The transcriptomic signature of myostatin inhibitory influence on the differentiation of mouse C2C12 myoblasts

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Original article

Abstract

GDF8 (myostatin) is a unique cytokine strongly affecting the skeletal muscle phenotype in human and animals. The aim of the present study was to elucidate the molecular mechanism of myostatin influence on the differentiation of mouse C2C12 myoblasts, using the global-transcriptome analysis with the DNA microarray technique. Treatment with exogenous GDF8 strongly affected the growth and development of C2C12 mouse myoblasts. This was manifested by the inhibition of proliferation and differentiation as well as the impairment of cell fusion. DNA microarray analysis revealed 778 genes regulated by GDF8 in differentiating myoblasts (436 down-regulated and 235 up-regulated). Ontological analysis revealed their involvement in 17 types of biological processes, 10 types of molecular functions and 68 different signalling pathways. The effect of GDF8 was mainly mediated by the disruption of the cell cycle, calcium and insulin signalling pathways and expression of cytoskeletal and muscle specific proteins. The identified key-genes that could play a role as GDF8 targets in differentiating myoblasts are: Mef2, Hgf, Ilb1, Itgb1, Edn1, Ppargc1a.

Key words: GDF8, myostatin, C2C12 differentiation, myogenesis, DNA microarray

Introduction

Myostatin also known as a GDF8 (growth and differentiation factor 8) is a cytokine which belongs to the TGF-β superfamily and is a strong negative regulator of skeletal muscle development. One of the main differences between GDF8 and the other TGF-β superfamily proteins is the fact that it is synthesized in a target tissue – muscles, whereas other factors from the family are produced in neighbouring tissues. GDF8 is synthesized in the muscle cells as a 376 aminoacid propeptide. Similarly to other TGF factors, myostatin aminoacid sequence includes a signal sequence for secretion, a proteolytic processing site and a carboxy-terminal region containing nine cysteine residues (McPherron et al. 1997). The myostatin gene was discovered by McPherron and Lee in 1997 when they bred double-muscled mice with damaged Gdf8 gene (Lee and McPherron 2001). The influence of GDF8 on skeletal muscle growth was examined in a mouse model expressing an insufficient myostatin level, which was manifested by an increased number of fibers (hyperplasia) and augmented fiber size (hypertrophy). The hypertrophic model of Gdf8 dysfunction
was also described in Belgian Blue and Piedmontese cattle demonstrating a double-muscle phenotype (Shi and Garry 2006). Previously published data suggest that the GDF8-related inhibition of myogenesis is exerted through binding to the activin type II receptors (ACT RIIB). Study on chicken myoblasts showed that the highest level of myostatin was observed during the differentiation and fusion of cells (Sato et al. 2006). A high level of exogenous myostatin added to cultured C2C12 cells inhibits their proliferation and differentiation through inactivation of the Rb protein by cyclin-dependent kinase 2 (CDK2) (Charge and Rudnicki 2004). Myostatin also inhibits the cell cycle both in progenitor cells during the embryonic development and in mature skeletal muscles through the induction of the p21 protein and suppression of the cyclin-dependent kinase CDK25 (Wagers and Conboy 2005). Moreover, GDF8 similarly to TGF-β1 causes a significant decrease in the level of myogenin, MyoD and other MRF factors leading to the inhibition of cell differentiation as a consequence of the inhibition of the MyoD-cyclin E complex (Langley et al. 2002). It is important to mention that in the myogenesis process many other factors play regulatory role, like decorin (DCN), follistatin (FST) and IGF I which (stimulate the proliferation and differentiation of myoblasts cells) and desmin (DES) which allows them to fuse. It is well known that myostatin is one of the key factors responsible for the control of skeletal muscle development.

Although biological effects of GDF8 are well described, its molecular mechanism of myogenesis inhibition is not completely elucidated. In the present study the DNA microarray technique was used to examine the whole-genome changes in the C2C12 cells transcriptome induced by exogenous GDF8 treatment as well as to identify a network of genes involved in the GDF8 inhibitory effect on differentiation of mouse C2C12 myoblasts.

