Transforming Growth Factor-β–Induced Inhibition of Myogenesis Is Mediated Through Smad Pathway and Is Modulated by Microtubule Dynamic Stability

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Abstract—The expression of muscle-specific genes associated with myogenesis is controlled by several myogenic transcription factors, including myogenin and MEF2D. Transforming growth factor-β (TGF-β) has been shown to inhibit myogenesis, yet the molecular mechanisms underlying such inhibition are not known. In the present study, TGF-β was shown to inhibit myogenin and MEF2D expression and myotube formation in C2C12 myoblasts cultured in differentiation medium in a cell density–dependent manner. Transfection of C2C12 cells with Smad7, an antagonist for TGF-β/Smad signaling, restored the capacity of these cells to differentiate in the presence of TGF-β or when cultured in growth medium at low confluence, conditions that hinder muscle differentiation. Moreover, nocodazole, a microtubule-destabilizing agent, enhanced the inhibition of myogenesis exerted by TGF-β, an effect that could be restored by tubulin-polymerizing agent taxol, both of which have been shown to affect Smad-microtubule interaction and regulate TGF-β/Smad signaling. Our results indicate that TGF-β inhibits myogenesis, at least in part, via Smad pathway, and provide evidence that low-dose pharmacological agents taxol and nocodazole can be used as a means to modulate myogenesis without affecting cell survival. (Circ Res. 2004;94:617-625.)

Key Words: myogenesis ■ transforming growth factor-β ■ Smad ■ signal transduction ■ microtubules

The satellite cell compartment provides skeletal muscle with a remarkable capacity for regeneration. It was shown that myoblasts that grew out of this compartment in vitro improved left ventricular function when injected into an experimentally infarcted myocardium.1 The mechanisms leading to the functional improvement after myoblast transplantation, however, are still unknown. Our working hypothesis is that injected myoblasts engraft and differentiate in such a way that they contribute to force generation by the injured myocardium. Hence, the study of myoblast differentiation has become a central target of our investigations.

Skeletal muscle differentiation (myogenesis) involves a cascade of muscle-specific gene expression that is regulated by members of the myogenic basic helix-loop-helix (bHLH) and myocyte enhancer factor-2 (MEF2) families of transcription factors, which themselves are controlled by numerous environmental cues, including growth factors.2 Transforming growth factor-β (TGF-β) has been shown to block the expression of MyoD and myogenin, two members of the bHLH family, thereby preventing the expression of downstream muscle-specific transcripts and myogenesis in skeletal myoblast cell lines.3 The molecular mechanisms/signaling pathways that mediate the TGF-β–induced ant.myogenic effects, however, remain unknown.

TGF-β elicits cellular effects by activating receptor-regulated Smad2 and Smad3 that form complexes with common partner Smad4, which accumulate in the nucleus and regulate transcription of target genes. Inhibitory Smad7 interferes with the signaling activity of these positive Smads in multiple fashions, limiting both the amplitude and duration of TGF-β–induced transcriptional responses.4 We have previously demonstrated that endogenous Smads 2, 3, and 4 bind microtubules (MTs) in several cell lines, and such binding provides a negative regulatory mechanism to control TGF-β activity. Disruption of the MT network by chemical agents, such as nocodazole and colchicine, leads to ligand-independent Smad nuclear accumulation and transcription of TGF-β–responsive genes and increases TGF-β–induced Smad activity.5 In the present study, we found that overexpression of Smad7 completely abolished TGF-β–induced inhibition of myogenic differentiation, indicating that the inhibitory effect of TGF-β is mediated, at least in part, through a Smad pathway. We showed that 54 nmol/L nocodazole, a dose that had little, if any, effect on MT spindle formation and cellular proliferation,6 enhanced TGF-β–induced inhibition of myogenesis in C2C12 cells. Furthermore, this effect could be blocked by pretreatment with 5 nmol/L MT-stabilizing agent paclitaxel (taxol). Our results provide
evidence, for the first time, supporting a role of the Smad pathway in mediating TGF-β-induced inhibition of MEF2 and myogenin. Furthermore, this inhibitory process is modulated by MT dynamics. These findings lay the foundation for the potential use of low-dose taxol and nocodazole in modulating myogenesis, particularly in the context of cellular cardiomyoplasty, where differentiation of myoblasts and stem cells into force-generating mature muscle cells is critical to their repair capacity.

