Low-magnitude vertical vibration enhances myotube formation in C2C12 myoblasts

Chau-Zen Wang,1,2 Gwo-Jaw Wang,2,3,4 Mei-Ling Ho,1,2 Yan-Hsiung Wang,2,5 Ming-Long Yeh,6 and Chia-Hsin Chen7,8,9

1Department of Physiology, Kaohsiung Medical University and 2Orthopaedic Research Center, Kaohsiung Medical University, Kaohsiung, Taiwan; 3Department of Orthopaedic Surgery, University of Virginia, Charlottesville, Virginia; 4Department of Orthopaedics, College of Medicine, Kaohsiung Medical University Hospital and 5School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan; 6Institute of Biomedical Engineering, National Cheng Kung University, Tainan, Taiwan; and 7Departments of Physical Medicine and Rehabilitation, Kaohsiung Municipal Ta-Tung Hospital, 8Department of Physical Medicine and Rehabilitation, and 9Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

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Low-magnitude vertical vibration enhances myotube formation in C2C12 myoblasts. J Appl Physiol 109: 840–848, 2010. First published July 15, 2010; doi:10.1152/japplphysiol.00115.2010.—Whole body vibration training is widely used in rehabilitation and sports activities to improve muscle strength, balance, and flexibility. However, the molecular mechanisms of vertical vibration (VV) training and their effect on the myogenesis of myoblasts remain undefined. This study was undertaken to address the hypothesis that VV can enhance the expression of ECM proteins and myogenic regulatory factors (MRFs) in myoblasts and, in turn, increase myotube formation.

Using real-time PCR, Western blot analysis, and immunofluorescence studies, we examined the effect of VV treatment with frequencies of 5, 8, or 10 Hz on the expression of ECM proteins and MRFs as well as myotube formation in C2C12 myoblasts. We showed that VV stimulation is safe and effective at stimulating myogenesis in C2C12 myoblasts. The levels of expression of the ECM proteins type I collagen and decorin were the highest after VV treatment at frequencies of 8 and 10 Hz. Expression of the MRFs MyoD and myogenin increased after VV stimulation in a time- and dose-dependent manner. The total number of myotubes formed, as well as the length and the average area of myotubes, were substantially increased following VV treatment at frequencies of 8 to 10 Hz. In conclusion, VV treatment at frequencies of 8 to 10 Hz can stimulate the expression of ECM proteins and MRFs in myoblasts and, in turn, increase myotube formation.

WHOLE BODY VIBRATION, myoblast; myogenesis; myotubes

Four MRFs (MyoD, Myf-5, myogenin, and MRF4) have been shown to induce myogenic conversion of nonmuscle cell lines, including fibroblasts, chondrocytes, and neurons (41). In addition, these MRFs are upregulated in satellite cells, the stem cells of adult skeletal muscles, after reactivation from a mitotically quiescent state following stress induced by exercise or trauma (28). Each MRF has been shown to play a specific role in myogenesis (1, 28, 41). Of interest, induction of MyoD expression is a key step in the commitment of somite cells to the myogenic lineage and has become a marker of activated and proliferating satellite cells (20). Myogenin, additionally, plays a critical role in the myogenesis of myoblasts and is a marker of terminal myoblast differentiation (33, 48). Previous gene knockout studies have shown that the myogenin gene product is required for myoblast formation during muscle development (7). Specifically, in differentiating C2C12 cells, myogenin promotes the fusion of myotubes and the formation of myofibers (33). A unique temporal expression pattern also exists for these MRFs, with early MyoD activation leading to subsequent myogenin expression (48). Thus, the expression of both MyoD and myogenin indicates a complete progression of myoblast proliferation, differentiation, and fusion into myotubes, which then leads to muscle regeneration and growth.