**Materials and Methods**

**Media and reagents**

DMEM with Glutamax, phosphate buffered saline (PBS) (pH 7.4), fetal bovine serum (FBS), horse serum (HS) and antibiotics: penicillin-streptomycin, fungizone and gentamycin sulphate were purchased from Gibco BRL (UK). Primary monoclonal rabbit anti-mouse MyHC (H-300) antibody was delivered by Santa Cruz Biotechnology Inc. (USA). Alexa Fluor 488 secondary antibody chicken anti-rabbit IgG and 7-aminoactinomycin D (7-AAD) were purchased from Sigma-Aldrich (Germany). Sterile conical flasks, and Lab-Tek chamber slides were supplied by Nunc Inc. (USA). Sterile Petri’s dishes and disposable pipettes were purchased from Corning Glass Co. (USA).

**Cell culture**

The mouse skeletal cell line C2C12 was purchased from the European Collection of Animal Cell Culture (UK). Cells were cultured as described before (Budasz-Świderska et al. 2005). Experimental medium was supplemented with 20nM GDF8. This concentration of GDF8 has been chosen on the base of our previous experiments, showing its inhibitory influence on myogenesis (Budasz-Świderska et al. 2005). Control medium did not contain any GDF8. On the sixth day, control and experiment cells were harvested for RNA isolation or fixed for staining for confocal microscopy.

**Experimental procedures**

For RNA isolation, C2C12 cells were cultured on Petri dishes in proliferation medium until 80% confluence. The medium was then replaced with a differentiation medium for the next 6 days. Pictures of each stage of differentiation were taken using contrast-phase microscopy. On the sixth day, cells were harvested and stored at -80°C until analysis. For confocal microscopy cells were cultured on 8-chamber Lab-Tek slides until they reached 80% confluence and then were differentiated with DMEM/2% HS for 6 days. Afterwards, cells were fixed by dipping in 0.25% paraformaldehyde.

**RNA isolation and validation,** were done as described previously (Sadkowski et al. 2008, 2009).

**Probe labelling, hybridization, signal detection and quantification**

Gene expression was evaluated using the Agilent whole mouse genome Unrestricted AMADID Release GE 4×44K oligonucleotide microarrays containing 35852 oligonucleotide probes that represent 25000 genes and 38000 transcripts. Total RNA from the cell cultures was extracted, amplified and labeled in accordance with the protocol for Agilent Gene Expression oligo microarrays (Version 5.7, March 2008). On microarray slide RNA from 4 experimental and 4 control cell cultures was used. Data from microarrays were analyzed by Gene Spring™ (Agilent) and differentially expressed genes were identified using t-test with P≤0.05 and a fold change ≥1.6 as the criteria for
significance. In the pathways analysis GeneSpring-Pathway Architect was used to identify the involved pathways.

Confocal Microscopy

The cells were fixed by dipping in 0.25% paraformaldehde and were then incubated in ice-cold 70% methanol. Samples were stored at -80°C until staining. Then cells were incubated for 1 h with the primary antibody (anti-MHC) diluted 1:250 with PBS and after triple washing they were incubated for 1 hour with Alexa Fluor 488 secondary antibody 1:500 (anti-rabbit) at 4°C in the dark. The cells were finally incubated for 30 min with 5 μg/ml of 7-aminoactinomycin D (7-AAD) at room temperature in the dark to stain the DNA of the nuclei. The slides before analysis were stored in the dark at 4°C for 24h. Images from confocal microscope FV-500 (Olympus Opticals, Germany) were analyzed using the MicroImage software (Olympus, Germany). For each culture stage, 15 pictures were taken.

Results

The influence of GDF8 on the differentiation of C2C12 myoblasts

GDF8 (20nM) caused a strong inhibitory effect on the differentiation of mouse C2C12 myoblasts, which was manifested by the impaired formation of myotubes. Contrary to GDF8-treated cells, in control cultures myotubes appeared in the 48h of the differentiation period. (Fig. 1a, upper vs lower panel). Inhibition of the differentiation of the C2C12 myoblasts by GDF8 was also shown by the immunofluorescence analysis of the expression of the myosin heavy chain protein in cultures on the sixth day of myoblasts differentiation. MyHC-related fluorescence was visible only on confocal images containing myotubes from the control cultures (Fig. 1b, upper panel). There was no MyHC expression in the GDF8-treated cultures (Fig. 1b, lower panel). Integrated optical density (IOD) was calculated using the MicroImage software and revealed a significantly (P<0.0001) lower expression of MyHC in the GDF8-treated myoblasts in comparison with control cells (Fig. 1c).

