Myostatin inhibits cell proliferation and protein synthesis in C2C12 muscle cells

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Taylor, Wayne E., Shalender Bhasin, Jorge Artaza, Frances Byhower, Mohd Azam, Darril H. Willard, Jr., Frederick C. Kull, Jr., and Nestor Gonzalez-Cadavid. Myostatin inhibits cell proliferation and protein synthesis in C2C12 muscle cells. Am J Physiol Endocrinol Metab 280: E221–E228, 2001.—Myostatin mutations in mice and cattle are associated with increased muscularity, suggesting that myostatin is a negative regulator of skeletal muscle mass. To test this hypothesis, we examined the effects of recombinant myostatin in mouse skeletal muscle C2C12 cells. Growth of these cells was inhibited by recombinant myostatin protein. These effects were dose dependent, and the inhibitory effects of both myostatin proteins were greater in C2C12 myotubes than in myoblasts. Neither protein had any significant effects on protein degradation or apoptosis. In conclusion, recombinant myostatin proteins inhibit cell proliferation, DNA synthesis, and protein synthesis in C2C12 cells, suggesting that myostatin may control muscle mass by inhibiting muscle growth or regeneration.

myoblast; myotube; growth differentiation factor 8; sarcopenia; growth differentiation factor

MYOSTATIN, a member of the transforming growth factor-β (TGF-β) superfamily, is a novel regulator of skeletal muscle growth (9, 19, 20). Inactivating mutations of the myostatin gene in cattle (4, 10, 13, 20) and mice (24) are associated with skeletal muscle hypertrophy. Similarly, mice made null for the myostatin gene by homologous recombination (19), or transgenic mice carrying dominant negative mutations of the myostatin gene, have increased muscle mass (28). The circulating concentrations of myostatin protein, measured by an immunoassay, are higher in patients with sarcopenia associated with acquired immunodeficiency (HIV) syndrome than in healthy young men (9). Furthermore, myostatin expression is increased in the atrophied muscles of rats exposed to hindlimb suspension or microgravity (3, 17, 26). Collectively, these data led us to hypothesize that the product of the myostatin gene might be an inhibitor of skeletal muscle growth in adult animals and might contribute to the multifactorial pathophysiology of sarcopenia associated with chronic illness (16, 22) and aging (2). However, the effects of myostatin protein on skeletal muscle growth have not been directly studied.

Because muscle mass represents the balance between muscle cell replication and protein synthesis and muscle protein breakdown and cell death (5), we considered the possibility that myostatin inhibits muscle growth by affecting one or more of these processes. We tested this hypothesis by expressing recombinant human full-length, 375-amino acid (aa) myostatin protein and determining its effects on cell proliferation, DNA and protein synthesis, protein degradation, and apoptosis in skeletal muscle cells. Because the 110-aa carboxy-terminal portion of the myostatin protein was proposed (19) to be the mature form of this protein, we also tested the effects of this recombinant protein. We used the C2C12 skeletal muscle cell line as the in vitro bioassay system, because this model has been used extensively in previous studies of the effects of muscle growth factors on muscle protein synthesis and degradation, cell replication, and apoptosis (6, 21, 23).

MATERIALS AND METHODS

Preparation of myostatin cDNA constructs in mammalian expression vectors. A human myostatin cDNA plasmid, pPCR-Mst(5a), was cloned and sequenced as described (9). The nucleotide sequence of this 2-kb Mst cDNA is identical to the published sequence (9, 19) except for two base changes at the 5′-end (A to G at positions 248 and 407, causing conser-
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Preparation of recombinant, full-length, 375-aa myostatin protein. Plasmids pDES-Mst along with pCoHygro were transfected by CaPO₄ into Drosophila S2 cells (15) (Schneider cells, Invitrogen), and stable transfectants were selected by growth for 6 wk in medium containing 300-μg/ml hygromycin B (GIBCO Life Technologies). To induce myostatin expression, the cells were grown for 24 h in medium containing 0.5 mg/ml CuSO₄.