In addition to MRFs, it has been shown that the ECM is required to ensure myoblast migration, proliferation, and differentiation (19). Similarly, the ECM has been shown to be capable of adapting to changes in the external environment, such as mechanical loading or inactivity and disuse, specifically with collagen levels responding to altered levels of physical activity (14, 19). Central to this regulatory role for collagen in muscle differentiation is the interaction between type I collagen and proteoglycans such as decorin (45). It has been shown that the decorin core protein can influence the rate and extent of collagen fibrillogenesis (40, 55). One possible mechanism for the role of decorin has been suggested in a recent study (18), which demonstrated that decorin enhances myoblast proliferation and differentiation by suppressing myostatin, a member of the TGF-β superfamily, that has inhibitory effects on myoblast proliferation and differentiation. Accordingly, expression of decorin and collagen I results in enhanced myoblast proliferation and leads to subsequent differentiation of myotube hypertrophy. Thus, measuring these ECM components, in addition to the expression of MRFs, can provide a
addition, the effect of VV treatment on the expression of the ECM components type I collagen and decorin was evaluated.

METHODS

Antibodies. Anti-Myo-D (C-20) and anti-myogenin (5-FD) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal sarcomeric myosin antibody (MF-20) was purchased from clone 8E5 (PY) (a gift from J. Engel, National Institutes of Health, NIH, Bethesda, MD). Mouse anti-β-actin was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell culture and VV stimulation. The mouse myoblast satellite cell line, C2C12 (CRL-1772; American Type Culture Convention, Manassas, VA), was maintained in DMEM (GIBCO-BRL, Grand Island, NY) containing 10% FBS (GIBCO-BRL) in a humidified atmosphere of 5% CO2 at 37°C. Cells were used before the 10th passage. To evaluate the effect of VV stimulation on the myogenesis of C2C12 myoblasts, cells were seeded at a high cell density (1 × 10^4 cell/cm^2). When cells grew to confluency, they were subjected to vibration stimulation, generated by a vertical platform (BodyGreen, Albany, North Shore, New Zealand), at a frequency of 5, 8, or 10 Hz with 0.4 mm amplitude for 10 min/day (Fig. 1A).

Quantitative real-time PCR. Total RNA was isolated from cells using Trizol reagent (GIBCO-BRL). Reverse transcription of the RNA into cDNA was performed using oligo(dt) primers, and the Moloney murine leukemia virus reverse transcriptase. Quantitative real-time PCR was performed in the q5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) using the q5 SYBR green supermix (Bio-Rad). Reactions were performed in a 25-μl mixture containing cDNA, specific primers for each gene and the q5 SYBR green supermix. Primer sequences used are shown in Table 1.

Table 1. Primer sequences for real-time PCR

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<thead>
<tr>
<th>Gene</th>
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<tr>
<td>MyoD</td>
<td>5′-GCTTCTATGCAGCAGCCACTCC-3′</td>
<td>NM_010866</td>
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<tr>
<td></td>
<td>5′-GCACATGCTCAGCTTCCAGCG-3′</td>
<td></td>
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<tr>
<td>Myogenin</td>
<td>5′-GCGAGCAAGGTGTTGGAAGGG-3′</td>
<td>NM_031189</td>
</tr>
<tr>
<td>Decorin</td>
<td>5′-ACAGACATCAGGTTAGAGGAGAAT-3′</td>
<td>NM_007833</td>
</tr>
<tr>
<td>Collagen</td>
<td>5′-TCAGGAGGGAGGATGCTGGG-3′</td>
<td>NM_007742</td>
</tr>
<tr>
<td>β-actin</td>
<td>5′-CCACGCCGAGAAGGAAATGAC-3′</td>
<td>NM_007393</td>
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Fig. 1. A: schematic diagram of the whole body vertical vibration (VV) device. B: quantification of cell viability after VV using the methylthiazoletetrazolium assay. C: cell cycle distribution of VV-treated cells was analyzed by using flow cytometry 24 h after treatment. Percentages of cells in G0/G1, S, and G2/M phase are presented. Data are means ± SD of 3 independent experiments.

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Specific PCR products were detected by measuring the fluorescence of SYBR Green, a double-stranded DNA-binding dye (30). After the real-time PCR reaction, a dissociation (melting) curve was generated to check the specificity of the PCR reaction. The relative mRNA expression level was calculated from the threshold cycle value of each PCR product and normalized to that of β-actin by using the comparative threshold cycle method (25). All real-time PCR experiments were performed in triplicate and repeated at least three times.

