Myostatin is an inhibitor of myogenic differentiation

RAMÓN RÍOS, ISABEL CARNEIRO, VÍCTOR M. ARCE, AND JESÚS DEVESA
Departamento de Fisiología, Facultad de Medicina, Universidad de Santiago de Compostela, San Francisco s/n, 15705 Santiago de Compostela, Spain

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During mammalian development, skeletal muscle cells arise from pluripotential mesenchymal precursors that become committed to the myogenic lineage upon expression of the muscle-specific basic helix-loop-helix (bHLH) transcription factors myoD and myf5. In response to differentiation signals, myogenin and muscle regulatory factor 4, also belonging to the bHLH family, execute the differentiation program that leads to the expression of muscle-specific proteins and myocyte fusion into multinucleated myotubes (18, 23). During this process, more cells have to be constantly generated to keep pace with embryonic growth, so muscle growth results from a balance between proliferation of precursor cells and their subsequent differentiation into muscle fibers. This process is tightly regulated in vivo through mechanisms that involve cell-cell and cell-matrix interactions, as well as extracellular secreted factors. Among the latter, several members of the transforming growth factor (TGF)-β superfamily of growth and differentiation factors have been shown to be potent regulators of muscle growth (1).

One of the TGF-β superfamily members that plays an essential role in regulating skeletal muscle growth is myostatin (MSTN) (15). During skeletal muscle development, MSTN expression is restricted initially to the myotome compartment of developing somites and continues to be limited to the myogenic lineage at later stages of development and in adult animals. Several murine (15, 27, 33) and bovine (4–6, 17) genetic models have clearly established the role of MSTN as a negative regulator of muscle fiber number. A reduction in muscle fiber number can result from either a decrease in myoblast proliferation or a delay in myoblast differentiation. The ability of MSTN to inhibit myoblast progression through the cell cycle has been recently demonstrated. Recombinant MSTN has been shown to reversibly inhibit C2C12 murine myoblast proliferation by arresting cells in the G1 and G2/M stages of the cell cycle (29). This arrest is probably mediated by the upregulation of the cyclin-dependent kinase (cdk) inhibitor p21cip1. In keeping with these findings, Taylor et al. (28) showed that MSTN inhibited proliferation, [3H]thymidine incorporation, and protein synthesis in C2C12 cells. Using a different approach, we have shown that transient transfection of C2C12 myoblasts with an expression vector encoding mouse MSTN cDNA not only inhibited cell proliferation but also reduced differentiation-associated cell death (25), probably by a mechanism involving also the upregulation of p21cip1, which has been previously shown to dramatically decrease the apoptotic rate of differentiating myoblasts (31).

Although MSTN expression has been reported to correlate with differentiation in several chicken muscles (10) and in C2C12 myoblasts (25), it is currently unknown whether MSTN plays any role in the regulation of the myogenic process. Moreover, such a role has been demonstrated for several other members of the TGF-β superfamily, such as TGF-β1 itself (14), activin (12), and bone morphogenetic protein (BMP)-2 (7), which have been shown to inhibit the differentiation of C2C12 myoblasts. An inhibitory effect on muscle development in vivo has been suggested for BMP-4, a close BMP-2 homolog (24). In all cases, the underlying mechanism explaining the inhibition of the myogenic program by TGF-β superfamily members involves the downregulation of the myogenic bHLH transcription factors myoD and myogenin.

In this report we show that the stable transfection of MSTN cDNA in C2C12 cells efficiently inhibits the
formation of multinucleated myotubes, reduces the mRNA levels of myoD and myogenin, and inhibits the activity of the myoD and myogenin downstream target, creatine kinase (CK). Therefore, we propose that MSTN negatively regulates muscle mass not only by decreasing the proliferation rate of myoblasts but also by inhibiting its terminal differentiation.

MATERIALS AND METHODS

Generation of stable clones of C2C12 cells expressing MSTN cDNA. The cloning of murine MSTN cDNA into the pBlue-script KS+ vector (Stratagene, San Diego, CA) has been previously described (25). The cDNA was further subcloned into the expression vector pcDNA 3.1 Zeo (Invitrogen, Barcelona, Spain). The generated construct was named pcDNA-MSTN.

