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Suppressed Cell Proliferation and Differentiation Following an Over-expression of Myostatin is Associated with Inhibited Expression of Insulin-like Growth Factor II and Myogenin in Rat L6 Myoblasts

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ABSTRACT : Myostatin (MSTN) and insulin-like growth factors (IGFs) are a known inhibitor and stimulators of proliferation and differentiation of muscle cells, respectively. The present study was performed to investigate the relationship of MSTN-induced growth inhibition to expression of the IGF system components and myogenin, a muscle cell-specific transcription factor, in rat L6 myoblasts. The L6 cells transfected with a green fluorescent protein-MSTN plasmid expression construct had a 47% less cell number than mock-transfected cells after 3-d serum-free culture, accompanied by delayed differentiation which was suggested by inhibited aggregation of cells. Moreover, cells transfected with the expression construct had decreased expression of IGF-II and myogenin genes, but not IGF-I or its receptor genes, as examined by reverse transcription-polymerase chain reaction. The reduced mitosis of the L6 cells transfected with the MSTN-expression construct increased following an addition of either IGF-I or IGF-II to the culture medium, but not to the level of mock-transfected cells. By contrast, myogenin gene expression in these cells increased after the addition of either IGF to the level of mock-transfected cells. Collectively, these results suggest that the inhibitory effect of MSTN on L6 cell proliferation and differentiation is likely to be partly mediated by serially suppressed expression of IGF-II and myogenin genes, not IGF-I gene. (**Key Words :** Myostatin, IGF, Myogenin, Myoblast, Proliferation, Differentiation)

INTRODUCTION

Muscle growth and development is regulated by a number of humoral factors including multiple growth factors (Florini, 1987; Florini and Magri, 1989). Growth factors, in general, have varying effects on the proliferation of muscle cells. For instance, transforming growth factor-β (TGF-β) inhibits muscle cell division, whereas fibroblast growth factor enhances this process. By contrast, most growth factors, including TGF-β, fibroblast growth factor and epidermal growth factor, are inhibitors of muscle cell differentiation.

Myostatin, also known as growth and differentiation factor-8, is a 25-kDa homodimeric TGF-β family peptide and is a negative regulator of hyperplasia as well as hypertrophy of the muscle cell (Langley et al., 2002; Lee, 2004; Bellinge et al., 2005; Yang et al., 2005). As such, a loss-of-function mutation of this regulatory peptide results in an increase in muscle mass which is known as double-muscling in Belgian Blue and Piedmont breeds of cattle (McPherron an Lee, 1997; Lee and McPherron, 1999). Similarly, suppression of endogenous myostatin activity by means of an over-expression of the TGF-β-binding protein "latency-associated peptide" (LAP) has been reported to a 5 to 20% increase in skeletal muscle mass of LAP-transgenic mice (Pirottin et al., 2005).

Insulin-like growth factor (IGF)-I and IGF-II are 7.5-kDa single-chain peptides which, unlike most other growth factors known to act on muscle cells, are potent stimulators of both cell proliferation and differentiation (Nissley and Rechler, 1984; Florini, 1987; Jones and Clemmons, 1995; Bach et al., 1995; Florini et al., 1996). These effects are mediated via IGF-I or type I IGF receptor. Moreover, it is known that both IGFs enhance differentiation by inducing the expression of myogenin and possibly other MyoD family of muscle cell-specific transcription factors in L6 myoblasts (Florini and Ewton, 1990; Florini et al., 1991a;

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Table 1. Sequences and annealing temperatures of PCR primers

cDNA	Direction	Sequence[a]	Annealing temp. (°C)	Location (#) in cDNA
Myostatin	Forward	5'-ATGATTCAAAAACCGCAAAT-3'	51	1-1,131
	Reverse	5'-TCACGAGCACCCACACCG GT-3'		
IGF-I	Forward	5'-GCT TGC TCA CCT TTACC AGC-3'	55	828-1,128
	Reverse	5'-AAT GTA CTT CCT TCT GGG TCT-3'		
IGF-II	Forward	5'-CAT GTC CCA CACTAA GGTCT-3'	55	1,748-2,314
	Reverse	5'-CCA GTG AAG AAA GAG TTTGG-3'		
IGF-I Receptor	Forward	5'-GCA TTG ACA TCC GCA ACG-3'	55	152-359
	Reverse	5'-ACT GTG AGG TTC GGA AGAGG-3'		
Myogenin	Forward	5'-AGT GAA TGC AAC TCC CAC AGC GCCT-3'	61	512-839
	Reverse	5'-TGG CTT GTG GCA GCC CAG GG-3'		
GAPDH	Forward	5'-TGC CGC CTG GAG AAA CCT GC-3'	57	1,582-1,753
	Reverse	5'-TGA GAG CAA TGC CAG CCC CA-3'		

[a] Sequence information was obtained from the Blast of NCBI (http://www.ncbi.nlm.gov).