Western blot analysis. Cells were washed twice with ice-cold PBS containing 1 mM sodium vanadate and then lysed in modified radio-immunoprecipitation assay buffer (150 mM NaCl, 1 mM EGTA, 50 mM Tris [pH 7.4], 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitor (Complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics, Taipei, Taiwan) and 1 mM sodium vanadate. The lysates were cleared by centrifugation at 14,000 rpm for 15 min at 4°C, subjected to SDS-PAGE, immunoblotted with antibodies as indicated, and were then developed with an enhanced chemiluminescence reagent (ECL System; Amersham Pharmacia Biotech).

Cell cycle analysis. Cells were detached and flushed into a single-cell suspension with Hank’s buffered solution. After centrifugation, cells were fixed with ice-cold 70% alcohol and treated with RNase (100 mg/ml in PBS) at 37°C for 1 h. Cells were then stained with propidium iodide (400 µg/ml in PBS) in the dark followed by filtration through a 41-µm pore size filter before analysis. The DNA content of individual cells was measured using flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) with excitation set at 488 nm. Data were analyzed using a cell-fit software program and represented as histograms.

Immunofluorescence. Cells on coverslips were washed three times with PBS and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After being washed three times with PBS, cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min, rinsed with PBS, and then immunostained with anti-myosin antibody (MF-20) for 1 h at room temperature. After being washed three times with PBS, the coverslips were exposed to Alexa Fluor 488-labeled secondary antibodies (Molecular Probes, Eugene, OR) for 1 h, and then the nuclei were stained with DAPI. The coverslips were mounted in anti-fade solution (Molecular Probes). Images of samples from three independent experiments were captured on a fluorescence microscope. A total of 15 representative images per sample were scored for myotube number, myotube length, myotube nuclei, and area occupied by myotubes relative to the total area using Image Pro Plus software (Media Cybernetics, Silver Spring, MD). The fusion index (%) was determined by dividing the number of nuclei within multinucleated myotubes by the total number of nuclei analyzed. In each case, staining without primary antibody was done with a side-by-side parallel specimen (as a negative control), which yielded a blank image.

Statistical analysis. Each value represents the mean ± SE of at least three independent experiments. One-way ANOVA or Student’s t-test was used to test for statistical differences. Statistical significance was set at P < 0.05.

RESULTS

Effect of VV stimulation on the viability and cell cycle profile of C2C12 myoblasts. To evaluate the cell viability of C2C12 myoblasts after VV stimulation, we seeded cells at a subconfluent cell density (7 × 10^4 cell/cm²) in each experimental group (0, 5, 8, and 10 Hz) to allow for cell proliferation. Under these cell culture conditions, confluent cell layers of C2C12 myoblasts were observed after 3 days of culture in each group, which indicates similar levels of cell proliferation. As shown in Fig. 1B, there was no significant difference in the cell viability between the VV-treated groups after 3 days. Furthermore, when cells were seeded at a higher density (1 × 10^4 cell/cm²), which allows for more rapid generation of high confluency, and were subsequently stimulated with VV (5, 8, and 10 Hz) for 3 days, the viability of C2C12 myoblasts was also not different from that of control cells (0 Hz) (data not shown). To investigate whether VV changes the cell cycle profile of myoblasts, we measured the cell cycle profile of C2C12 myoblasts 24 h after vibration treatment. As shown in Fig. 1C, there was no significant difference in the cell cycle profile of VV-treated cells compared with control cells.