The mouse myoblast C2C12 cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO2 atmosphere. All cell culture reagents were purchased from Life Technologies (Invitrogen). Cells were transfected by means of the Lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol with 1 μg of either the pcDNA-MSTN plasmid or the control plasmid (pcDNA 3.1 Zeo alone). Clones were selected in medium supplemented with 250 μg/ml Zeocin (Invitrogen). MSTN overexpression was assessed by RT-PCR (25). To verify whether MSTN was being processed and secreted properly, conditioned media from control and MSTN cDNA transfected clones were separated by 15% SDS-PAGE under reducing conditions and transferred onto a nitrocellulose membrane by electroblotting. The membrane was then blocked overnight at 4°C in Tris-buffered saline (TBS) buffer with 0.1% Tween 20 and 0.2% casein and then incubated for 1 h at room temperature with an anti-MSTN antibody (1:500) raised against the COOH terminus of MSTN (C-20 antibody; Santa Cruz Biotechnology, Heidelberg, Germany). The membrane was washed with TBS-Tween 0.1% and further incubated with protein A-horseradish peroxidase (HRP) conjugate (Amersham Pharmacia Biotech, Freiburg, Germany) at 1:1,000 dilution for 1 h at room temperature. After washing, HRP activity was detected by using the enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech).

Cell proliferation and apoptosis assays. For the proliferation assay, 4 × 10^4 cells were seeded in triplicate in 35-mm diameter dishes. Cells were cultured in DMEM supplemented with 10% FBS. At 24, 48, and 72 h, cells were washed with phosphate-buffered saline buffer (PBS), harvested after a 5-min incubation with 0.25% trypsin (Invitrogen), and counted on a Neubauer chamber.

For the quantification of apoptosis, 6.5 × 10^4 cells were seeded in 15-mm dishes (n = 6). Cells were incubated for 24 h in DMEM containing 10% FBS and then changed to 1% FBS. After an additional period of 72 h, cells were stained with 50 nM Hoechst 33258 (Sigma, St. Louis, MO). Three random fields of each of the six replicates were photographed at a ×40 magnification with a DP10 microscope digital camera (Olympus Optical, Tokyo, Japan). Hoechst-positive condensed nuclei were counted in each of the fields. A parallel experiment was performed to assay apoptosis with the Cell Death Detection ELISA (Roche Molecular Biochemicals, Mannheim, Germany), following manufacturer’s instructions. The assay is based on the quantitative determination of oligonucleosomes released into the cytoplasm of apoptotic cells with monoclonal antibodies directed against DNA and histones.

RT-PCR analysis. The effects of MSTN cDNA overexpression on the expression of myoD and myogenin were assayed by RT-PCR. Cells (2 × 10^6) were seeded in 60-mm plates. After a 24-h incubation in 10% FBS-DMEM, differentiation was induced by shifting the medium to 1% FBS-DMEM. To test whether the effect of MSTN was reversible, we incubated cells in the presence of the antibody raised against the COOH-terminal region of MSTN (C-20; Santa Cruz Biotechnology). An antibody directed against the pro-region of MSTN (N-19; Santa Cruz Biotechnology) was used as a control. Cells were harvested at the indicated times, and total RNA was extracted by means of the Trizol reagent (Invitrogen). Total RNA (1 μg) was reverse transcribed for 1 h at 37°C with 200 units of MMLV reverse transcriptase (Invitrogen), followed by 5 min at 95°C, in a 30-μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 5.5 mM MgCl2, 0.5 mM each dNTP, 40 units of RNaseOUT recombinant ribonuclease inhibitor (Invitrogen), and 1.7 μg/μl random primers (Invitrogen). Three microliters of the RT reaction were amplified by PCR with 1.25 units of Taq polymerase (Invitrogen) in fifty microliters of a reaction mixture containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM MgCl2, 0.2 mM each dNTP, and 0.4 μM each oligonucleotide primer. The housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as a load control. The oligonucleotide sequences (with product length and GenBank accession nos. for murine sequences) were as follows: HPRT (139 bp; NM013556), upper 5'-AGTCCCCAGCTGTGATTA-3', lower 5'-AGCAAGTCTTCTGATCCTGT-3'; myoD (528 bp; M84918), upper 5'-GATTGGAATGAGGGATACAC-3', lower 5'-GACATATGCTTTTCTTGGG-3'; myogenin (424 bp; M98500), upper 5'-GCTACGCTCCCCATACCAG-3', lower 5'-ATGTAATGGAGTGGGA-3'. All the oligonucleotide primer pairs were designed to amplify a region including at least one intron (assuming conservation of exon-intron junctions between murine myoD and myogenin) so that amplimers arising from genomic DNA contamination could be easily distinguished from those originated from genuine cDNA amplification. The conditions of the PCR reactions were 28 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min, followed by a final amplification step of 72°C for 10 min. The PCR products were resolved on 2% agarose gels stained with ethidium bromide.