Materials and Methods

Cell Culture and Myogenic Differentiation

Mouse skeletal myoblast cell line C2C12 was obtained from American Type Culture Collection (Rockville, Md). The cells were maintained at subconfluent densities in growth medium (GM) consisting of DMEM containing 1g/L glucose supplemented with 10% fetal bovine serum (FBS), 0.5% chick embryo extract, and 1% penicillin/streptomycin. To induce terminal myogenic differentiation, cells were seeded in T75 flasks containing GM. After overnight incubation, cells were switched to differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum for further culture.

Smad7 Transfection

To generate stable transfectants, C2C12 cells were transfected with pcDNA3 plasmid containing human Smad7 cDNA tagged with the FLAG epitope under the control of cytomegalovirus (CMV) promoter and encoding neomycin resistance gene using FuGene 6 (Life Technologies). Transfected C2C12 cells were cultured in growth medium containing G418 (800 μg/mL). After 14 to 21 days, G418-resistant clones were isolated, expanded, and screened by Western blotting using antibodies against Smad7 and Flag, respectively. A total of 20 FLAG- and Smad7–expressing clones were isolated. Such stably transfected clones were maintained in GM medium containing 400 μg/mL of G418.

Immunoblotting

Cell extracts were separated by SDS-PAGE electrophoresis in nonreducing conditions. Proteins on the gel were transferred to a nitrocellulose membrane. For the detection of endogenous and exogenous Smad7 expression, the membrane was first probed with a rabbit polyclonal anti-Smad7 antibody (Santa Cruz Biotechnology, Inc) and was then stripped off and reprobed with a mouse monoclonal anti-FLAG antibody (M2, Sigma). For the detection of Smad2 activation, the membrane was probed with a rabbit anti-phospho-Smad2 antibody (Upstate Biotechnology) and was then stripped off and reprobed with Smad2 antibody. For the detection of myogenin, MEF2D, and Runx2, the membrane was first probed with a rabbit anti-myogenin antibody (Santa Cruz Biotechnology, Inc), and then was stripped off and reprobed with a mouse anti-MEF2D antibody (Transduction Laboratories) or a rabbit anti-Runx2 antibody (Alpha Diagnostic). Incubation with corresponding HRP-conjugated secondary antibodies was performed, followed by chemiluminescence (ECL) detection after washing.

Immunofluorescence Double and Triple Labeling

Cells fixed in 4%paraformaldehyde were permeabilized with 0.2% Triton X-100. Cells were incubated with a rabbit anti-myogenin antibody (Santa Cruz Biotechnology, Inc) or a mouse anti-MEF2D antibody (Transduction Laboratories) followed by incubation with FITC-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies, respectively. For triple labeling, cells were then incubated with a mouse anti-Flag antibody (Sigma). After washing, the cells were incubated with Texas Red-conjugated goat anti-mouse secondary antibody. Thereafter, cells were washed and incubated with Hoechst 3342 (Jackson Immunoresearch Laboratories) to stain the nucleus. Fluorescent microscopic visualization was performed after stringent washing.

Results

Expression of Myogenin and MEF2D Is Induced in Differentiation Medium and by Cell-to-Cell Contact

It has been demonstrated that switching from growth medium (GM, containing 10% FBS) to differentiation medium (DM, containing 2% horse serum) induces myogenic protein expression and myotube formation in cultured myoblasts. To reproduce the phenomenon in our experimental system and to study the role of cell density in myogenesis, we first examined myogenin and MEF2D expression by Western blot in C2C12 myoblasts before and after culture medium switch. As expected, switching culture medium from GM to DM resulted in a time-dependent induction of myogenin and MEF2D expression in cultured C2C12 cells. Maximum induction of myogenin and MEF2D proteins occurred at 48 and 72 hours after medium switch, respectively (Figure 1A). We then studied the effect of cell confluence on myotube formation in C2C12 myoblasts cultured in the absence of medium switch (GM). Expression of myogenin and MEF2D was induced in C2C12 myoblasts cultured in GM when the cell density reached ≥80% confluence, and the expression level of myogenin increased in a cell density–dependent manner (Figure 1B). Because cell-to-cell contact induces growth inhibition, it is likely that cell density–dependent induction of myogenin and MEF2D observed in C2C12 cells cultured in GM is mediated by cell cycle arrest.