Effect of VV stimulation on type I collagen and decorin expression. Type I collagen and decorin are the major components of the ECM in muscle structure and are important for the myogenic differentiation of myoblasts (32). We investigated whether VV stimulation regulated type I collagen and decorin expression in C2C12 myoblasts. It has been reported that myogenic differentiation can be induced by a reduction in serum concentration (31). In this study, we cultured C2C12 cells in complete medium; thus, vibration-induced effects on myogenesis could be measured independently of mitogen deprivation-induced effects. The gene expression of type I collagen and decorin in C2C12 myoblasts with VV stimulation (0, 5, 8, and 10 Hz) was determined using real-time PCR for up to 3 days (Figs. 2). Overall, the expression of type I collagen and decorin significantly increased within 3 days after VV stimulation. The gene expression of type I collagen in VV-stimulated groups (5, 8, and 10 Hz) was about threefold higher than...
that of the control group (0 Hz) (Fig. 2A). Notably, expression of type I collagen was highest in the 5 Hz VV-stimulated group, although all VV-treated groups had levels of type I collagen expression that were significantly higher than that of control cells. The gene expression of decorin was also significantly increased from days 1 through 3 in VV-treated groups (5, 8, and 10 Hz), compared with that of the control group (Fig. 2B), with the highest decorin expression in the 10 Hz VV-stimulated group.

VV stimulation augments the expression of MyoD and myogenin. MRFs, including MyoD and myogenin, are the master regulators in the early and terminal differential stages of myogenesis (31, 39). To determine whether VV stimulation promoted myotube formation via regulation of MyoD or myogenin expression, we compared the expression of MyoD and myogenin in C2C12 cells with or without VV stimulation. Real-time PCR analysis showed that MyoD and myogenin gene expression increased in C2C12 cells in a dose-dependent manner with VV stimulation (Fig. 3). In the 5 Hz VV-treated group, gene expression of MyoD was significantly greater than control at days 1 and 3 (Fig. 3A). In addition, MyoD gene expression in the 8 and 10 Hz VV-treated groups was higher than that of the 5 Hz VV-stimulated group (Fig. 3A). In the 5 Hz VV-treated group, gene expression of myogenin was significantly greater than that of the control group at days 1 and 3 (Fig. 3A). In addition, MyoD gene expression in the 8 and 10 Hz VV-treated groups was higher than that of the 5 Hz VV-treant group (Fig. 3A). In the 5 Hz VV-treated group, gene expression of myogenin was significantly greater than that of the control group at days 2 and 3, but was lower than those of the 8 and 10 Hz VV-treated groups (Fig. 3B). In cells with 8 Hz VV stimulation, myogenin gene expression was increased about twofold over that of control (0 Hz) cells at days 1 and 3 and threefold higher than that of the control group at day 2. In cells that received 10 Hz VV stimulation, myogenin gene expression was about twofold higher than that of the control group at day 1 and fourfold higher than control cells at days 2 and 3.

We confirmed the expression of MyoD and myogenin in VV-stimulated C2C12 cells using Western blot analysis (Fig. 4A). Consistent with the real-time PCR results, quantitative Western blot results showed that the expression of MyoD (Fig. 4B) and myogenin proteins (Fig. 4C) was significantly increased in VV-treated groups (5, 8, and 10 Hz) in a dose-dependent manner (Fig. 4B).

VV stimulation enhances myotube formation. Multicellular myotubes are formed when C2C12 myoblasts differentiate and fuse with each other (51). To investigate the myogenic effect of

![Fig. 3. Effect of VV stimulation on MyoD (A) and myogenin (B) gene expression. Cells were cultured for 3 days with or without VV stimulation. The mRNA expression of MyoD (A) and myogenin (B) were determined using real-time PCR. Data are means ± SD of 3 independent experiments. **P < 0.01, ***P < 0.001 for VV-treated vs. control cells (0 Hz).](http://jap.physiology.org/)

![Fig. 4. Effect of VV stimulation on the protein expression of Myo-D and myogenin at day 3. The cells were cultured for 3 days with or without VV stimulation. A: protein expression of MyoD and myogenin were determined by Western blot analysis. The blots were quantified, and the ratio of myogenin vs./H9252-actin (B) and MyoD vs./H9252-actin (C) were measured and expressed as ratios compared with control cells (0 Hz) for which the ratio was defined as 1. Data are means ± SD of 3 independent experiments. *P < 0.05, **P < 0.01 for VV-treated vs. control cells (0 Hz).](http://jap.physiology.org/)
VV stimulation in C2C12 myoblasts, confluent cells were stimulated with VV for 3 days and cultured for an additional 3 days (total of 6 days) or 6 days (total of 9 days) to examine myotube formation by immunofluorescence. Immunofluorescence of VV-stimulated C2C12 cells was performed using antibodies directed against the muscle marker sarcomeric myosin (MF-20), which binds to the myosin heavy chain of vertebrate striated muscle cells. Figs. 5A and 6A show a culture

