

THE EFFECTS OF MYOSTATIN GENE INHIBITION BY AN ANTISENSE OLIGODEOXYNUCLEOTIDE AND ITS IMMUNOCYTOCHEMICAL LOCALIZATION IN C₂C₁₂ MYOBLASTS

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Summary: C₂C₁₂ skeletal muscle cell line as a representative of satellite cells has been used extensively in the effects of muscle growth factors on muscle cell replication and differentiation. Myostatin, a recently identified member of transforming growth factor- β family, has been widely accepted as a negative regulator of skeletal muscle growth. The purpose of the present study was to determine whether myostatin inhibition by an antisense oligodeoxynucleotide (ODN) alters proliferation and/or differentiation rates and its location in C₂C₁₂ satellite cells. C₂C₁₂ cells were transfected with one microgram of ODNs for corresponding antisense and control groups via lipofectin reagent. Standard immunocytochemistry technique with 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used to localize unprocessed myostatin protein in C₂C₁₂ cells. Myostatin inhibition stimulated myoblast differentiation to give rise to myotubes. Positive staining for unprocessed myostatin was mainly detected in the cytoplasm of myotubes and myoblasts.

Keywords: Myostatin, C₂C₁₂ myoblasts, immunocytochemistry, antisense oligos.

Antisens Oligodeoxynucleotide Aracılığı ile Myostatin Geni İnhibisyonunun C₂C₁₂ Miyoblast Hücreleri Üzerine Etkisi ve Myostatin'in İmmunositokimyasal Lokalizasyonu

Özet: Büyüme faktörlerinin, kas hücrelerinin proliferasyonu ve diferansiyasyonu üzerine etkilerini araştırmak üzere, satellit hücrelerini temsilen C₂C₁₂ çizgili kas hücreleri (miyoblast) yaygın bir şekilde kullanılmaktadır. Transforming growth faktör ailesinin son yıllarda izole edilmiş bir üyesi olan myostatin'in çizgili kas büyümesini engelleyen bir faktör olduğu kabul edilmektedir. Bu çalışmanın amacı, antisense oligodeoxynucleotide (ODN) tarafından ekspresyonu inhibe edilen myostatin geninin, C₂C₁₂ satellit hücrelerinin proliferasyonu ve diferansiyasyonu üzerine etkisini araştırmak ve myostatin proteininin lokalizasyonunu belirlemektir. C₂C₁₂ hücreleri antisens ve kontrol gurubu (biri mikrogram ODN) için ayrı ayrı olmak üzere, Lipofectamin aracılığı ile transfekte edildi. Proses edilmemiş (aktif hale gelememiş) myostatin proteininin, C₂C₁₂ hücrelerinde lokalizasyonunu tespit için standart immunositokimya tekniği (3,3'-diaminobenzidine tetrahydrochloride; DAB) kullanıldı. Myostatin inhibisyonunun miyotüp oluşumunu hızlandırdığı gözlemlendi. Proses edilmemiş myostatin proteini yaygın olarak miyoblast ve miyotüplerin sitoplazmasında bulundu.

INTRODUCTION

Satellite cells located between the basal lamina and sarcolemma of the muscle fibers¹ are precursor cells of vertebrate skeletal muscle. All known mammalian skeletal muscles contain satellite cells and they make up 2 to 7% of the nuclei associated with a particular fiber¹. These cells are responsible for postnatal muscle growth and regeneration during normal development, as well as in experimentally-induced pathological conditions and in various muscle diseases. These cells are also considered mitotically quiescent until stimulated by growth factors to proliferate, differentiate and fuse to existing muscle fibers². We used the C₂C₁₂ skeletal muscle cell line as a representative of satellite cells, since it is a pure, immortalized mouse satellite cell line, which is morphologically similar to primary satellite-cell

cultures³. Additionally, this model has been used extensively in previous studies of the effects of muscle growth factors on muscle cell replication, differentiation and apoptosis^{4,5}.

Myostatin, a recently identified member of TGF- β family, has been shown to negatively regulate skeletal muscle growth⁶. The main source of myostatin synthesis and secretion is skeletal muscle tissues. Inactivating mutations of the myostatin gene in cattle^{7,8} and mice⁶ are associated with skeletal muscle hypertrophy. Similarly, myostatin null mutation mice showed a dramatic increase in skeletal muscle mass, primarily due to increased number of muscle fibers⁶. It has also been shown that the addition of recombinant myostatin protein inhibits cell proliferation, DNA synthesis, and protein synthesis in C₂C₁₂ muscle cells⁹.