TGF-β Inhibits C2C12 Myoblast Myogenic Differentiation in DM but not in GM

To examine the inhibitory effects of TGF-β on cell cycle withdrawal-induced myogenic differentiation, C2C12 myoblasts were initially cultured in GM, and then switched to DM for 48 hours in the presence or absence of 12.5 ng/mL TGF-β, and the expression of myogenin and MEF2D proteins in these cells was examined using Western blotting. As shown in Figure 1C, TGF-β exposure resulted in a marked decrease in myogenin expression in C2C12 cells (70% to 90% confluence); however, TGF-β treatment did not elicit detectable changes in the same conditions but at lower cell density (50% confluence). A similar cell density–dependent pattern of expression in response to TGF-β treatment was observed for MEF2D (data not shown).

To determine the time course by which TGF-β affects myogenin expression under myogenic differentiation conditions, we exposed C2C12 cells (80% confluence) already cultured in GM for 12 hours to TGF-β for various periods, myogenin expression was examined at 6, 12, 24, and 48 hours by Western blot analysis. The inhibitory effect on myogenin was not evident at 6 hours, but became apparent at 12 hours after TGF-β treatment. More pronounced inhibition of myogenin expression was observed at 24 and 48 hours, perhaps due to the combined effects of TGF-β–induced synthesis inhibition and protein turnover (Figure 1D).

We also examined the regulatory effects of TGF-β on cell contact-induced myogenic differentiation in C2C12 myoblasts cultured in GM. TGF-β treatment for 48 hours in
C2C12 cells cultured in normal serum and at 90% confluence did not suppress myogenin expression (Figure 1C), nor did it affect myogenin expression in these cells cultured in the same condition but with 50% confluence (data not shown). Collectively, these data support the notion that TGF-β/H9252 inhibits cell cycle withdrawal-induced myogenic differentiation in C2C12 myoblasts in a cell density- and time-dependent manner, but does not affect cell-to-cell contact-induced muscle differentiation of these cells cultured in the presence of optimal serum conditions.

Although evidence supporting the notion that TGF-β-induced inhibition of myogenesis is mediated by downregulating the levels of myogenic transcription factor expression is compelling, it was recently shown that TGF-β induced the translocation of MEF2 from nucleus to the cytoplasm in multiple myogenic cell types, making it unavailable for participation in an active transcriptional complex—a mechanism proposed to underlie the inhibition of transcriptional activation of downstream muscle-specific genes. To determine whether TGF-β might affect the cellular distributions of MEF2D and myogenin, in addition to their expression levels, in our experimental system, we performed immunofluorescence staining using C2C12 cells cultured in GM and DM in the presence or absence of TGF-β for 48 hours. Consistent with our Western blot data, the levels of myogenin and MEF2D were undetectable in C2C12 cells cultured in GM or in DM treated with TGF-β (data not shown). A predominant, if not exclusive, nuclear staining of these two factors was demonstrated in cells cultured in DM without TGF-β incubation. To establish an experimental system where MEF2D and myogenin were present at the time of TGF-β exposure to facilitate the study of their relocation, we exposed C2C12 cells already cultured in DM for 12 hours (and therefore expressed considerable levels of myogenin and MEF2D) to TGF-β for 30 minutes and 2 hours. Indeed, a portion of MEF2D, but not myogenin, was exported from the nucleus to the cytoplasm at 30 minutes on TGF-β exposure. A substantial amount of MEF2D, however, still remained in the nucleus (Figures 2A through 2F). At 2 hours after TGF-β treatment, most, if not all, MEF2D returned to the nucleus (Figures 2G through 2I). These results are consistent with many other reports, indicating that regulation of myogenic transcription factor expression plays a primary role in mediating TGF-β-induced myogenesis inhibition, whereas MEF2 relocalization represents a secondary and transient phenomenon that may contribute to TGF-β activity, but to a much less degree. Hence, in the following experiments, we focused our investigation on myogenin and MEF2D expression, rather than their translocation.