CK activity. Cells (3.5 × 10^5) were seeded in triplicate in 35-mm plates, incubated for 24 h in 10% FBS-DMEM, and then shifted to medium containing 1% FBS to induce its differentiation. The cells were harvested by trypsinization at the indicated times, washed with PBS, centrifuged, and stored as a pellet at −20°C until the assay was performed. Briefly, the cells were sonicated in saline buffer, and CK activity was measured by a modification of the spectrophotometric method described by Olivier (22) and Rosalki (26) in a Dimension Clinical Chemistry System (Dade Behring, Newark, NJ). The amount of total protein in the samples was determined with the pyrogallol red/molybdenum method, described by Fujita et al. (2).

Statistical analysis. A Mann-Whitney test was used to evaluate the statistical significance of the data.

RESULTS

Transfected C2C12 cells overexpress a biologically active MSTN. To investigate the autocrine effects of MSTN on the myogenic process in vitro, we generated stable clones of C2C12 cells overexpressing murine MSTN cDNA. Stable transfectants were tested for MSTN overexpression by RT-PCR analysis of total
RNA. As shown in Fig. 1A, the amount of MSTN mRNA is considerably higher in cells transfected with the pcDNA-MSTN plasmid than in control cells. According to our previously published results (25), only a faint band can be detected in cells transfected with the pcDNA 3.1 Zeo control plasmid. Moreover, Western blot analysis of the conditioned medium of transfected cells showed a band migrating at the predicted size (12 kDa) for the monomeric processed form of MSTN (Fig. 1B), thus demonstrating that C2C12 cells are capable of secreting and proteolytically processing MSTN. Finally, to further validate our model we determined whether the MSTN overexpressed in C2C12 cells was biologically active by investigating its effects on cell proliferation and cell survival. Whereas proliferation was inhibited (Fig. 1C), survival was enhanced (Fig. 1, D and E) in MSTN-transfected C2C12 cells. Because these effects have been previously attributed to MSTN (25, 28, 29), it is likely that they are produced by the overexpressed peptide. Furthermore, the effect of MSTN on the proliferation rate of the myoblasts is also consistent with the fact that double-muscled cattle, where the phenotype for disruption of the \textit{mstn} gene was first observed, present increased muscle fiber number.

**Overexpression of MSTN in C2C12 cells inhibits myogenic differentiation.** The ability of MSTN to inhibit myoblast differentiation is shown in Fig. 2. Incubation of C2C12 cells transfected with the control plasmid for 5 days in differentiation medium resulted in their fusion into postmitotic syncytial myotubes. In contrast, when C2C12 cells transfected with the pcDNA-MSTN plasmid were incubated in differentiation medium, they remained as mononuclear cells even after 5 days (Fig. 2B). The ability of MSTN to inhibit myoblast differentiation is shown in Fig. 2. Incubation of C2C12 cells transfected with the control plasmid for 5 days in differentiation medium resulted in their fusion into postmitotic syncytial myotubes. In contrast, when C2C12 cells transfected with the pcDNA-MSTN plasmid were incubated in differentiation medium, they remained as mononuclear cells even after 5 days (Fig. 2B).
mid were incubated under the same conditions, the formation of multinucleated myotubes was suppressed.