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In recent years, antisense oligodeoxynucleotides (ODN) have been extensively used as molecular probes to better understand the interaction between proteins and nucleic acids at molecular level. They have also been used as therapeutic and diagnostic agents¹⁰. For example, the antisense oligos are used to block gene expression by binding especially with complimentary sequences of target mRNA. Thus, in the present study, an ODN, which is short sequence of DNA designed to inhibit the expression of myostatin, was introduced to determine whether myostatin absence alters proliferation and/or differentiation rates of those satellite cells.

MATERIALS and METHODS

Cell culture: Mouse skeletal muscle C₂C₁₂ cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 4 mM glutamine and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin). All reagents for cell culture were purchased from life technologies (Life technologies Inc. Rockville, MD, USA). Cells were plated in 6-well plates at a density of 4 X 10⁵ cells/well and allowed to attach for 12 h in a CO₂ incubator at 37°C. The cell collection procedure was the replicates of three.

Antisense oligonucleotid addition: Oligodeoxynucleotides were synthesized by solid phase phosphoramidite chemistry and bioactivity of these sequences were verified in chinese hamster ovary cell lines by Dr. Lee Liang (Metamorphix Inc., Baltimore, MD, USA). C₂C₁₂ cells seeded on rat tail collagen-coated cover slips were transfected with one microgram ODNs for corresponding antisense and control groups. Lipofectin reagent (Gibco BRL, Rockville, MD, USA) was used as a carrier to introduce ODNs into satellite cells according to supplier's instructions. The sequence of antisense ODN complementary to the RSRR translation codon region of mouse myostatin mRNA was 5'-AGCAAGATCTCGTCCAGTCTC -3'. An oligonucleotide consisting of the scrambled sequence of 5'TAGCTTTTGCATCATCTTAAC -3' was used as a negative control.

Immunocytochemistry: The anti-myostatin mouse polyclonal antibody was kindly provided by Dr. Lee Liang (Metamorphix Inc., Baltimore, MD, USA). The uniqueness of this antibody is that it can only recognize the myostatin in which cleavage site at the RSRR region was inhibited by an antisense ODN.

C₂C₁₂ cells were grown with the ODN's for 24 h before processing for microscopy. All the cover slips were dried at room temperature and were rinsed twice in phosphate-buffered saline (PBS) and incubated with 0.2% Triton-X100 in PBS and 10% fetal calf serum (FCS) for 20 min. The slides were then preincubated with 50% FCS for 30 min and again with polyclonal antibodies to myostatin with 1:25 dilution in PBS and 10% FCS for 45 min at 37 °C. After washing three times, the slides were incubated with secondary anti-IgG mouse antibody. Myostatin immunostaining was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Life technologies, MA, USA) and the cover slips were counterstained with hematoxylin. Microscopic images were captured with an Olympus BX51 microscope (Olympus Optical Co., Germany) equipped with an Olympus camera and processed using Camedia master software.

RESULTS and DISCUSSION

After 24 h *in vitro* incubation with an antisense ODN, most of the cells in experimental group demonstrated positive staining for unprocessed myostatin as detected by immunocytochemical methods (Fig. 1), whereas no positive myostatin staining was detected in scrambled ODN control group (Fig. 2). Positive staining for unprocessed myostatin was mainly detected in the cytoplasm of myotubes and myoblasts (Fig. 1). Positive staining was also detected in the nuclei of both myoblasts and myotubes (Fig. 1). Intriguingly, myostatin inhibition caused rapid myotube formation in an antisense ODN transfected C₂C₁₂ cells (Fig. 1). These results were partially in agreement with findings of Artaza¹¹ who demonstrated that expression of myostatin mRNA was found in myotubes but not in myoblasts, and myostatin protein was detected in the cytoplasm of myotube. They have also shown that myostatin protein was expressed mostly in the nuclei of polynucleated C₂C₁₂ fibers which were in agreement with the findings of the present study.

Myostatin is synthesized in skeletal muscle as a 376 amino acid propeptide, which gives rise to 15 kDa active, processed and mature protein⁶. Therefore, after myostatin has been proteolytically-processed at the carboxy-terminal site (RSRR), it has become biologically active protein. In the current study, an antisense ODN was used to inhibit myostatin gene expression at the RSRR cleavage site, which

ultimately gave rise to unprocessed myostatin protein. Myostatin protein level was found to be higher in the slow-fiber-type dominated muscles (soleus) than in the fast-fiber-type dominated muscles (tibialis anterior) of rats¹². In contrast, myostatin mRNA and protein level were higher in fast-fiber-dominated muscles (gastrocnemius/plantaris) than that of slow-fiber-dominated muscles (soleus) of mice¹³. Satellite cells isolated from pectoralis major (predominantly fast fibers) and biceps femoris (predominantly slow fibers) muscles of 5-wk-old broilers, myostatin mRNA expression was nearly identical, with the exception of significant increase when fusion started in biceps femoris satellite cells¹⁴. In the light of these findings, it is possible to postulate that myostatin may be one of the major determinants of muscle fiber type in any given muscle. Thus, the localization of myostatin and its function may vary in different skeletal muscle cell lines.

