Geranylgeraniol-induced Myogenic Differentiation of C2C12 Cells

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Abstract. *Background: Geranylgeraniol (GGOH) is a C20 isoprenoid found in fruits, vegetables, and grains, including rice. As a food substance, GGOH is categorized as ‘Generally Recognized as Safe’. GGOH is an intermediate product in the mevalonate pathway and acts as a precursor to geranylgeranylpyrophosphate. Materials and Methods: C2C12 mouse myoblasts derived from muscle satellite cells were used. Quantitative reverse-transcriptase polymerase chain reaction, western blotting analysis, and immunocytochemical analysis were performed to respectively assess mRNA expression, protein levels, and the number of myofibers. Results: GGOH reduced the expression levels of skeletal muscle atrophy-related ubiquitin ligases in myofibers derived from C2C12 cells. GGOH induced myogenic differentiation of C2C12 cells via geranylgeranylation. GGOH did not adversely affect the proliferation of C2C12 cells. Conclusion: GGOH induces myoblast differentiation in C2C12 cells.*

Satellite cells are skeletal muscle stem cells residing beneath the basal lamina that provide myonuclei for postnatal muscle growth, repair, and regeneration in adults. Satellite cells are

activated in response to muscle injury, proliferate extensively and then differentiate into myoblasts. This is accompanied by the transcriptional up-regulation of myogenic differentiation (*MYOD*) and other myogenic differentiation marker genes, such as myogenin (*MYOG*), creatine kinase M-type (*CKM*), and myosin heavy chain (*MYHC*) (1-3). Insulin-like growth factors (IGFs) are essential for skeletal muscle development, regeneration, and hypertrophy – processes which all require satellite cell activation and differentiation (4-6). The autocrine action of IGF2 is especially critical for the differentiation of satellite cells *in vitro* (7). C2C12 cells are a murine myoblast cell line derived from satellite cells (8). C2C12 cells are commonly used as an *in vitro* model of muscle regeneration due to their ability to transition from a proliferative phase into differentiated myofibers, similar to satellite cells, upon adequate stimulus (3).

Statins act by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the first step of the isoprenoid biosynthetic pathway and the rate-limiting step of cholesterol biosynthesis (9). Statins are used as a frontline therapy for lowering plasma cholesterol and preventing cardiovascular disease (10-13). Statins are generally safe and effective. However, they may induce a variety of skeletal muscle-associated, dose-dependent adverse reactions that range from muscle pain to muscle cell damage and severe rhabdomyolysis (14-17). These statin-associated muscle side-effects are prevalent in about 10% of patients (18). Statin-associated muscle disorders are likely due to inhibition of the synthesis of crucial intermediary molecules such as geranylpyrophosphate and geranylgeranylpyrophosphate (GGPP) (19-21). Treatment of C2C12 cells with GGPP was found to reverse the suppressive effect of statin on myotube formation (22). Geranylgeraniol (GGOH), a precursor to GGPP, reduced

This article is freely accessible online.

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Key Words: Sarcopenia, myogenesis, statin, geranylgeranylation, C2C12 cells.

muscle cell damage induced by statin treatment *in vitro* (23). Thus, GGOH seems to have protective effects on skeletal muscle. However, the extent of this potentially beneficial effect remains unknown. In this study, the effect of GGOH on myogenesis in C2C12 cells was investigated.

Materials and Methods

Cell culture, transfection, and skeletal muscle differentiation. C2C12 murine myoblasts were purchased from American Type Culture Collection (Manassas, VA, USA). C2C12 cells were maintained as previously described (24) and cultured in the presence of 0, 5, 10, 50, or 100 μ M GGOH (Sigma–Aldrich Chemicals, St. Louis, MO, USA) and 100 μ M of the geranylgeranyltransferase I inhibitor *N*-[4-[2(*R*)-amino-3-mercaptopropyl]amino-2-(1-naphthalenyl)benzoyl]-L-leucine methyl ester trifluoroacetate salt (GGTI-298) (Tocris Bioscience, Bristol, UK). Skeletal muscle differentiation in C2C12 cells was induced by culturing cells with Dulbecco's modified Eagle's medium supplemented with 2% horse serum (Biosera, Kansas City, MO, USA) for 5–8 days (3).