The influence of GDF8 on the transcriptomic profile of differentiating C2C12 myoblasts

Comparison of the transcriptomic profiles between cells treated with GDF8 (20nM) and control cultures revealed 778 genes with statistically significant differences in expression between those two cultures, with at least an 1.6-fold change. Moreover, 1586 genes with at least 1.3-fold change were detected. Computational analysis of the results obtained using the GeneSpring software showed that GDF8 treatment caused down-regulation of 543 genes and up-regulation of 235 genes with at least an 1.6 fold change. The ontological analysis of these regulated genes using the Panther and KEGG enabled us to classify the identified genes in relation to a biological process, molecular function, and metabolic pathway.

Classification of the identified genes using the Panther software revealed 489 mapped transcript IDs. Analysis of the molecular function showed 10 groups of genes, significantly affected by GDF8 treatment (Fig. 2). The majority of genes were associated with the following molecular functions: binding processes (172) with the main groups: protein binding (110) including nucleic acid binding (67) and calcium ion binding (20); catalytic activity (161) with the main group showing hydrolase activity (69); structural molecule activity (66) containing a large group of structural constituent of cytoskeleton (50); enzyme regulator activity (40) with G-protein coupled receptor activity (12) and 38 genes with a transcription regulator activity.

Classification of genes according to biological processes in which they are involved revealed 17 processes significantly affected by GDF8 treatment (Fig. 3). The highest number of genes was involved in: cellular process (237) with a large group of 159 genes involved in cell communication; metabolic process (230) with the main group of regulating genes for nucleic acids (92), protein metabolism (101) and carbohydrate metabolism (32); cell communication (159) and developmental process (118).

Functional analysis of the regulated genes using the KEGG Brite database showed that the majority of the identified genes encodes proteins playing enzymatic role (102), the majority of them are either kinases or peptidases; receptors and channels (47) with big group of G-protein coupled receptors; cytoskeleton proteins (28) with the main group of actin cytoskeleton proteins; chromosome – especially histones and centromere proteins (24); ubiquitin system (22) and transcription factors (21).

Analysis of the obtained results using the Pathway Architect (GeneSpring-Agilent) revealed connections between the products of genes identified in this study and genes previously described in the literature. This enabled us to create a network of reciprocal interactions between protein products of the investigated genes. In this network there are also proteins of special significance, which formed junctions converg-
Fig. 1. a – Six day differentiation process of C2C12 myoblasts (100x magnification). Control culture stimulated to differentiation in DMEM/2% HS for 48, 72, 96 and 144 hours (upper panel). Experimental culture maintained in the above mentioned medium supplemented with 20nM of GDF8 (lower panel); b – Images from confocal microscopy showing expression of MyHC (AlexaFluor 488 – green) and the DNA of nuclei (7-AAD – red) after six days of differentiation in control (upper panel) and GDF8 treated culture (lower panel); c – Influence of exogenous GDF8 on the MyHC protein level in differentiating C2C12 myotubes. Results are showed as mean ± SEM of MyHC integrated optical density (IOD). Means were calculated as a mean value of 15 digital images. Values a and b indicate a significant statistical difference (P=≤0.0001) between experimental culture treated with 20nM GDF8 and control culture analyzed with the t-Student test (n=4).
Fig. 2. Numbers of identified genes of which expression was changed in C2C12 cells under GDF8 treatment, according to their molecular function. Analysis performed with the Panther classification software.

Fig. 3. Numbers of identified genes, of which expression was changed in C2C12 cells under GDF8 treatment, according to the biological process they were involved in. Based on Panther classification software.