The complex cascades of processes including myogenic transcription factor's expression and activity, cell cycle withdrawal, myoblast fusion in myotubes regulates muscle growth. It has been found that myostatin inhibits cell proliferation and differentiation¹⁵. It has been also indicated that inhibition of myostatin synthesis leads to enhanced cell cycle withdrawal and consequently stimulates myoblast differentiation¹⁵. The results obtained in the current study suggested that the exogenous antisense ODN addition was successfully repressed myostatin expression in C₂C₁₂ cell line and then attenuation of myostatin expression led differentiation of the myoblast to myotubes. *In vitro* studies displayed that recombinant myostatin inhibited the proliferation of C₂C₁₂ myoblasts¹⁶. The inhibitory effect of myostatin was reversible, as myoblasts maintained their ability to proliferate after myostatin was removed. Extra myostatin specifically upregulated P²¹, a cyclin-dependent kinase inhibitor, and decreased the amount of cyclin-dependent kinases (especially cdk2), a family of enzymes that catalyze events required for cell cycle transition, in C₂C₁₂ cells^{16,17}. Both groups suggested that myostatin blocked the myoblast transition in the G1/S and/or G2/M phases of the cell cycle. Thus, in terms of my findings, it needs to be determined whether increased differentiation rates in an antisense ODN added cell culture was due to either the increased number of myoblasts or direct effect of myostatin on differentiation genes such as myogenin. In conclusion, the result of the present study contributes to better understanding of myostatin function and potential consequences of its inhibition in C₂C₁₂ myoblasts which in turn could be beneficial to human health and food animal agriculture.

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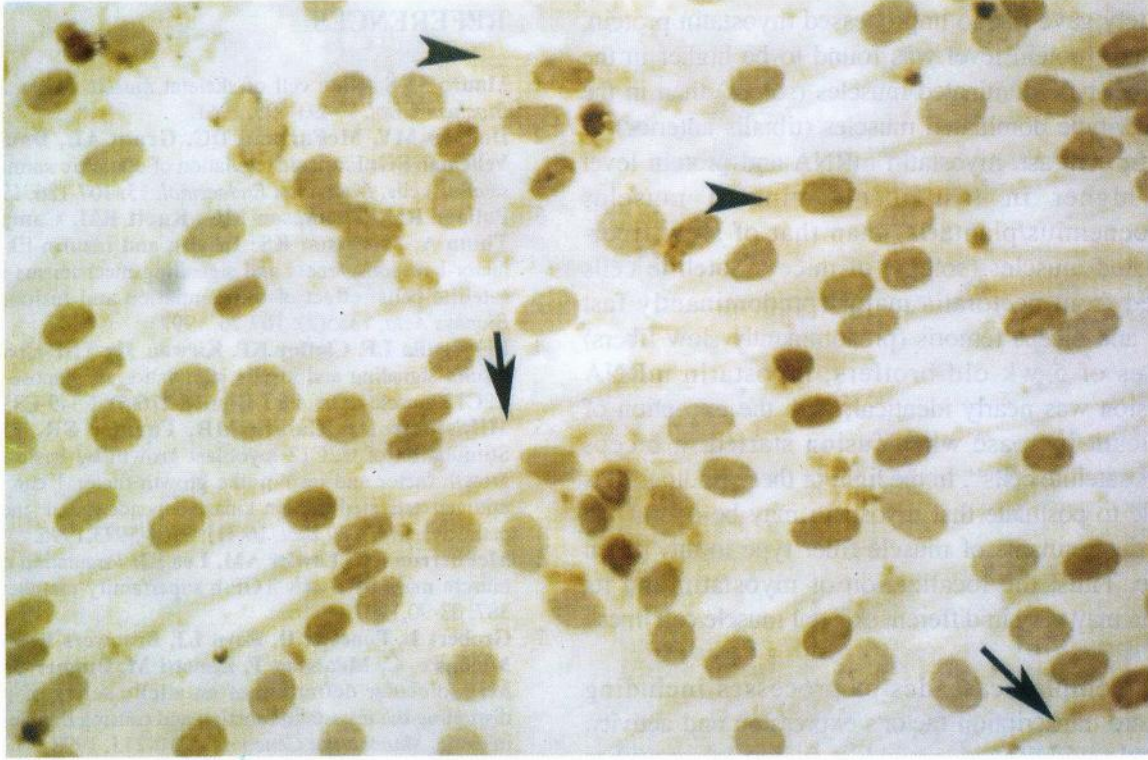