Recombinant full-length myostatin protein, Mst375D, was purified from the insect cell pellet (5 g) by lysis in 8 M urea, 20 mM sodium phosphate, 500 mM NaCl, and 30 mM imidazole, pH 6.0. Bound protein was eluted with a buffer containing 6 M urea, 20 mM sodium phosphate, 500 mM NaCl, and 50 mM EDTA, pH 5.3. Fractions containing the highest amounts of Mst375D protein, as assessed by SDS-PAGE, Coomassie blue staining, and Western blots, were pooled and concentrated on Centricon-10 (YM-10, Millipore) and were purified by preparative HPLC [Delta Pack C₁₈ PrepLC, 15 μm, 300 Å (Waters)], with the...
use of 0.1% trifluoroacetic acid (TFA). Myostatin eluted in flow through, and smaller contaminating proteins were bound to the column. Eluted material was checked on an SDS gel, concentrated on Centricron-10, and loaded on a Sephacryl S-200 HR (Sigma) column equilibrated with 10 mM HEPES, pH 7.4, 150 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride (PMSP). Eluted protein peaks were analyzed by SDS-PAGE and concentrated on Centricron-10, and glycerol was added to 10%. Myostatin protein fractions were dialyzed extensively at 4°C against PBS buffer. Final protein estimation was performed by the microbicinchoninic acid method (Pierce) and by immunoblotting.

Preparation of recombinant Mst110EC protein. The construct for Mst110EC was expressed in E. coli strain BL21(DE3) as protein contained in inclusion bodies. The cells resuspended in 8 M urea, 0.5 M l-arginine, 0.1 M Tris, 10 mM NaCl, 0.5 M l-arginine, 0.1% (3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, 10 mM EDTA, 5 mM reduced glutathione, 2.5 mM oxidized glutathione, pH 8.0), incubated for 3 days at 4°C, clarified by centrifugation, dialyzed exhaustively against 10 mM HCl, and lyophilized. The Mst110EC protein was further purified by HPLC on a Poros R2/M column with 0.1% TFA in 0–80% linear acetonitrile gradient. Fractions eluting at 50% acetonitrile were pooled and lyophilized. The protein was estimated by HPLC and SDS-PAGE to be 99% pure, and its identity was confirmed by NH2-terminal Edman sequencing and amino acid compositional analysis. The pure lyophilized protein was stored at −80°C in 50% ethanol + 3 M HCl.

Protein gel electrophoresis and Western blot. Purified myostatin proteins (0.3–3 μg) or SDS extracts of cultured cells and skeletal muscle (30 μg protein) were treated as described (9), separated by SDS-PAGE on a 12–20% gradient Tris-glycine gel (ReadyGel, Bio-Rad, Hercules, CA), and detected by Coomassie blue and silver staining (Silver Stain Plus, Bio-Rad). Recombinant myostatin proteins were detected by Western blotting to nitrocellulose with polyclonal antibody B against myostatin (9) (1:1,000) or mouse monoclonal antibody against the Myc epitopes (1:3,000, Invitrogen) as primary antibodies and antibody to IgG linked with horseradish peroxidase (HRP) as the second antibody. Additionally, a direct staining procedure was applied for the detection of the carboxy-end polyhistidine tag with a monoclonal antibody to polyhistidine (C0OH terminal) coupled to HRP (1:2,000, Invitrogen). Blots were developed with an enhanced chemiluminescent substrate for HRP and exposed to film (ECL hyperfilm, Amersham).

C2C12 cell proliferation assay. Mouse skeletal muscle cell line C2C12 (ATCC) was propagated as myoblasts in DMEM plus (DMEM containing 4 mM glutamine and antibiotics) with 10% PBS, and incubated at 37°C at 10–50% confluence on the appropriate plates for each assay. For differentiation into myotubes, the myoblasts were plated at ~90-100% confluence; after 2 days, the medium was changed to DMEM plus with 5% horse serum. The myotubes began to form in 2–4 days, and multinucleated muscle fiber cultures were used at 7–10 days. C2C12 cell proliferation was determined in 96-well plates by the Formazan dye assay (Promega). Cells were grown at initial densities of 400, 800, 1,600, or 3,200 cells/well; then, after 1 day, they were treated with recombinant myostatin proteins in varying concentrations for 72 h. After 3 days of incubation, Formazan substrate buffer was added to the cultures for 3 h at 37°C, and the absorbance at 492 nm was read by an ELISA plate reader. For cell counting, the cells were removed by trypsinization, and the number of viable cells was counted in a hemocytometer with the use of trypan blue staining.