Discussion

GDF8 (myostatin) is one of the key regulators of skeletal muscle growth and development. It is a very interesting cytokine because of its potent intramuscular action which is still not fully understood. In the present study we showed that the potent GDF8-related inhibition of cell differentiation (Fig. 1) was accompanied by a significant change in the expression of hundreds of genes (Table 1). The bioinformatic analysis of the transcriptomic profiles of cells treated with exogenous GDF8 revealed 778 transcripts with a different expression (at least a 1.6-fold change) when compared to control culture. Since the majority of the GDF8-regulated genes were down-regulated, we hypothesized that GDF8 mainly shows an inhibitory effect on the gene expression. Comparative analyses of our results using diverse bioinformatical databases

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allowed us to clarify the mechanism by which GDF8 inhibits myoblasts differentiation. The ontology analyses of the regulated genes were performed using the Pathway Architect (GeneSpring) software as well as the Panther and KEGG online databases. We designed a network of interactions for regulated genes, which are particularly important due to their location at the intersection of many metabolic pathways which could play a key role as GDF8 targets during the differentiation of myoblasts (Fig. 4). The proteins encoded by these genes are localized in different cell compartments and are involved in different molecular functions but they could create a functional network, which regulates GDF8-dependent inhibition of proliferation and differentiation of muscle cells. Interpretation of this type of high-throughput data analysis is not simple due to the lack of knowledge about the function of many regulated in myogenesis genes and difficulties to assess whether they are regulated by GDF8 directly or indirectly.

Exogenous GDF8 changed the expression of many genes and influenced many metabolic and signalling pathways. From our point of view the most important are these which are related with tissue development (since GDF8 inhibited the development of muscle tissue), with calcium homeostasis (calcium is a key signalling molecule within muscle cells), with development of muscle-specific diseases, with insulin signalling (insulin is a key hormone regulating muscle cell growth and development) and with the cell cycle (since inhibition of the differentiation of myoblasts must be connected with the cell cycle regulation) (Fig. 5).

GDF8 treatment decreased the expression of genes encoding cytokines and their receptors i.e. Hgf (↓1.75) and Ilb1 (↓2.06). HGF is a multi-functional cytokine playing a major role in embryonic organ development and is one of the most important growth factors engaged in the regeneration of organs and satellite cell activation in skeletal muscles (Shi and Garry...
The transcriptomic signature of myostatin inhibitory influence...

Fig. 5. Network of the main identified cell processes inhibited by GDF8 which lead to the suppression of differentiation and metabolism regulation of myoblasts.

ILBI is an important mediator of the inflammatory response, involved in a variety of cellular activities, including cell proliferation, differentiation and apoptosis. Those two genes have a particularly wide spectrum of connections with other GDF8-regulated genes, which assures that Hgf and Ilb1 could be a target for GDF8 by which the latter inhibits the differentiation of myoblasts. Recent studies demonstrated that HGF triggers the activation and entry of myoblasts into the cell cycle in response to mechanical perturbation, and the subsequent expression of myostatin may signal a return to cell quiescence (Yamada et al. 2009).

GDF8 treatment resulted in the down-regulation of many genes specific for the first stage of differentiation process which were earlier described by other authors: Mef2 and Myh-1,-2,-8,-6,-14 genes, Fgf-1,-5,-9,-11,-13,-15,-18, Myf6, Csrp3, Olr1, Igb1bp2, Igb1, Galnt5, Galnt6 and up regulation of Neo1, Lfng (Janot et al. 2009). We also observed an up-regulation of the following genes: Ccna2, Ccnb1, Ccnb2, Ccne1, Ccni, Ceng and Cdk-4,-6,-3. The overexpression of cdk/cyclins has been reported to inhibit the activity of MyoD and to prevent myogenic differentiation by different modalities (De Falco et al. 2006). GDF8 treatment resulted in an increased expression of some genes typical for undifferentiated cells, namely Igfbp5, Myl2, Tnii3, Ckm (Shen et al. 2003). Another response to GDF8 treatment was the increased expression of Pax7 and Sox8. The down-regulation of Pax7 by constant MyoD expression is described to be the initial signal for differentiation of the cells (Diel et al. 2008). All these transcriptomic changes indicate that GDF8-treated myoblasts did not reach the status of differentiated muscle cells.