Mangiacapra et al., 1992), whereas myostatin suppresses differentiation by down-regulating MyoD gene expression in C2C12 myoblasts (Langley et al., 2002). It is thus highly probable that the expression of myostatin, myogenin, IGFs and their receptor be inter-related in differentiation and proliferation of muscle cells. The present study was therefore undertaken to delineate the relationship between expressions of these components associated with the muscle growth and development in L6 myoblasts.

MATERIALS AND METHODS

Preparation of a myostatin expression construct

Total cellular RNA was extracted from skeletal muscle of an adult Sprague-Dawley rat using Trizol (Invitrogen, Carlsbad, CA, USA) as previously described (Lee et al., 2001). A 1,131-bp cDNA fragment coding for the entire rat myostatin peptide was synthesized by reverse transcription-polymerase chain reaction (Table 1) and inserted into the pcDNA3.1/NT-GFP-TOPO plasmid expression vector (Invitrogen) following manufacturer's instruction. The fidelity of the expression construct was confirmed by restriction digestion and nucleotide sequencing of the cDNA insert (data not shown).

L6 cell culture and transfection of the myostatin expression construct

The rat L6 myoblasts (Yaffe, 1968) obtained form ATCC (Rockville, MD, USA) were cultured in 100-mm culture flasks in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 IU/ml penicillin and 50 μg/ml streptomycin (Invitrogen) up to 60% confluency at 37°C in humidified atmosphere with 5% CO_2. The cells were washed with PBS, treated with 0.25% trypsin and harvested. 4×10^5 cells were transfected with 5 μg of the myostatin expression construct or the vector for 20 msec with 200 pulses using a square wave generator (Model BTX-930; Gentronics, San Diego, CA, USA) in 2-ml serum-free DMEM containing varying concentrations of IGF-I or IGF-II (Gropep, Adelaide, Australia) in 35-ml culture dishes. Transfected cells were incubated for 72 h at 37°C.

In cell proliferation assay, cultured cells were detached with trypsin/EDTA solution and counted in triplicate using a hemocytometer.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA of cultured cells was extracted using Trizol. The RT-PCR for myostatin, IGF-I, IGF-II, IGF-I receptor and myogenin, as well as glyeraldehyde-3-phosphate dehydrogenase (GAPDH) as control, was performed using respective primer pairs (Table 1) as described previously (Lee et al., 2001). The PCR conditions were: 94°C 5 min → {94°C 1 min→annealing 1 min→72°C 1 min}×35 cycles→72°C 5 min→4°C, with the annealing temperatures as indicated in Table 1. The PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide for visualization. The extent of target gene expression were quantitated using densitometry with each group normalized to its respective GAPDH control.

Statistical analysis

Data of the cell proliferation assay were analyzed by the ANOVA procedure of SAS (SAS Institute, Inc., Cary, NC, USA). Numbers of cells of the treatment groups were compared with that of the mock transfection by the Duncan's multiple-range test.

RESULTS

Transfection of the myostatin expression construct

Following transfection of the green fluorescent protein-

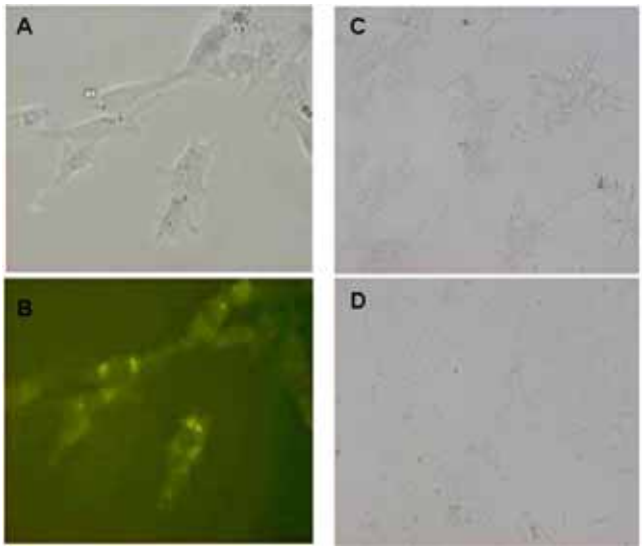

Figure 1. Effect of myostatin over-expression on cellular development in L6 myoblasts. After transfecting the L6 cells with a green fluorescent protein-myostatin plasmid expression construct (panels A, B and D) or the vector (panel C), the cells were incubated in serum-free DMEM and microscopically examined under bright field (panels A, C and D) or through FITC filter (panel B) after 24-h (panels A and B) or 72-h (panels C and D) culture. Note the green fluorescence in panel B and also aggregated vs. dispersed cells in panels C and D, respectively.