Reverse transcription and quantitative polymerase chain reaction (qPCR) analysis. Total RNA was isolated from C2C12 cells using FastGene RNA Basic Kit (Nippon Genetics, Tokyo, Japan) and then reverse-transcribed into cDNA using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). SYBR green-based qPCR was performed in 96-well plates using PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA, USA) with QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific). Values were normalized to β -actin (*Actb*) using the $2^{-\Delta\Delta C_t}$ method (25). The following primers were used for qPCR analyses: qPCR for murine *Fbxo32*: forward: agtgaggaccgctactgtg, reverse: gatacaacgcttgccaatct; tripartite motif containing 63 (*Trim63*): forward: tgacatctacaagcaggagtg, reverse: tcgtctctgtgttccttcg; murine *Igf2*: forward: cctccttaccacacttcaggt, reverse: aagagatgagaagcac caacatc; murine *Myog*: forward: ccttgctcagctccctca, reverse: tgggagtgctactcctgg; murine *Myod*: forward: agcactacagtggcgactca, reverse: ggccgctgtatccatcat; murine *Ckm*: forward: cagcacagacag acactcagg, reverse: gaactgtgtt gggtgttgc; and murine *Actb*: forward: aaggccaaccgtgaaagat, reverse: gtggtacgaccagagcatcac.

Proliferation assay. C2C12 cells were cultured in the presence of GGOH at concentrations of 0, 5, 10, 50, or 100 μ M for 1, 2, or 3 days. Proliferation of C2C12 cells was assessed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's protocol.

Immunocytochemical analysis. C2C12 cells were fixed by 4% formaldehyde for 10 min and incubated with primary antibodies for 1 hour at room temperature following blocking/permeabilization with phosphate-buffered saline containing 0.3% Triton X100 and 5% goat serum for 30 min at room temperature. Mouse monoclonal antibody to MYHC (R & D Systems, Minneapolis, MN, USA) was used for immunocytochemistry. The target protein was visualized using an Alexa 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) and imaged with an ABZ-9000 microscope (Keyence, Tokyo, Japan).

Western blot analysis. The following mouse monoclonal antibodies were used for western blot analysis: anti-MYOG (Santa Cruz

Figure 1. Geranylgeraniol (GGOH) reduces the expression levels of skeletal muscle atrophy-related ubiquitin ligases in myofibers derived from C2C12 cells. C2C12 cells were cultured with 2% horse serum for 5 days and then treated with or without (Ctrl) 50 μ M GGOH for another 3 days. Total RNA was isolated, then *F*-box protein 32 (*Fbxo32*) (A) and tripartite motif containing 63 (*Trim63*) (B) mRNA levels were analyzed using quantitative polymerase chain reaction. All data are expressed as the mean $\pm$ SD ($n=3$). *Significantly different at $p<0.01$ versus vehicle-treated cells. Similar results were obtained by three independent experiments.

Biotechnology, Santa Cruz, CA, USA), anti-MYHC (R & D systems), and anti-ACTB (Sigma–Aldrich Chemicals). The target proteins were detected using an anti-mouse or anti-rabbit IgG antibody conjugated with a horseradish peroxidase (Cell Signaling, Beverly, MA, USA) and visualized by using ImmunoStar LD (WAKO, Osaka, Japan).

Statistical analysis. Comparisons were made using an unpaired Student's *t*-test; the results are shown as the means $\pm$ S.D. Statistical significance was defined as $p<0.05$.

Results

Firstly, it was confirmed whether GGOH protects skeletal muscle fibers derived from C2C12 cells from atrophy. Treatment of cells with 50 μ M GGOH for 3 days reduced the expression levels of muscle atrophy-related ubiquitin ligase *Fbxo32* (Figure 1A) and *Trim63* (Figure 1B).

Next, we examined the effect of GGOH on skeletal muscle differentiation in C2C12 cells. qPCR analysis revealed that although 50 μ M GGOH treatment for 2 days did not change the expression level of *Myod* (Figure 2A), it did enhance the induction of early-stage myogenic marker genes such as *Myog* and *Ckm* (Figure 2B and C). GGOH treatment also led to a dose-dependent increase in the protein level of MYOG (Figure 2F) as well as of the late myoblast marker MYHC (Figure 2G and H). In addition, GGOH dramatically stimulated the expression level of *Igf2* in C2C12 cells (Figure 2D). The