Smad7 Abrogates TGF-β Inhibition of Cell Differentiation

We wanted to determine whether TGF-β-induced inhibition of myogenic differentiation in C2C12 myoblasts cultured in...
DM was mediated via the Smad pathway. We reasoned that, if it were the case, the inhibitory effect of TGF-β on muscle differentiation and the formation of myotubes, would be reversed by Smad7 overexpression. To determine the effect of Smad7 (inhibitory Smad) on TGF-β–induced inhibition of myogenic differentiation, we established stably transfected C2C12 myoblasts overexpressing FLAG-tagged Smad7. C2C12 cells were transiently transfected with an expression vector containing full-length human Smad7 coding sequence under the CMV promoter (pFLAG-Smad7) at different doses (3, 6, and 12 μg/mL). Transient transfection resulted in a dose-dependent increase in Smad7 expression at 72 hours compared with cells transfected with the same vector containing no cDNA insert (Figure 3A). Cells were passaged and subjected to G418 selection at a concentration of 800 μg/mL for 2 to 3 weeks, 24 hours after transfection. G418-resistant clones were screened by immunoblotting with an anti-FLAG antibody and an anti-Smad7 antibody to examine the expression of transfected Smad7. In total, 20 G418-resistant clones were positive for both antibodies, and they were considered as Smad7 expressers. Examples of three positive clones are shown in Figure 3B. All of these clones were isolated, and clone 19 showing abundant overexpression of exogenous Smad7 was selected for detailed analysis as described later.

C2C12 cells stably transfected with Flag-tagged Smad7 or control cells were induced to undergo differentiation using medium switch in the presence or absence of TGF-β. As expected, myogenin and MEF2D expression was not detected in control C2C12 cells cultured in GM with <70% confluence. Forty-eight hours after medium switch, marked myogenin and MEF2D expression was induced, and such induction was inhibited by TGF-β (Figure 3C). In contrast, myogenin and MEF2D expression was detected in cells overexpressing Flag-tagged Smad7 even in GM at low density (40% to 60% confluence). When these cells were switched to DM, a noticeable increase in the expression of these two proteins was observed. Addition of TGF-β did not inhibit myogenin and MEF2D expression (Figure 3C).

Next, we examined the expression of MEF2D, myogenin, and Smad7 in C2C12 cells using immunofluorescence triple labeling. In control C2C12 cells, where Flag-tagged Smad7 was absent, nuclear staining of myogenin and MEF2D was only detected when these cells were cultured in DM, but not in GM or when treated with TGF-β (Figures 4A through 4L). In cells stably transfected with Flag-tagged Smad7, nuclear staining of myogenin and MEF2D were detected in the presence and absence of TGF-β, irrespective of culture media (Figures 4M through 4X). Taken together, these data demonstrate that Smad7 antagonizes the inhibitory effects exerted by TGF-β on the expression of myogenin and MEF2D in C2C12 myoblasts cultured in DM. The data also indicate that blockade of Smad pathway enhances myogenic protein expression in GM.