[^H]Thymidine incorporation into DNA. Cells cultures in 48-well plates were incubated in T-labeling medium (Leu-labeling medium described below plus 160 μg/ml of unlabeled leucine) containing 2 μCi/ml [^H]thymidine (ICN no. 24066) in the absence or presence of myostatin proteins. The cells were harvested at 24, 48, and 72 h, washed with PBS, and lysed with 120 μl of buffer with 1% Triton X-100 + 1% SDS. To complete cell disruption, extracts were frozen and thawed, denatured by NaOH added to 0.1 M, incubated for 30 min at 25°C, and then neutralized with HCl. For analysis of both 14C-labeled protein and/or T-labeled DNA by TCA precipitation, 20 μl of cell lysate were spotted onto GF/C filters (Whatman). Filters were dried, washed three times in 5% TCA, rinsed in ethanol, dried, and placed in 3 ml of scintillation fluid (Scinti-Safe, Fisher) and counted in a β-counter (Beckman, Fullerton, CA).

Protein synthesis and degradation. For measurement of the rate of protein synthesis, cells in 48-well plates were incubated in Leu-labeling medium (RPM1 1640 medium with no leucine, containing 4 mM glutamine, insulin, transferrin, selenium, and 1 μg/ml of added leucine, with 10% dialyzed FBS). Quadruplicate wells containing 0.2–12 μg/ml of myostatin proteins were incubated for 40 h and then pulse-labeled with 0.2 μCi/ml of [1-14C]leucine (ICN) for 2.5 h. Medium containing dialyzed horse serum was used in place of FBS for myotube induction. To measure the rate of protein degradation, cells were first pulse-labeled with [14C]leucine in Leu-labeling medium for 16 h. The cells were then transferred into T-labeling medium with and without myostatin protein at 6 μg/ml, but without [1-14C]leucine; excess leucine was added at time 0 to prevent further labeling or reutilization of [14C]leucine. Cells were harvested at 1-day intervals over the 3-day chase period. At the completion of both procedures, the cells were treated and counted as above.

Apoptosis. The apoptotic index was determined by the TdT-mediated dUTP nick end labeling (TUNEL) method, based on the ability of terminal TdT to catalyze addition of digoxigenin-dUTP and dATP to 3’-OH ends of cleaved DNA. The cells were placed on removable plastic slides within 8-well chambers and were incubated with or without myostatin proteins for 1–3 days and then fixed in 10% paraformaldehyde. The slides were treated with proteinase K and H2O2, followed by addition of primary and secondary antibodies. The slides were stained with 3’-3-diaminobenzidine (DAB) and counterstained with 0.5% methyl green. The apoptotic index was calculated by dividing the number of apoptotic cells by the number of nuclei in the microscope field.

Statistical analyses. The data are presented as means ± SD. All data points represent the means of 4 wells. To determine the effects of myostatin on DNA synthesis (Fig. 4) and protein degradation (Fig. 6), we used a two-way ANOVA, with incubation time and myostatin (±) as the two factors. To determine the effects of myostatin on protein synthesis, a one-way ANOVA was used. The cell growth curves (Fig. 3) in the absence and presence of graded concentrations of recombinant myostatin proteins were analyzed by a multifactor ANOVA. If ANOVA revealed significant overall effects, comparison between groups was performed by t-matrix analysis. The P values were adjusted for multiple comparisons using Boneferroni’s correction.
RESULTS

Expression of recombinant full-length myostatin proteins. The recombinant full-length Mst375D protein was expressed from a cDNA construct subcloned from our previously reported clone (9) into the expression vector pCDNA3 in both the sense (Mst) and antisense (Mst[A]) orientation. In addition, the sense cDNA was fused with a Myc-His tag [Mst(H)] at its carboxy terminus to facilitate identification of the recombinant protein with the use of anti-Myc or anti-His antibodies (Fig. 1A). Myostatin gene expression from these plasmids was seen both in vitro and in vivo (Fig. 1, B and C). When Mst cDNA constructs were transcribed and translated in a cell-free system, we detected the 45-kDa full-length protein, as expected. The translation of the Mst(H) plasmid yielded a slightly longer 50-kDa protein with a His tag. No specific protein band was detected from the antisense construct (Fig. 1B).