Analyses of the signalling pathways with the KEGG database enabled us to select GDF8-related genes which are important for the development of muscle cells and play a crucial role as marker genes for skeletal muscle dystrophy (Dmd ↓ 2.12, Dmpk ↓ 1.34, Tcap ↓ 2.57, Capn3 ↓ 1.68, Cav3 ↓ 1.67, Col6a2 ↓ 1.26, Myot ↓ 2.42, Neb ↓ 1.96, Pomt1 ↓ 1.80, Tpm3 ↓ 1.34, Tn ↓ 1.98) and HCM (Hypertrophy Cardiomyopathy) (Myl2 ↓ 4.18, Mybpc3 ↓ 3.27, Tnni3 ↓ 2.93, Dmd ↓ 2.12, Igfa9 ↓ 1.98, Tn ↓ 1.98, Igb1 ↓ 1.90, Igb6 ↓ 1.74, Galnc1s ↓ 1.68, Tpm3 ↓ 1.52, Tpm3 ↓ 1.34, Cacna2d1 ↓ 1.28, Igb6 ↓ 1.28, Tnc ↑ 1.40, Igb4 ↑ 1.21, Igb3 ↑ 1.43, Edn1 ↑ 1.85) (Morimoto 2008, Ho 2010). Based on the GeneSpring signalling pathways and literature we found that another two genes correlated with this muscle disease. Mef2c (↑ 2.19) and Mef2d (↓ 1.26) were down-regulated (Bachinski et al. 2010, Konno et al. 2010). MEF2 is a calcium-dependent transcription factor, regulator of the cell division, differentiation and death related with myotonic dystrophy and other neuromuscular disorders. In our study we observed that 4 important MEF2 target genes during muscle diseases, namely Atp2a2 (↓ 1.50), Myh6 (↓ 2.01), Myom1 (↓ 1.96), MyoT (↑ 2.42) were down-regulated but no changes were observed in the level of the four well known Mef2 regulators (Hdac4, Neoa2, Nfat5 and Pp3c5a). However, we observed changes in the expression of other genes from the Hdac group: Hdac3 ↑ 1.09 (and its regulator Dbc1 ↑ 1.47), Hdac7a ↑ 1.29, Hdac9 ↑ 1.68 (Bachinski et al. 2010).

Analysis with the Panther and KEGG databases showed that GDF8 down-regulated 12 genes involved in the calcium signalling pathway. It acted on different levels: on the receptors, binding proteins and the Ca2+-dependent proteins (Fig. 6). The decrease in the expression is observed among genes connected with receptor channels, both voltage-operated (Cacna1s) and receptor-operated channels as well as cholinergic and purinergic receptors (P2rx1, Ptgfr and Erbb3). These genes were described before by Berridge (2003) as important elements of the calcium signalling pathway. Another group of genes with a decreased expression were the ones encoding calcium ion binding proteins (F2, Camk2b, Hgf, Myl6b, Cd93, Myl2, Casq1, Prkaa2, Efemp1, Tesc, Wifi and Capn3). Decrease in the expression also was observed for the Atp2a1 gene encoding endoplasmic reticulum ATP-ase, responsible for Ca2+ transport. The inhibition of the calcium signalling pathway by GDF8 could disturb the proper functions of many calcium dependent transcription factors like MEF2, CREBs, and many kinases and cyclins factors (Iseenberg 2004, Bachinski et al. 2010).