Figure 1. Immunocytochemical staining of unprocessed myostatin expression after stable transfection with an antisense ODN for 24 h. The cytoplasmic (arrow heads) and nuclear brown staining represents unprocessed myostatin expression. Arrows indicate myotube formation. Bar: 50 μ m.

Şekil 1. Bir antisense ODN ile 24 saat transfekte edildikten sonra proses olmamış myostatin'in immunositokimyasal boyanması. Sitoplazmik (ok başı) ve çekirdeksel kahverengi boyanma proses edilmiş myostatin'i göstermektedir. Oklar miyotüp oluşumunu belirtmektedir. Bar: 50 μ m.

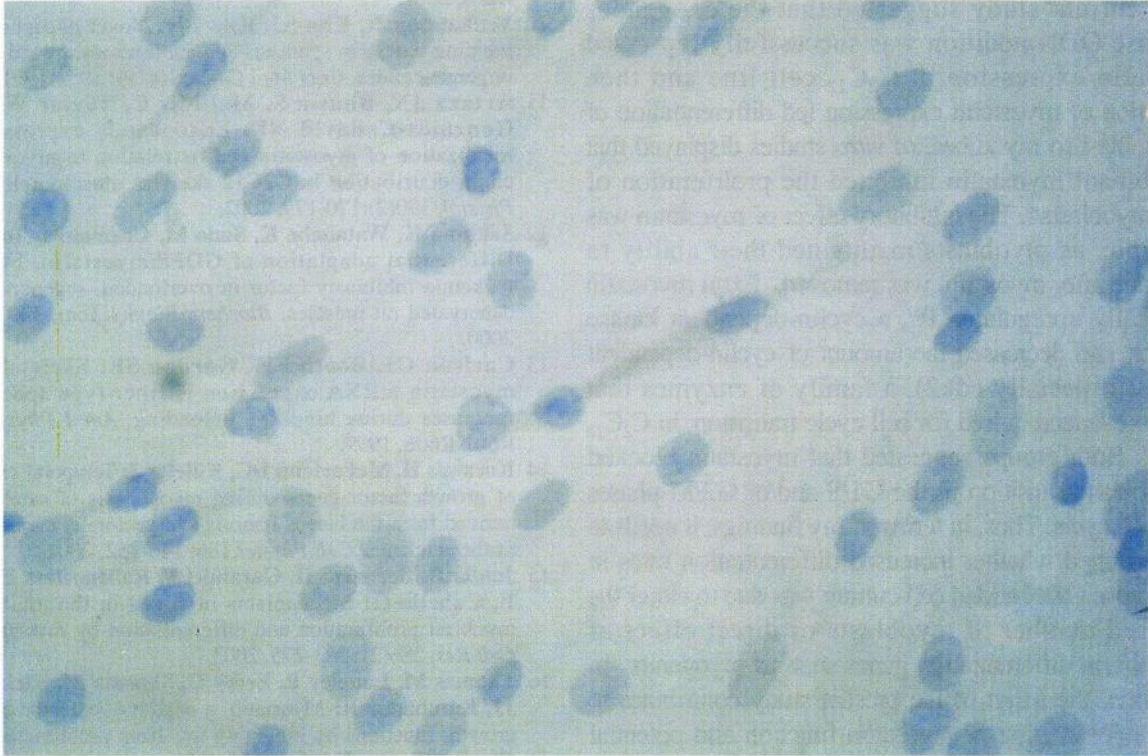


Figure 2. Immunocytochemical staining of unprocessed myostatin expression after stable transfection with a scrambled ODN, as a control, for 24 h. No positive staining was observed for unprocessed myostatin expression. Bar: 50 μ m.

Şekil 2. Kontrol olarak, baz karışımından oluşan bir ODN ile 24 saat transfekte edildikten sonra proses olmamış myostatin'in immunositokimyasal boyanması. Proses edilmemiş myostatin'i gösterir herhangi bir boyanma gözlemlenmedi. Bar: 50 μ m.