When CHO cells were transfected with the pcDNA3-Mst(H) plasmid, the full-length myostatin protein with the Myc-His tag could be detected by Western blotting by use of antibodies against myostatin (Fig. 1C, top), the His tag (Fig. 1C, middle), and the Myc epitope (Fig. 1C, bottom). Myostatin proteins were seen in both monomeric (50–55 kDa) and dimeric (100–110 kDa) forms, expressed from the Mst(H) plasmid. As expected, full-length myostatin protein lacking the His tag was detected only with anti-myostatin antibody. No comparable bands were detected in the vector control with anti-myostatin, anti-His, or anti-Myc antibodies (Fig. 1C).

To produce the full-length 375-aa myostatin protein with a His tag (Mst375D), the cDNA coding region was subcloned into an insect expression vector. This DES culture system allows for a more faithful processing of recombinant animal proteins in S2 insect cells than in bacterial systems. Myostatin expression was detected as a 45- to 50-kDa band in induced, but not in uninduced, S2 cells and was verified by Western immunoblotting with antibodies against myostatin (B) and the poly-His tag (Fig. 2A). Recombinant myostatin was purified by nickel affinity columns, reverse phase HPLC, gel filtration, and dialysis and was characterized by gel electrophoresis and staining with Coomassie blue (Fig. 2B, left) and silver staining (Fig. 2B, middle) and Western blot analysis with anti-myostatin antibody (Fig. 2B, right). We detected a 45-kDa monomer and a 90-kDa dimer by Coomassie blue and silver staining and by Western blot with anti-myostatin antibody. In addition, a 22-kDa myostatin peptide band was also detected by silver staining and Western blot with anti-myostatin antibody. One silver-stained 55-kDa band, which did not react with the anti-Mst(B) antibody, may be a nonmyostatin contaminant.

Expression of the 110-aa carboxy-terminal myostatin protein in E. coli. The recombinant 110-aa carboxy-terminal protein was produced in E. coli (Mst110EC) by use of the Mst110 coding region fused with a start codon and NH3-terminal polyhistidine tag. The Mst110EC protein was expressed in E. coli, refolded, and purified by HPLC. On SDS gel electrophoresis, this protein was visualized as a 15- to 17-kDa monomer and a 30-kDa dimer by silver staining and immunoblotting with the anti-myostatin antibody (Fig. 2B). On the basis of the intensity of the major Coomassie-stained 15-kDa band, this Mst110EC protein is >95% pure.

Effects of Mst375D and Mst110EC proteins on muscle cell replication and DNA synthesis. To measure myostatin effects on cell number (Fig. 3A), C2C12 myoblasts were grown in media containing recombinant Mst375D (top) or Mst110EC (bottom) protein at increasing concentration from 0 to 6 μg/ml. The wells were harvested each day for 3 days, and the cell number per well was counted. In the absence of myostatin protein, the C2C12 myoblast cell number increased progressively from days 1 to 3 (Fig. 3A). Graded concentrations of both the Mst375D and the Mst110EC proteins dose dependently inhibited the growth of C2C12 myoblasts. After 3 days, the increase in cell number was inhibited by 55 ± 5% in the presence of 4 μg/ml Mst375D (Fig. 3A, top) and by 37 ± 5% in the presence of 4 μg/ml Mst110EC (Fig. 3A, bottom).

In C2C12 myoblasts, cell proliferation, as assessed by the Formazan assay, was also inhibited in a dose-dependent manner by recombinant myostatin proteins.
After 3 days' growth in the presence of 4 mg/ml recombinant proteins, the cell titer in wells with initial cell density of 3,200 cells/well was inhibited by 63±9% by Mst375D (Fig. 3B, top), and 34±3% by Mst110EC (Fig. 3B, bottom). In CHO cells, a nonmyogenic cell line, Mst375D, had significantly less effect, inhibiting cell titer by 25% at 4 mg/ml concentration.