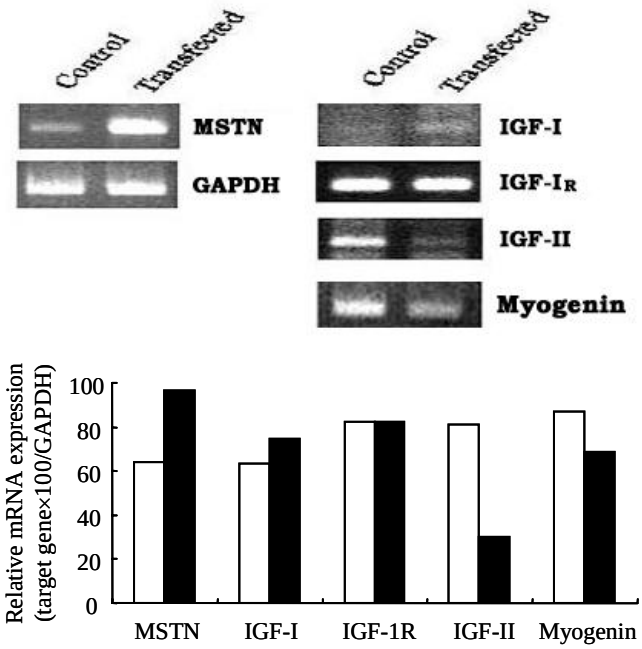

Figure 2. Effects of over-expression of myostatin on the expression of genes coding for IGFs, IGF-I receptor (IGF-I_R) and myogenin. Rat L6 myoblasts were transfected with a GFP-myostatin (MSTN) plasmid expression vector and cultured for 72 h, followed by RT-PCR as described in Materials and Methods. The PCR products were electrophoresed on agarose gel and visualized by ethidium bromide staining (upper). The extent of target gene expression was quantitated using densitometry with each group normalized to its respective GAPDH control (lower). Blank and filled boxes indicate control (Mock-transfected) and MSTN transfected groups, respectively. Data shown are representative of three independent experiments.

myostatin (GFP-MSTN) plasmid expression construct, L6 cells exhibited the expected fluorescence emitting from expressed GFP under the fluorescence microscope (Figure 1B). The transfection efficiency, which was assessed by the ratio of fluorescence-emitting cells to total cells 24 h after the transfection, was typically 35 to 40% under the present experimental condition. The mock-transfected cells aggregated presumably for myotube formation following 72-h serum-free culture (Figure 1C), whereas the cells transfected with the expression construct remained dispersed (Figure 1D).

Effects of myostatin on expression of the IGF system components and myogenin

Relative MSTN mRNA abundance of the L6 cells transfected with the expression construct, as expected, was much greater than that of mock-transfected cells as examined by RT-PCR (Figure 2). Over-expression of MSTN resulted in decreased expression of IGF-II and myogenin genes. However, expression of IGF-I and its receptor genes barely changed. Relative intensities of the RT-PCR bands of Figure 2 are shown for quantifying the results.

Effects of IGFs on MSTN-induced inhibition of proliferation and myogenin gene expression of L6 cells

The number of cells decreased 47% following the transfection of the MSTN expression construct compared with that of the mock transfection in the 3-day L6 cell culture (Figure 3). Both IGF added to culture medium enhanced the cell proliferation of the MSTN-over-expressing cells, although the dose-responsiveness was somewhat different between the two peptides at concentrations of 50, 100 and 200 ng/ml. The effect of IGF-I was biphasic, i.e. mitogenic only at 100 ng/ml, whereas IGF-II was effective only at the highest concentration (200 ng/ml). However, the total cell number never reached that of mock-transfected cells following the addition of either IGF in the MSTN-over-expressing cells.