To confirm that myogenic gene expression correlated with morphological changes occurring during muscle differentiation, we examined myotube formation, a morphological marker for myoblast differentiation, in control and Smad7–transfected C2C12 cells. In control C2C12 cells, myotube formation was observed 3 days after such cells were transferred from GM to DM, a phenomenon that was abrogated by the addition of TGF-β to the medium (Figures 5A through 5C). In cells stably transfected with Smad7, multinucleated myotubes were detected even when the cells were cultured in GM, and myotube formation further increased 3 days after these cells were switched from GM to DM. In contrast to control C2C12 cells, the addition of TGF-β had no effect in myotube formation in the stably transfected cells (Figures 5D through 5F). These data demonstrate that Smad7 antagonizes TGF-β–induced inhibition of morphological changes associated with muscle differentiation in C2C12 cells switched to DM. Furthermore, consistent with myogenic protein expression, blockade of Smad pathway enhances myotube formation in C2C12 cells cultured in GM.
To provide additional evidence that TGF-β-induced inhibition of myogenic differentiation required the activation of Smad signaling, and to confirm that the ability of Smad7 to block the TGF-β effects in C2C12 myoblasts was through the suppression of Smad activation, we examined the phosphorylation state of Smad2 by immunoblotting analysis using an antibody that only recognizes phosphorylated Smad2. Smad2 was phosphorylated in control C2C12 cells cultured in both GM and DM after exposure to TGF-β. In the absence of TGF-β, phosphorylation of Smad2 was not detected in these control cells. In C2C12 cells stably transfected with Flag-tagged Smad7, no Smad2 phosphorylation was detected regardless of TGF-β treatment (Figure 5G). Smad7 transfection did not alter the level of Smad2 expression (Figure 5G). Together, these data indicate that the activation of Smad pathway is required, but not sufficient, for TGF-β to inhibit cell cycle withdrawal-induced myogenic differentiation. Additional factors, such as cell density, also play a role through a TGF-β-independent pathway.

Disruption of the MT Network Enhances TGF-β-Induced Inhibition of Myogenic Differentiation
Because MTs function as a negative regulator toward TGF-β/Smad signaling pathway, we reasoned that disruption of MTs with nocodazole might affect cell cycle withdrawal-induced myogenic differentiation in C2C12 myoblasts. As expected, nocodazole at 5 μg/mL—a dose we originally used to disrupt Smad/MT complex and activate Smad pathway—resulted in decreased myogenin expression in C2C12 Cells cultured in DM even in the absence of TGF-β. In the presence of TGF-β, myogenin expression was almost completely abolished (Figure 6A). It was previously shown that microtubule dynamics could be altered by as little as 54 nmol/L of nocodazole—a dose that had little effect on cell growth and MT spindle formation. Because high-dose nocodazole is associated with many nonspecific effects, affecting cell survival and rendering data interpretation complicated, we tested if the low-dose nocodazole would similarly induce the activation of Smad pathway and the inhibition of myogenic protein expression. Indeed, 54 nmol/L nocodazole resulted in Smad2 phosphorylation and inhibition of myogenin and MEF2D expression to a similar degree as 5 μg/mL of the agent (Figure 6B). Furthermore, low nocodazole exerted a similar synergistic effect with TGF-β to that observed with 5 μg/mL (Figure 6B). Hence, nocodazole-induced Smad2 phosphorylation and inhibition of myogenic differentiation can be achieved with a very low dose, indicating that MT-Smad interaction is very sensitive to the dynamic stability changes of MTs.

Taxol Blocks Nocodazole Enhancement of TGF-β Inhibition in Myogenic Differentiation
When the stability of MTs is not perturbed by nocodazole, we did not anticipate much effect of taxol, a MT stabilizing agent, on TGF-β–induced Smad activation and inhibition of C2C12 cell differentiation. As shown in Figures 6A and 6B,
addition of 1 μmol/L (high dose) or 5 nmol/L (low dose) taxol in C2C12 cells treated with and without TGF-β, had little effect on the phosphorylation of Smad2 and the expression of myogenin and MEF2D. In contrast, treatment of C2C12 cells with high- and low-dose taxol for 1 hour before exposure to nocodazole, alone or in combination with TGF-β (12.5 ng), for 18 hours, blocked high-dose and low-dose nocodazole-enhanced TGF-β activity (Smad activation and myogenic differentiation inhibition), respectively (Figures 6A and 6B). Furthermore, high-dose taxol was able to block the effects exerted by both high- and low-dose nocodazole (data not shown), whereas low-dose taxol failed to inhibit high-dose nocodazole–induced Smad2 phosphorylation and myogenesis inhibition (data not shown). These data suggest that C2C12 myoblasts with a stable MT network display a constant rate of TGF-β–induced Smad pathway activation and myogenic inhibition that is unaffected by taxol. When the MT network is destabilized by nocodazole or other environ-