To determine the effects of recombinant myostatin on DNA synthesis, C2C12 cells, grown as 35% confluent myoblasts or differentiated myotubes, were treated with or without 6 mg/ml of Mst375D protein in medium containing [3H]thymidine. As expected, the rate of [3H]thymidine incorporation into DNA was higher in proliferating myoblasts than in differentiated myotubes (Fig. 4). DNA synthesis, measured by [3H]thymidine incorporation, was inhibited in both myoblasts and myotubes by myostatin protein. The inhibition of DNA synthesis by 6 µg/ml of full-length myostatin protein after 3 days of incubation was more pronounced in myotubes (63±8%) than in myoblasts (33±13%) (Fig. 4).

Effects of recombinant myostatin proteins on muscle cell protein degradation and apoptosis. To determine the effects of recombinant myostatin proteins on muscle protein breakdown, we prelabeled cell proteins with [14C]leucine and then prevented further incorporation of [14C]leucine into protein by addition of excess leucine in the medium. The remaining 14C counts in the protein fraction were chased during 3 days of incubation (Fig. 5).

Effects of recombinant myostatin proteins on muscle cell protein synthesis. The full-length Mst375D protein inhibited protein synthesis, measured by incorporation of [14C]leucine, in a dose-dependent manner in both myoblasts and myotubes (Fig. 5). The inhibition of the rate of protein synthesis was 59±6% in myoblasts incubated with 6 µg/ml of full-length Mst375D protein for 40 h; in myotubes, the inhibition was 75±1% at this concentration (Fig. 5, top). The Mst110EC protein also inhibited protein synthesis in differentiated myotubes, as measured by [14C]leucine incorporation. The inhibition was 39±8 and 71±3%, respectively, in the presence of 2 and 6 µg/ml of Mst110EC (Fig. 5, bottom).

Effects of recombinant myostatin proteins on muscle cell proliferation. C2C12 myoblasts were grown for 24, 48, or 72 h in media containing 0, 0.5, 2, 4, and 6 µg/ml Mst375D (Fig. 3A, top) or Mst110EC (Fig. 3A, bottom). Data for cell count/well are means ± SD (n = 4 wells at each time point and concentration). ANOVA for Mst concentrations of 2, 4, and 6 µg/ml vs. none (0) at 48 and 72 h show a significant effect of P < 0.001. B: cell titer measured by Formazan dye assay. C2C12 cells were plated at various initial densities of cells/well (x-axes) for 1 day and then incubated for 3 days in media containing increasing concentrations of Mst375D (top) or Mst110EC (bottom), followed by Formazan assay. Data are means ± SD (n = 4 wells at each point). ANOVAs for Mst375D concentrations of 2, 4, and 6 µg/ml vs. none (0) at 800 or 1,600 initial cells show a significant effect of P < 0.001, and at 3,200 cells show P < 0.04.

(Fig. 3B). After 3 days' growth in the presence of 4 µg/ml recombinant proteins, the cell titer in wells with initial cell density of 3,200 cells/well was inhibited by 63±9% by Mst375D (Fig. 3B, top), and 34±3% by Mst110EC (Fig. 3B, bottom). In CHO cells, a nonmyogenic cell line, Mst375D, had significantly less effect, inhibiting cell titer by <25% at 4 µg/ml concentration.

Effects of recombinant myostatin proteins on muscle cell protein degradation and apoptosis. To determine the effects of recombinant myostatin proteins on muscle protein breakdown, we prelabeled cell proteins with [14C]leucine and then prevented further incorporation of [14C]leucine into protein by addition of excess leucine in the medium. The remaining 14C counts in the protein fraction were chased during 3 days of in-

Fig. 3. Effects of recombinant myostatin proteins on C2C12 myoblast cell proliferation. A: cell number measured by direct count of viable cells in a hemocytometer. C2C12 myoblasts were grown for 24, 48, or 72 h in media containing 0, 0.5, 2, 4, and 6 µg/ml Mst375D (top) or Mst110EC (bottom). Data for cell count/well are means ± SD (n = 4 wells at each time point and concentration). ANOVA for Mst concentrations of 2, 4, and 6 µg/ml vs. none (0) at 48 and 72 h show a significant effect of P < 0.001. B: cell titer measured by Formazan dye assay. C2C12 cells were plated at various initial densities of cells/well (x-axes) for 1 day and then incubated for 3 days in media containing increasing concentrations of Mst375D (top) or Mst110EC (bottom), followed by Formazan assay. Data are means ± SD (n = 4 wells at each point). ANOVAs for Mst375D concentrations of 2, 4, and 6 µg/ml vs. none (0) at 800 or 1,600 initial cells show a significant effect of P < 0.001, and at 3,200 cells show P < 0.04.