Effects of IGFs on the expression of myogenin in MSTN-over-expressing cells were next examined by RT-PCR. The reduced myogenin gene expression following the transfection of the MSTN-expression vector increased to the level of mock-transfected cells when either IGF was added to the culture medium to a concentration of 100 ng/ml (Figure 4). However, IGF-I receptor was not affected by the transfection of MSTN or the addition of IGF. Relative intensities of the RT-PCR bands of Figure 4 are

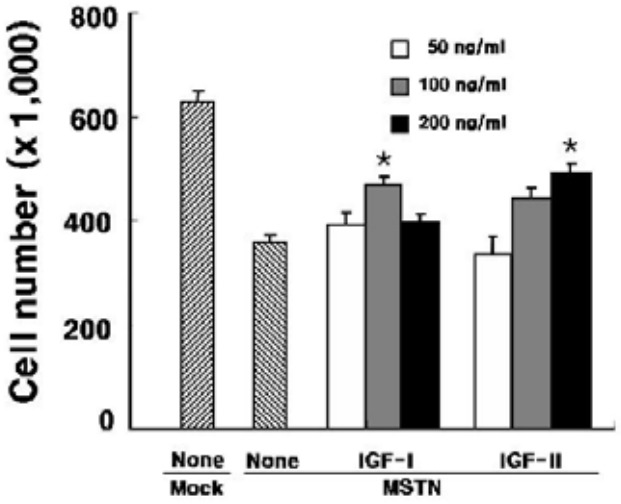

Figure 3. Effects of IGF-I and IGF-II on proliferation of L6 cells transfected with a green fluorescent protein-myostatin (GFP-MSTN) expression construct. Four-hundred thousand L6 cells, which had been transfected with a GFP-MSTN plasmid expression construct, were cultured for 72 h in the presence of an indicated concentration of IGF-I or IGF-II. For comparisons, cells which had been transfected with the vector (Mock) or the expression construct (MSTN) and cultured in the absence of IGF (None) were included. Data represent means and SE of four or five separate cultures. * $p<0.05$ compared with that of cells transfected with the expression construct and cultured in the absence of IGF.

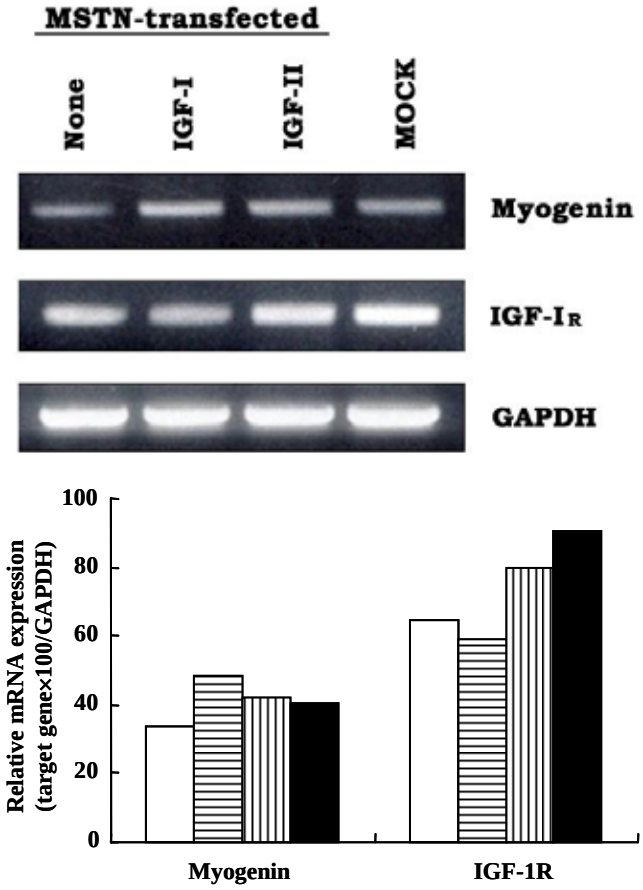

Figure 4. Effects of IGF-I and IGF-II on expression of myogenin and IGF-I receptor genes. L6 cells, which had been transfected with a GFP-MSTN expression construct, were incubated for 72 h in the absence (None) or presence of 100 ng/ml IGF-I or IGF-II, and total RNA was collected to examine the expression of myogenin and IGF-IR mRNA using RT-PCR as described in Materials and Methods. The PCR products were electrophoresed on agarose gel and visualized by ethidium bromide staining (upper). The extent of target gene expression was quantitated using densitometry with each group normalized to its respective GAPDH control (lower). Blank, boxes filled with horizontal lines, boxes filled with vertical lines and filled boxes indicate none, IGF-I, IGF-II and Mock, respectively. Data shown are representative of three independent experiments.

shown for quantifying the results.

