we examined the regulation of the expression of SMC markers by TGF-β1 in rat peritoneal macrophages. We demonstrated that TGF-β1 increases the expression of SMC markers, such as α-SM actin, calponin, and SMemb. Furthermore, we showed that the inhibition of TGF-β signaling by SB-431542 or Adeno-Smad7 reverses the stimulatory effect of TGF-β1 on the expression of SMC markers. Further elucidation of the pathways for the transdifferentiation of macrophages into SMC-like cells will not only illuminate an interesting area of developmental biology but also may reveal potential therapeutic targets for the treatment of vascular remodeling and post-angioplasty restenosis.

Acknowledgements

We thank Dr. Takeshi Imamura (Chief of the Department of Biochemistry, the JFCR Cancer Institute, Tokyo) and Dr. Kohei Miyazono (Professor of the Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo) for providing Adeno-Smad7.

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