The effect of MSTN overexpression on myoblast differentiation was further investigated by assessing its effects on two early markers of myogenic differentiation: the bHLH family transcription factors myoD and myogenin. Myoblasts were induced to differentiate by lowering mitogen concentration in media from 10% to 1%. RT-PCRs of different cycle number were performed to ensure that all the assays were in the linear range of amplification (Fig. 3A). As Fig. 3B clearly indicates, MSTN cDNA overexpression decreased the mRNA levels of both transcription factors at the two time points tested. Note that when the medium was shifted to DMEM containing 1% FBS (differentiation medium), the cells had been incubated in growth medium (DMEM + 10% FBS) for 24 h, so MSTN could also exert its effect at time 0. As has been previously reported, myoD mRNA levels do not change with the induction of differentiation, whereas myogenin mRNA

Fig. 2. Overexpression of MSTN inhibits myotube formation in C2C12 cells. Representative microphotographs show C2 1.7 and C2 2.8 clones incubated for 5 days in differentiation medium. Lens magnification, ×100.

Fig. 3. Overexpression of MSTN inhibits myoD and myogenin gene expression in C2C12 cells. A: amplification plots for HPRT, myoD, and myogenin. Samples were amplified for 20, 25, 30, 35, or 40 cycles. At 28 cycles, all the PCRs were within the linear range of amplification. B: RT-PCR analysis of the effects of MSTN cDNA overexpression on the amount of myoD and myogenin transcripts. Cells (2 × 10⁶) were seeded in 60-mm plates, and after a 24-h incubation in 10% FBS-DMEM, differentiation was induced by shifting the medium to 1% FBS-DMEM. Cells were harvested at the indicated times, and RT-PCR (28 cycles) was performed with 1 μg of total RNA. The housekeeping gene HPRT was used as a control. Note that the MSTN effect at time 0 is due to its action during the 24-h incubation in 10% FBS-DMEM. C: effect of MSTN was counteracted in 2 additional clones by incubating the cells with an antibody against the COOH-terminal region of MSTN (anti C-MSTN). The antibody was added after 24 h of incubation in 1% FBS-DMEM, and cells were harvested 24 h later. An antibody raised against the NH2-terminal propeptide (anti N-MSTN) was used as a control. RT-PCR was performed as described in B.
levels increase as differentiation takes place. To test whether the effect of MSTN overexpression was reversible, two additional clones of C2C12 cells transfected either with the empty vector (1.8) or myostatin cDNA (2.5) were incubated for 24 h in low-serum medium and then treated with an antibody directed against the COOH-terminal myostatin peptide. After an additional 24-h incubation, the expression of myoD and myogenin was assessed by RT-PCR. As shown in Fig. 3C, the inhibition of overexpressed myostatin with the antibody against the COOH-terminal region almost completely reversed its negative regulatory effects on myoD and myogenin transcription. In contrast, when cells were treated with an antibody against the NH2-terminal region of myostatin (this region is not contained in the mature processed form of the protein), the inhibition of the differentiation process was not reverted.

The inhibitory action of MSTN cDNA overexpression on C2C12 cell differentiation was also evaluated by investigating its effect on the activity of the muscle-specific gene CK. Myoblast differentiation was induced as explained above, and CK activity was determined. As Fig. 4 shows, MSTN cDNA overexpression resulted in decreased CK activity at all time points studied.

DISCUSSION

To our knowledge, this is the first report in the literature demonstrating the inhibitory effect of MSTN on myogenesis. In the same direction, Oldham et al. (21) recently reported that myoD expression is increased in double-muscled cattle fetuses. This increase is probably caused by the lack of an active biological form of MSTN in these animals, thus suggesting a role for MSTN in the negative regulation of the differentiation process in vivo. Surprisingly, no upregulation of myogenin was found in this case. The reason for this discrepancy is unknown.