Fig. 4. Effects of recombinant myostatin proteins on muscle cell protein synthesis. The full-length Mst375D protein inhibited protein synthesis, measured by incorporation of [14C]leucine, in a dose-dependent manner in both myoblasts and myotubes (Fig. 5). The inhibition of the rate of protein synthesis was 59±6% in myoblasts incubated with 6 µg/ml of full-length Mst375D protein for 40 h; in myotubes, the inhibition was 75±1% at this concentration (Fig. 5, top). The Mst110EC protein also inhibited protein synthesis in differentiated myotubes, as measured by [14C]leucine incorporation. The inhibition was 39±8 and 71±3%, respectively, in the presence of 2 and 6 µg/ml of Mst110EC (Fig. 5, bottom).
and the slopes of the $^{14}$C decay curves were used as a measure of protein degradation. Neither the Mst375D protein (6 μg/ml, Fig. 6, left), nor the Mst110EC protein (6 μg/ml, Fig. 6, right) had any significant effect on the rate of protein degradation in myotubes (bottom graphs). A slight increase in protein degradation rate by myostatin treatment cannot be ruled out for myoblasts (top graphs).

To assess any effects of the recombinant proteins on cell death, we used trypan blue staining and apoptosis assays. Neither the Mst375D protein nor the Mst110EC protein had any significant effect on apoptosis, as assessed by the TUNEL assay (data not shown). Similarly, cell death detected by Trypan blue staining of cells was not significantly different in myostatin-treated cells and control cells (~2.5%). To test for reversibility of myostatin’s effect, we tested the growth rate of cells pretreated with the protein. After precubation of C2C12 cells with Mst375D at 6 μg/ml for 3 days, there was a 45% reduction in cell number compared with control cells. When these cells were extensively washed to remove myostatin and then replated and grown in normal media for 3 days, the growth rates (as determined by Formazan assay) of myoblasts pretreated with recombinant myostatin protein were not significantly different from those of cells pretreated with media (data not shown). These data indicate a reversible inhibitory effect of recombinant protein on cell proliferation.

DISCUSSION

Our data represent the first successful expression of the human myostatin proteins and demonstration of their biological activity in an in vitro bioassay system. Both the Mst375D protein and the Mst110EC protein spontaneously form homodimers in solution that are not easily dissociable under mild-to-moderate denaturing conditions. The human myostatin gene encoding the 375-aa protein is expressed in vitro and in vivo as a 45- to 55-kDa protein, which dimerizes spontaneously into a 90- to 110-kDa complex. The recombinant 110-aa, carboxy-terminal, myostatin protein is expressed in E. coli as a 15-kDa protein that dimerizes into a 30-kDa complex. This is consistent with experience with other members of the TGF-β superfamily that also form homo- or heterodimers (18). The molecular masses, estimated for the full length 375-aa (45 kDa) and 110-aa (15 kDa) myostatin proteins [both having His-6 tags (0.74 kDa)], are in agreement with the sizes predicted theoretically for human Mst375D (42.750 kDa) and the Mst110EC protein (12.563 kDa).

Both the full-length protein and the 110-aa carboxy-terminal myostatin protein dose dependently inhibit the growth of C2C12 skeletal muscle cells, decreasing
proliferation of cells, and inhibiting DNA and protein synthesis in both myoblasts and particularly in myotubes. Recombinant myostatin protein has little effect on protein turnover or apoptosis in C2C12 cells. Collectively, these data suggest that myostatin protein inhibits muscle mass by its inhibitory effects on muscle cell replication and protein synthesis. By analogy with other members of the TGF-β family, it is possible that myostatin might exert its effects by binding to a specific receptor of the serine-threonine kinase family on the membrane of skeletal muscle cells; this, however, remains to be demonstrated. The recombinant full-length myostatin protein produced a greater inhibition of DNA synthesis in myotubes than in myoblasts; we do not know the exact reasons for this, but it could be due to higher levels of expression of the putative receptors in myotubes than in myoblasts. The question of whether the general inhibition of protein synthesis by myostatin also specifically affects regulation of the actin or myosin contractile proteins in myotubes needs to be studied further.