Figure 2. Geranylgeraniol (GGOH) induces myogenic differentiation of C2C12 cells via geranylgeranylation. C2C12 cells were treated with or without (Ctrl) 50 μ M GGOH for 2 days. Myogenic differentiation (*Myod*) (A), myogenin (*Myog*) (B), creatine kinase, M-type (*Ckm*) (C), and insulin-like growth factor-2 (*Igf2*) (D) mRNA levels were analyzed using quantitative polymerase chain reaction. All data are expressed as the mean $\pm$ SD ($n=3$). *Significantly different at $p<0.01$ versus vehicle-treated cells. Cells were treated with 0, 5, 10, 50, or 100 μ M GGOH for 3 (F) or 5 (G) days. The protein levels of MYOG (F) and myosin heavy chain (MYHC) (G) were assessed by western blotting analysis. Immunocytochemical analysis was performed using antibody to MYHC on day 5. Scale bar represents 10 μ m (H). Cells were treated with or without 50 μ M GGOH in the presence or absence of 100 μ M GGTI-298 for 3 days. The protein levels of MYOG were determined by western blotting analysis (I). Similar results were obtained by three independent experiments (F-I). Myogenic differentiation of C2C12 cells was induced by the treatment with Dulbecco's modified Eagle's medium supplemented with 2% horse serum (Myo-Media) (A-F).

positive effect of GGOH on the induction of *Myog* was blocked by the addition of the geranylgeranyl transferase inhibitor GGIT-298 (Figure 2I), suggesting that the augmentative effect of GGOH on myogenic differentiation is via geranylgeranylation. GGOH did not adversely affect the proliferation of C2C12 cells (Figure 3).

Discussion

In the present study, we examined the effect of GGOH on fiber degradation, differentiation, and proliferation in C2C12 cells.

GGOH is a C20 isoprenoid found in fruits, vegetables, and grains, including rice. As a food substance, GGOH is categorized as 'Generally Recognized as Safe' (26). GGOH is an intermediate product in the mevalonate pathway and

acts as a precursor to GGPP. In the cell, GGOH is thought to be subsequently converted into the pyrophosphate moiety, GGPP, by two successive monophosphorylation events (27). GGPP induces geranylgeranylation, which is necessary for the membrane localization of intracellular proteins, particularly the small GTP-binding proteins rat sarcoma virus oncogene (RAS), ras homolog family member (RHO), Rac family small GTPase (RAC) and Rap GTPase (RAP) (28). In our study, GGTI-298, an inhibitor of geranylgeranylation, eliminated GGOH-induced myoblast differentiation of C2C12 cells (Figure 2I), suggesting that GGOH-induced myogenesis requires geranylgeranylation of certain protein(s). Further experiments are needed to elucidate the mechanism that underlies GGOH-induced myoblast differentiation.

Inhibition of the isoprenoid biosynthetic pathway by statins may be involved in the occurrence of statin-associated

Figure 3. Geranylgeraniol (GGOH) does not affect the proliferation of C2C12 cells. C2C12 cells were cultured in the presence of 0, 5, 10, 50, or 100 μ M GGOH for the indicated times. The proliferation of cells was assessed using a Cell Counting Kit-8. All data are expressed as the mean $\pm$ SD ($n=3$).

muscle problems (19, 21), suggesting that GGOH may be a potential drug for the prevention or treatment of statin-associated muscle problems without interfering with the beneficial plasma cholesterol-lowering effects of statins.

Skeletal muscle atrophy, such as observed during sarcopenia, is characterized by an associated loss of skeletal muscle mass and a decrease in muscle strength that impairs healthy life expectancy (29). The prevalence of sarcopenia is approximately 10% among adults aged 60 years or older (30), resulting in a cost of over US\$18 billion in 2001 alone (31). Thus, there is an unmet and urgent need for strategies that will prevent the loss of skeletal muscle fibers and stimulate the proliferation and differentiation of skeletal muscle stem cells such as satellite cells for improving skeletal muscle mass and function.

In conclusion, we demonstrated that in C2C12 murine skeletal muscle myoblasts cells, GGOH reduced the expression of muscle atrophy-related genes and enhanced myogenic differentiation. Since GGOH is categorized as 'Generally Recognized as Safe' and can be taken orally (32), upon the establishment of a suitable protocol for GGOH treatment, our data will provide the basis for a new potential product for the treatment of muscle atrophy.

Conflicts of Interest

The Authors declare that they have no conflict of interests in regard to this study.

Acknowledgements

This study was supported by The Japan Health Foundation (S. K.).

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Received August 15, 2018
Revised September 20, 2018
Accepted September 26, 2018