DISCUSSION

Myostatin is an established negative regulator of proliferation and differentiation of muscle cells (Lee et al., 2004). It is poorly understood, however, by what mechanisms myostatin regulates these cellular events. By contrast, both IGF-I and IGF-II enhance proliferation and differentiation of muscle cells and the mechanisms of these peptides also have been well documented (Jones and Clemmons, 1995; Florini et al., 1996). IGF-I is more potent than IGF-II in mitogenic activity, whereas the reverse is true in inducing differentiation (Ewton et al., 1987, 1994; Florini et al., 1991b). Collectively, these reflect the fact that although both IGFs act during early and late stages of muscle development, cellular proliferation and differentiation are still mutually exclusive.

As an initial step to understand the action of myostatin in muscle cell proliferation and differentiation, effects of over-expressed myostatin on the expression of IGFs, IGF-I receptor and myogenin in L6 myoblasts were investigated in the present study. Over-expression of myostatin by transfection with the GFP-MSTN expression construct, as expected, resulted in a remarkable decrease in cell number compared with mock-transfected control after 72-h serum-free culture, accompanied by decreased expression of IGF-II gene, but not IGF-I or its receptor gene. The MSTN-induced down-regulation of cell proliferation was only partially restored by addition of IGF-I or IGF-II to the culture medium, which suggests that MSTN may suppress L6 cell proliferation not only by down-regulating IGF-II gene expression but also by unidentified mechanism(s). It was also noteworthy that IGF-I exhibited a biphasic concentration-dependent mitogenic effect vs. the mitogenic effect of IGF-II only at the highest concentration in these cells transfected with the MSTN-expression construct. This

indicates that IGF-I is more potent than IGF-II in mitogenic activity in L6 cells, which apparently reflects the fact that the former has greater affinity for IGF-I receptor than the latter (Ewton et al., 1987; Florini, 1987).

Concomitant with the inhibited mitosis and IGF-II gene expression in L6 cells transfected with the MSTN-expression construct were decreased myogenin gene expression and presumptively suppressed differentiation as indicated by the inhibited aggregation of cells preceding myotube formation. This suggests that the apparently suppressed differentiation in these cells may have partly resulted from a cascade of inhibited expression of IGF-II and myogenin genes since myogenin, which is up-regulated by IGF (Florini and Ewton, 1990; Florini et al., 1991a), is a potent inducer of terminal differentiation in L6 myoblasts. In support of this suggestion, if not in confirmation, myogenin gene expression increased by addition of either IGF to the culture medium of the cells transected the MSTN expression construct. Myf-5 is another MyoD family muscle cell-specific transcription factor known to be expressed to a significant extent in L6 cells, but this is only required for the induction of myogenin and is actually down-regulated during terminal differentiation (Mangiacapra et al., 1992; Florini et al., 1996).

The actions of IGFs are modulated by six known IGF-binding proteins (IGFBPs) *in vitro* as well as *in vivo* (Rechler and Clemmons, 1998; Hwa et al., 1999). The IGFBPs can either enhance or inhibit IGF actions depending on its class as well as culture conditions. Moreover, IGFBPs can also modulate cell proliferation and differentiation in an IGF-independent manner depending on the cell type. In this regard, Xi et al. (2004) have reported that exogenous IGFBP-3 suppressed proliferation of L6 cells independently of the IGF ligand, although expression of this class of IGFBP was not detected in this cell type. Ewton and Florini (1995) have detected the expression of IGFBPs-4, -5 and -6 in L6 cells, but these workers have not elucidated the roles of these IGFBPs. It thus remains to be studied whether or how myostatin affects the expression of IGFBPs in L6 myoblasts as related to cell proliferation and differentiation.

In summary, over-expression of MSTN caused a decrease in cell proliferation accompanied by decreased expression of IGF-II and myogenin genes in L6 myoblasts. This suggests that the inhibitory effect of MSTN on proliferation of this cell type partly lies in its suppression of IGF-II expression which results in decreased expression of myogenin causing an inhibition of differentiation as well. However, because the MSTN-induced suppression of cell proliferation was only partially restored by addition of either IGF to the culture medium, the inhibitory effect of MSTN on cell proliferation is also likely to be mediated via unidentified factor(s) other than the IGF system component.

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