These effects of recombinant myostatin on protein synthesis and cell proliferation do not represent non-specific toxic effects. First, morphological evaluation of cells and the use of the trypan blue dye did not reveal evidence of cytotoxicity at the concentrations used. Second, muscle cell apoptosis and protein degradation were not affected. Third, the inhibitory effects of the recombinant, full-length protein on cell proliferation were reversible upon removal of the protein. Also, the inhibitory effects of the full-length protein were substantially greater in C2C12 muscle cells than in a non-myogenic CHO cell line, providing evidence of preferential effects on muscle cells.

Although our data provide evidence that both the full-length, 375-aa protein and its 110-aa carboxy-terminal fragment are biologically active in this bioassay, the myostatin concentrations required for biological effects in this system are higher than those of TGF-β required for biological effects in other systems (11). We do not know how myostatin gene product is processed, what the mature form of myostatin is, or whether it undergoes posttranslational modification. It is possible that further processing of the full-length 375-aa protein, posttranslational modifications, and protein folding in the skeletal muscle may be essential for optimal biological activity. The expression systems used to produce the recombinant proteins may not be capable of appropriate processing, postranslational modification, and folding. The 110-aa carboxy-terminal protein was proposed (19), on the basis of studies in CHO cells, to be the mature form of myostatin; this remains to be established in human skeletal muscle. Although the amino acid sequence of the 110-aa peptide is identical in the mouse and the human, the mouse and the human myostatin proteins differ by 13 aa in the amino-terminal region of myostatin. It is possible that the full-length human myostatin protein might be less active than its murine homolog in its effects on the mouse C2C12 cell growth.

Our data show that anti-myostatin antibody recognizes the full-length myostatin protein and the 110-aa carboxy-terminal protein. The data presented in this and previous publications (3, 9, 17, 26) suggest that the 28- to 30-kDa myostatin-immunoreactive protein in the skeletal muscle is formed either by dimerization of the 110-aa carboxy-terminal protein or by its association with another muscle protein. We have shown that the dimerization occurs spontaneously with purified recombinant 110-aa myostatin protein. Further support for the hypothesis that the 28- to 30-kDa myostatin-immunoreactive band in the skeletal muscle and plasma of mice and humans is a product of the myostatin gene is provided by observations that the intensity of this band is decreased by previous incubation of the antibody with the 16-aa myostatin peptide B against which the antibody was generated and that this band is observed only in the extracts of skeletal muscle and plasma (9). TGF-β, inhibin B, and activin A do not cross-react significantly with this antibody (9); GDF-11 has considerable homology with myostatin (8), but its mRNA is not expressed in skeletal muscle. Collectively, these data suggest that the 30-kDa, myostatin-immunoreactive protein in the skeletal muscle, which is recognized by our antibody B, results from dimerization of its 110-aa carboxy-terminal fragment.

These data represent the first demonstration that the products of the human myostatin gene have biological effects in this in vitro model system; these data need further confirmation by in vivo experiments. Although C2C12 cells have been widely used for studying the effects and mechanisms of action of many growth factors (6, 21), they do not fully represent in vivo physiology. It is possible that the effects of myostatin proteins in vivo might differ because of the differences in hormonal and growth factor milieu that are prevalent in the human skeletal muscle (1, 12, 14, 25). It is also possible that the effects of myostatin proteins in skeletal muscle cells might be altered by processing, postranslational modification, folding, and the concomitant action of other muscle growth factors such as growth hormone, insulin-like growth factor I (IGF-I) and testosterone.

The regulation of myostatin expression is not well understood. The human myostatin promoter region contains sequences for potential binding by muscle-specific transcription factors including MyoD and myocyte enhancer factor 2 (Ref. 7; Ma K, and Taylor W, unpublished observations) and also for possible regulation by cytokines and growth factors. The role of these elements in the developmental and hormonal regulation of myostatin expression remains to be studied.