Two different ways of inhibiting the myogenic process have been described. One is the use of mitogens, which tend to delay myogenic differentiation by retaining the cells in a proliferative state (19). On the other hand, the factors belonging to the TGF-β superfamily, along with MSTN, inhibit both proliferation and differentiation (14). However, some striking differences exist among members of the superfamily. Although both TGF-β and activin, on one side, and BMP-2 and related factors, on the other, inhibit myotube formation in C2C12 myoblasts, the latter can also induce this cell line to express osteoblast phenotypes, such as alkaline phosphatase (ALP) activity (7). According to our own unpublished observations, MSTN is unable to induce ALP activity in C2C12 cells, not a surprising finding considering the phenotype of mstn-null mice, which show no defects in osteogenesis (15). Differences also exist between TGF-β/activin and BMP with regard to the regulation of adipogenic differentiation. MSTN has also been reported to be expressed in adipose tissue (15) and, along with TGF-β1 and TGF-β2, strongly inhibited adipogenesis (9, 30). In contrast, both BMP-2 and BMP-4 have been shown to induce adipogenic conversion in the pluripotential mesenchymal cell line 10T1/2 at lower concentrations than those needed for transdifferentiation into osteoblasts (30). Again, these results indicate that the effect of MSTN is similar to that of TGF-β/activin and different from that of BMPs.

The major difference in signaling between TGF-β/activin and BMP occurs at the level of the receptor-regulated Smad (R-Smad) that is activated by type I receptors before forming a complex with the common Smad4 and translocating to the nucleus, where they regulate the transcription of target genes. R-Smads 2 and 3 transduce TGF-β and activin signals, whereas
BMP signaling uses R-Smads 1, 5, and 8 (reviewed in Ref. 13). This differential activation of R-Smads seems to mediate the differential effects of both pathways on myogenic differentiation, since forced expression of R-Smads 1, 5, and 8 or R-Smads 2 and 3 mimics the effects of BMPs and TGF-β/activin, respectively, on C2C12 cells (8, 32). Although MSTN signaling pathway has not been elucidated at the biochemical level, several lines of evidence, apart from their common effects on myogenesis and adipogenesis, suggest that MSTN may share TGF-β/activin signaling pathway. First, MSTN clusters with TGF-βs and activins in a phylogenetic tree of all known TGF-β superfamily members (20). Second, it has been recently demonstrated that MSTN is able to bind activin receptor type IIb (ActRIIb) and that transgenic mice overexpressing a dominant negative form of ActRIIb under the control of a muscle-specific promoter exhibit a dramatic increase in muscle mass (11), similar to that of mice lacking MSTN (15). In keeping with this hypothesis, a high degree of similarity also exists between the phenotypes of mice either lacking ActRIIb or growth and differentiation factor (GDF)-11, thus suggesting that GDF-11 and activin may as well share a common receptor and signaling pathway (16). GDF-11 has a 90% identity with MSTN in its COOH-terminal region, and it has also been shown to inhibit myogenesis in the developing chick limb by a mechanism involving downregulation of myod expression (3). A major difference exists between MSTN and the rest of the TGF-β superfamily factors with regard to its restricted expression pattern during development. Whereas MSTN expression is confined to developing skeletal muscles, other members of the TGF-β superfamily that negatively regulate myogenesis are expressed in neighboring tissues. This type of communication, characteristic of BMPs and GDF-11, seems to be involved in the establishment of boundaries between adjacent territories. For instance, GDF-11, which inhibits avian limb muscle differentiation, is expressed in nonmuscle progenitor mesenchymal cells from the progress zone, which will later differentiate into skeletal structures (3). Similarly, BMP-4 signals emanate from the dorsal ectoderm, the neural tube, and the lateral plate mesoderm, and its negative effect on myod expression is counterbalanced in the dorso-medial lip of the dermatomyotome (where muscle progenitors first initiate the expression of myogenic bHLH transcription factors) by noggin (24). Considering the differences stated above, together with our findings, it is likely that MSTN regulates myogenic differentiation via an autocrine/paracrine mechanism. We therefore propose a model, depicted in Fig. 5, in which MSTN expressed in the course of myogenic differentiation regulates the deposit of muscle mass during development by inhibiting both the proliferation of myoblasts and their differentiation into multinucleated myotubes.

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REFERENCES