Fig. 5. Effect of VV stimulation on myotube formation at day 6. A: fluorescence images of myosin-stained C2C12 myoblasts with or without VV stimulation. Green, myosin (MF20); blue, nuclear staining with 4,6-diamidino-2-phenylindole (DAPI). Bar = 100 μm. Quantification of myotube numbers (B), the number of myotubes of different lengths (C), average myotube length (D), average area of individual myotubes stained with MF-20 (E) and % fusion index (F). Data are means ± SD of 3 independent experiments. **P < 0.01, ***P < 0.001 for VV-treated vs. control cells (0 Hz).
Fig. 6. Effect of VV stimulation on myotube formation at day 9. A: fluorescence images of myosin-stained C2C12 myoblasts with or without VV stimulation. Green, myosin (MF20); blue, nuclear staining with DAPI. Bar = 100 μm. Quantification of myotube numbers (B), number of myotubes of different lengths (C), average myotube length (D), average area of individual myotubes stained with MF-20 (E), and % fusion index (F). Data are means ± SD of 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 for VV-treated vs. control cells (0 Hz).
of multicellular myotubes with antimyosin staining at day 6 and day 9, respectively. Only myotubes show myosin staining, while undifferentiated myoblasts show little or no myosin staining. The nuclei of all cells are labeled with DAPI. The nuclear (cell) numbers were similar in each VV-treated group, consistent with the cell viability results in Fig. 1B.

To assess the inductive effect of VV stimulation on myotube formation in myoblasts, the number, length, fusion index, and area of multinucleated fused myotubes were measured. In the control group (0 Hz), there was minimal myotube formation (in terms of number), and the myotubes were less organized (length and thickness) than those formed in the groups with VV stimulation (5, 8, and 10 Hz) (Figs. 5 and 6). In all VV-treated groups, the majority of the cells cultured for 9 days showed self-assembly of individual myotubes (Fig. 6), which were longer and thicker than those observed at day 6 (Fig. 5). At day 6 (Fig. 5B), total myotube numbers in the VV-treated groups of 5, 8, and 10 Hz were 1.3-, 2.5-, and 2.5-times greater (20 ± 1.7, 25 ± 4.6, and 34 ± 4.7, respectively) than that of the control group (0 Hz; 15 ± 1.4). At day 9 (Fig. 6B), total myotube numbers in the VV-stimulated groups of 5, 8, and 10 Hz were 1.1-, 1.5-, and 1.2-times, greater (32 ± 2.4, 44 ± 2.6, and 36 ± 1.9, respectively) than that of the control group (0 Hz; 31 ± 8.7).