Muscle mass in humans and animals reflects a balance between anabolic factors such as IGF-I/growth factor I/growth hormone, testosterone, nutrition, and exercise, and catabolic factors such as glucocorticoids, thyroid hormones, mediators of systemic inflammatory response, and cytokines. Sarcopenia that occurs in association with aging and chronic illness is a complex process that involves changes in systemic metabolism.
and intramuscular gene expression (2). Myostatin should be added to the list of catabolic factors that inhibit muscle growth and contribute to the multifactorial pathophysiology of muscle loss that occurs in association with aging and chronic illnesses such as HIV infection. This proposal is supported by observations that myostatin expression increases in rat hindlimb muscles that undergo atrophy in response to microgravity (17) or hindlimb suspension (3, 26) and in wounded or regenerating muscle tissues (27). Surprisingly, some myostatin is expressed in fibroblasts infiltrating the wound (27), suggesting that it is a result of an inflammatory process occurring at an early stage in muscle regeneration. Myostatin may also function as a chalone to limit muscle growth in a homeostatic process (5). This is also consistent with the hypermuscularity seen in transgenic mice expressing the myostatin protein mutated in the RSRR protease cleavage site (28), in which the full-length mutant precursor functioned in a dominant negative manner to inhibit the activity of endogenous myostatin. Our data suggest that increased expression of myostatin could result in loss of muscle mass by inhibition of muscle protein synthesis. In addition, myostatin-induced inhibition of DNA synthesis and cell replication could impair the ability of muscle cells to regenerate and restore muscle mass during a catabolic illness or aging. Our in vitro studies set the stage for further in vivo testing of the function of myostatin in adult animals.

This study was supported by research grants from the National Institute of Aging (1R01 AG-14369); the Food and Drug Administration Orphan Drug Program (OPD-1397); National Institute of Diabetes and Digestive and Kidney Diseases (1R01 DK-49296); General Clinical Research Center (MO-05433); Research Center in Minority Inst. (RCMI; P20 RR-11045–01, RCMI Clinical Research Center in Minority Health and Health Disparities (1RO1 DK-49296); National Institute of Aging (1RO1 AG-14369); the Food and Drug Administration. Myostatin may also function as a chalone to limit muscle growth in a homeostatic process (5). This is also consistent with the hypermuscularity seen in transgenic mice expressing the myostatin protein mutated in the RSRR protease cleavage site (28), in which the full-length mutant precursor functioned in a dominant negative manner to inhibit the activity of endogenous myostatin. Our data suggest that increased expression of myostatin could result in loss of muscle mass by inhibition of muscle protein synthesis. In addition, myostatin-induced inhibition of DNA synthesis and cell replication could impair the ability of muscle cells to regenerate and restore muscle mass during a catabolic illness or aging. Our in vitro studies set the stage for further in vivo testing of the function of myostatin in adult animals.

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5. Del Aguila LF, Claffey KP, and Kirwan JP. Molecular definition of an allelic series of mutations that myostatin expression increases in rat hindlimb muscles by inhibition of muscle growth and contribute to the multifactorial pathophysiology of muscle loss that occurs in association with aging and chronic illnesses such as HIV infection. This proposal is supported by observations that myostatin expression increases in rat hindlimb muscles that undergo atrophy in response to microgravity (17) or hindlimb suspension (3, 26) and in wounded or regenerating muscle tissues (27). Surprisingly, some myostatin is expressed in fibroblasts infiltrating the wound (27), suggesting that it is a result of an inflammatory process occurring at an early stage in muscle regeneration. Myostatin may also function as a chalone to limit muscle growth in a homeostatic process (5). This is also consistent with the hypermuscularity seen in transgenic mice expressing the myostatin protein mutated in the RSRR protease cleavage site (28), in which the full-length mutant precursor functioned in a dominant negative manner to inhibit the activity of endogenous myostatin. Our data suggest that increased expression of myostatin could result in loss of muscle mass by inhibition of muscle protein synthesis. In addition, myostatin-induced inhibition of DNA synthesis and cell replication could impair the ability of muscle cells to regenerate and restore muscle mass during a catabolic illness or aging. Our in vitro studies set the stage for further in vivo testing of the function of myostatin in adult animals.

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