Figure 4. Immunofluorescence examination of myogenin expression in relation to culture medium, TGF-β treatment, and Smad7 overexpression. All experiments were repeated 3 times to confirm reproducibility. Triple labeling of myogenin (green), human Smad7 (red), the nucleus (blue/white), and overlay of 2 colors (green and red) of nontransfected C2C12 myoblasts cultured in GM (A through D) and DM in the absence (E through H) or presence of TGF-β (I through L) demonstrates the induction of myogenin by DM and the inhibitory effect of TGF-β in such induction. In Smad7-transfected C2C12 cells, myogenin expression is present in cells cultured in GM (M through P), is increased in DM (Q through T), and is unaffected by TGF-β exposure (U through X). Scale bars=30 μm.

Figure 5. Myotube formation and Smad2 phosphorylation in C2C12 myoblasts in relation to culture medium, TGF-β treatment, and Smad7 overexpression. Myotube formation is rare in C2C12 cells cultured in GM (A) and is induced in DM (B), which is inhibited by TGF-β treatment (C). Cells from Smad7-transfected clone 19 show extensive myotube formation even when cultured in GM (D), and such myotube formation is further increased when switched to DM (E), a phenomenon that cannot be inhibited by TGF-β (F). Smad phosphorylation is detected in cells treated with TGF-β regardless of culture media (G).
Discussion

The development of skeletal muscle is a multistep process in which pluripotent mesodermal cells give rise to myoblasts that subsequently withdraw from the cell cycle and differentiate into myotubes. These stages are controlled by the MyoD and myocyte enhancer factor 2 (MEF2) families of transcription factors, which interact with one another to establish a unique transcriptional code for activation of skeletal muscle-specific genes. It has been shown that such muscle differentiation–specific gene expression occurs in a stereotypical pattern. Within 24 hours of switching to differentiation medium or serum withdrawal, proliferating myoblasts initiate the expression of myogenin, followed by the expression of MEF2 family of transcription factors, including MEF2D. Consistent with these observations, we showed in the present study that cell cycle withdrawal induced myogenin and MEF2D protein expression, peaking at 48 and 72 hours after medium switch, respectively. Furthermore, we demonstrated, for the first time, that the expression of myogenin and MEF2D transcription factors and myotube formation were induced in C2C12 myoblasts cultured in normal serum but with high cell density, indicating that cell-to-cell contact serves another trigger for muscle differentiation, in addition to cell cycle exit. Considering that cell-to-cell contact can induce growth inhibition, these two triggers may work through the same pathway.

Multiple growth factors and cytokines have been shown to regulate the expression of myogenic factors and influence myogenesis. Indeed, at least two models have been proposed for the generation of different fates from multipotent stem cells, both of which are based on the regulation of expression levels of critical transcription factors. According to the divergence model, a lineage determination gene, such as myogenin or Runx2, which is specific for myogenic and osteogenic differentiation, respectively, is induced in uncommitted multipotent stem cells and converts them to the corresponding committed monopotent progenitor cells or mature cells. In the transdetermination model, the determination gene expressed initially is downregulated and another

Figure 6. Alterations of microtubule dynamics affect myogenin, MEF2D and Runx2 expression, and Smad2 phosphorylation. Treatment of C2C12 cells with 5 μg/mL nocodazole and 1 μmol/L of taxol affects myogenin expression (A, high dose). A similar effect on myogenin, MEF2D, Smad2 phosphorylation, and Runx2 is observed with 45 nmol/L nocodazole and 5 nmol/L taxol (B, low dose). Nocodazole and taxol treatment in Smad7-transfected C2C12 cells fails to elicit any effects on myogenin expression (C). A β-actin blot, serving as control for equal loading, is presented in each of the above 3 experimental conditions. All experiments were repeated 2 times to confirm reproducibility.