The functional analyses of the regulated genes revealed two other pathways that should be considered as mostly affected by exogenous myostatin treatment, namely glucose metabolism pathway and insulin pathway. Among the regulated genes there was Ecdn (↑ 1.85) which impairs insulin-stimulated glucose uptake (Shemyakin et al. 2010). This confirms our hypothesis that GDF8 action could occur through the
insulin signalling pathway. EDN1 is a potent vasoconstrictor and mitogen playing a role in the development of hypertrophy and in the progression of heart failure through the mitogen-activated protein kinase (MAPK) pathway involved in various cellular functions, including cell proliferation, differentiation and migration (Choukroun et al. 1998). Our analyses using the KEGG database revealed that GDF8 increased Edn1 expression. This was accompanied by a simultaneous decrease of the MAPK signalling cascade and G-protein related signalling genes. GDF8 regulated also genes involved in glucose metabolism. It was observed at the level of insulin synthesis (Ins2), glycolysis (Pparg1a) and glycogenesis (Pygm, Prkaa2). Pparg1a ($\downarrow_{1.97}$) is one out of the potential key genes which modulates transcription of genes specific for oxidative phosphorylation in heart and skeletal muscles (Mootha et al. 2003). In case of genes related to this process we observed down-regulation of Cox7a1, Cox8b, Aptbvd2, Atp6v1c2, which were not described earlier as connected with the GDF8 action. There was also a decrease in the expression of lipogenesis inhibitors (AMPK Prkaa2) and Slec4a, a GLUT4-associated gene, which are responsible for enhancement of glucose uptake. These results are convergent with Guo (2009) and McPherron (2002) who observed in double-muscled mice fat tissue accumulation and increase of insulin sensitivity decreased. Our results also indicate that myostatin can disrupt the cell cycle leading to inhibition of cell proliferation and differentiation. GDF8 regulated many genes linked with chromosome organization (Hist1h1b, Hist1h2ak, Hist1h2af, Hist1h2ai, Ncapg, Top2a, Cenpa, Cenpf, Cdeca8, Aurkb, Spc25, Bub1b, Bub1, Ttk, Sgo1, Esco2, Chaf1b, Hdac9, Cac5, Nuf2, Kif2c, Sgo2, Aurka, Ptk1, Cenpm), replication (Ccnb1, Top2a, Ccnb2, Exo1) and DNA repair and recombination (Rad54b, Polq, Exo1). We observed a changed expression of many transcription factors: Sim1, Rarg, Mef2c, Cux2, Hnf1b, Irf6, Ttp63, Esrrg, Zscan4c, Csp3, Arx, Tbx15 (down-regulated) and Msc, Mxd3, Wt1, Foxd1, Myb, Nr1i2, Otx1, Pax6, Foxm1 (up-regulated). It suggests that at the transcriptional level GDF8 inhibits muscle growth and development at the level of replication, protein synthesis and cell arrest in a manner similar to that mediated by TGF-$\beta$1 – cytokine that we examined before (Wicik et al. 2010). It is known that GDF8 mediates the cell cycle but the knowledge about the inhibition of cell proliferation and differentiation is still obscure (Thomas et al. 2000, Joulia et al. 2003). It also seems to be important to point out that GDF8 significantly regulated Gdf1 ($\downarrow_{1.36}$), Gdf5 ($\downarrow_{2.06}$), Gdf6 ($\downarrow_{1.61}$), Nodal ($\downarrow_{1.08}$) and Mdfic ($\downarrow_{1.25}$) which are positive myogenesis regulators; GDF8 inhibitors Cav3 ($\downarrow_{1.67}$) and Fst ($\downarrow_{1.33}$) and Tgfbl1

Fig. 6. Network of GDF8- regulated genes products (dark grey) with fold-change at least 1.6 and gene products known from literature (light grey) involved in the calcium signalling pathway. The analysis was performed using the KEGG database.
(↑ 1.26) Smad5, (↑ 1.16) and Smad6 (↑ 1.16) playing an important role in TGFβ1 signal transduction (Ostrander et al. 2009).

Conclusions

In conclusion, we observed the effects of exogenous myostatin on many levels and the inhibition of muscle differentiation mediated by this cytokine occurred through transcriptional regulation of replication and inhibition of binding protein synthesis. Based on the obtained results and available literature we hypothesize that the main inhibitory effect of myostatin is exerted by 1) down-regulation of a wide spectrum of genes involved in the developmental processes and down-regulation of key muscle gene: Mef2, Hgf, IIb1, Itgb1, Edn1, Ppargc1a; 2) impairment of cell communication and inhibition of muscle cytoskeleton compounds synthesis; 3) impairment of the calcium signalling pathway and insulin pathway.

The implementation of microarray technique allowed us to perform the comprehensive analysis of GDF8 action on differentiating myoblasts and showed myostatin-dependent signalling pathways. It could be of interest to compare our results with the results of GDF8 deprivation in the culture of differentiating myoblasts to better understand this cytokine mechanism of action and its primary and secondary interactions.

All of the 728 identified genes are presented in Table 1 available at the website http://www.sadkowski.info/knf/microarray/gdf8/index.html

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