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TGF-β—suggesting that TGF-β-mediated by Smad proteins, but evidence has emerged that myogenic differentiation of C2C12 cells is independent of Smad signaling. Indeed, TGF-β-induced activation of p38 MAP kinase, but not Smads, is required for TGF-β-induced apoptosis, and epithelial-to-mesenchymal transition in mouse mammary epithelial (NMuMG) cells. We demonstrated that overexpression of Smad7 blocked TGF-β-induced inhibition of myogenin and MEF2D expression and myogenic differentiation in C2C12 myoblasts. Furthermore, we provided evidence that exogenous Smad7 inhibited Smad2 phosphorylation, a critical step for the propagation of TGF-β signaling from the plasma membrane to nucleus. These observations represent the first evidence indicating that the TGF-β-induced inhibitory effect on myogenesis is, at least in part, mediated through the Smad pathway. Interestingly, Smad7-overexpressing C2C12 myoblasts displayed an overall increased propensity for muscle differentiation, as evidenced by the expression of myogenin and MEF2D and myotube formation even in cells cultured in GM with low cell density, suggesting that endogenous TGF-β/Smad signaling may prevent the commitment of myoblasts to terminal differentiation. Alternatively, Smad7 may stimulate muscle differentiation through mechanisms independent of Smad2 phosphorylation. The undetectable Smad2 phosphorylation in the cells cultured in GM at low density seems to support the latter possibility. Nonetheless, our data support the notion that exogenous Smad7 expression may be used as a means to promote skeletal muscle differentiation.

We have previously demonstrated that microtubules serve as a negative regulator for TGF-β/Smad signaling by forming a complex with endogenous Smad2, Smad3, and Smad4, sequestering the receptor-regulated Smads away from the TGF-β receptor. Destabilization of the MT network by nocodazole disrupts the complex between Smads and MTs and increases TGF-β-induced Smad2 phosphorylation and transcriptional response. In the present study, we found that 54 nmol/L nocodazole, 1/300 of the dose (16.7 μmol/L) used to induce TGF-β signaling in our previous study, decreased myogenin and MEF2D expression in C2C12 cells even in the absence of TGF-β. In the presence of TGF-β, nocodazole treatment almost completely abolished myogenin and MEF2D expression. Preincubation of cells with taxol at 5 nmol/L, 1/200 of the original dose (1 μmol/L) used for our TGF-β signaling study, abrogated nocodazole-induced myogenesis inhibition in the absence of TGF-β, and the synergistic enhancement of such inhibition in the presence of TGF-β. Furthermore, 1 μmol/L (high dose) taxol was able to block the effects exerted by both high- and low-dose nocodazole, whereas 5 nmol/L (low dose) taxol only inhibited 54 nmol/L (low-dose), but not 5 μg/mL (high-dose), nocodazole-induced myogenesis inhibition, indicating that MT dynamic stability and TGF-β/Smad signaling is modulated by the relative balance between MT stabilizing and destabilizing agents. Instructively, these effects are associated with concordant changes in the phosphorylation state of Smad2. Because it was shown that 54 nmol/L nocodazole had little effect on microtubule spindle formation, our data thus indicate that the binding between Smads and MTs is sensitive to changes in the dynamic stability of MTs at levels below the threshold necessary to induce tubulin depolymerization with resultant mitotic block, providing a new level of understanding regarding the role of Smad-MT interaction in TGF-β signaling, particularly in regulating muscle differentiation. Importantly, these data suggest that MT interacting agents may be used to modulate muscle differentiation at low doses without significantly affecting cell survival. Autologous cell transplantation for the treatment of damaged myocardium after myocardial infarction is be-
coming an increasingly promising strategy. Over the past decade, multiple cell types, including myoblasts (satellite cells originated from skeletal muscle), fetal cardiomyocytes, and bone marrow stem cells have been used in animal studies, and clinical trials to determine the safety of myoblast injection into damaged myocardium are presently being conducted. A key event that affects the efficacy of myoblast transplantation is the efficient differentiation of locally injected cells into force-generating myocardial/muscle cells. Novel findings presented in this study, characterizing the molecular pathway of, and the regulatory effects of microtubules on, TGF-β-induced myoblast differentiation, should have a significant impact on the emerging field of cell transplantation in the treatment of heart disease.

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