Moreover, the myotubes formed in the 8 and 10 Hz VV groups were not only longer but also thicker than those in the 5 Hz and control groups. At day 6 (Fig. 5, C and D), average myotube lengths were 170 ± 22 and 171 ± 26 μm in the 8 and 10 Hz VV-treated groups, respectively, compared with 151 ± 25 μm in the 5 Hz VV-treated group and 135 ± 12 μm in the control (0 Hz) group. At day 9 (Fig. 6, C and D), the myotube lengths were 288 ± 19, 333 ± 51, and 324 ± 39 μm in the 5, 8, and 10 Hz VV-treated groups, respectively, which were significantly greater than the length of 178 ± 23 μm measured in the control (0 Hz) group. We also measured the average area of individual myotubes stained with myosin antibody (MF-20). At day 6 (Fig. 5E), the average areas of individual myotubes stained with MF-20 were 3,599 ± 399 and 3,862 ± 348 μm² in the 8 and 10 Hz VV-stimulated groups, respectively, which were significantly greater than the areas of 2,800 ± 388 μm² in the 5 Hz VV-treated group and 2,383 ± 370 μm² in the control (0 Hz) group. At day 9 (Fig. 6E), the average areas of individual myotubes stained with MF-20 were 6,851 ± 1,330 and 6,177 ± 2,179 μm² in the 8 and 10 Hz VV-treated groups, respectively, which were significantly greater than the areas of 4,285 ± 891 μm² in the 5 Hz VV-treated group and 2,846 ± 110 μm² in the control (0 Hz) group. Furthermore, the fusion indices of all of the VV-treated groups (5, 8, and 10 Hz) were significantly higher than that of the control group (Figs. 5F and 6F). These results indicate that VV stimulation, especially in the 8 and 10 Hz VV-stimulated groups, was able to markedly enhance cell fusion and myotube formation in C2C12 myoblasts.

PI3-kinase inhibitor (LY294002) efficiently suppressed the VV-induced myotube formation. To determine whether VV stimulation promoted myotube formation via regulation of PI3-kinase signaling, we compared the myotube formation in C2C12 cells with (10 Hz) or without (0 Hz) VV treatment using the PI3-kinase inhibitor (LY294002). The results showed that LY294002 efficiently suppressed the VV-induced (10 Hz) myotube formation in C2C12 cells (Figs. 7).

DISCUSSION

Mechanical load is widely used in rehabilitation and sports activities to improve joint flexibility in humans. Many studies are designed to investigate the cellular effects of mechanical load such as magnetic stimulation (52) or flow stress (5). Mechanotransduction from a mechanical load acting on a cell can initiate a sequence of signaling events that lead to changes in transcription, translation, or cell proliferation (37). Mechanical load on skeletal muscle has been shown to increase muscle IGF-I mRNA expression (27). Muscle fiber properties and sarcomere length can also be regulated by different mechanical loads (46). It is thus clear that mechanical stimulation plays an important role in initiating optimal changes in gene expression in myocytes. Previous studies indicate that short bouts of passive stretching, a procedure commonly used to improve joint range-of-motion in humans, increased MyoD gene expression in rat muscle (35, 53). A recent study using C2C12 cells showed that the application of mechanical stretching increased the expression of MyoD and myogenin in early times (12 h) after the stretching and that this effect gradually decreased thereafter (1). The results of our study showed that the application of VV, a type of mechanical load, can promote MRF (MyoD and myogenin) expression in a time- and dose-dependent manner and enhance myogenesis in C2C12 satellite myoblasts. The molecular mechanisms of VV-induced myogenesis in C2C12 cells may include enhancement of the expression of ECM components (type I collagen and decorin) and MRFs (MyoD and myogenin) in myoblasts; these factors may, in turn, increase myotube formation, which may enhance muscle activities (42).

There are various methods of delivering VV to the body, such as via a seesaw, horizontal, or vertical platform (34). The optimal frequency for whole body training varies between studies (9). One recent study has shown that low-magnitude mechanical load with

Fig. 7. Effect of the PI3-kinase inhibitor LY294002 on myotube formation in C2C12 cells with (10 Hz) or without (0 Hz) VV treatment. A: vehicle control (DMSO) treatment. B: LY294002 treatment. Green, myosin (MF20); blue, nuclear staining with DAPI.
a frequency over 30 Hz is effective in osteoporosis (15). Moreover, lower frequencies, from 4–20 Hz, offer increased muscle force, which contributes to spinal stability (4). Another study, using 8 Hz vibration in a training program, showed enhancement in muscle activity and strength (44). Accordingly, we used the VV model with frequencies from 5 to 10 Hz, which provided adequate and balanced transmission of force throughout the target cells for simulating and studying the mechanisms of whole body vibration training on enhancing muscle activity. In this study, we demonstrate that both MyoD and myogenin play important roles in the development and maintenance of striated muscle during myogenesis, with expression levels of both factors increasing in proportion to the number of days in culture and degree of VV stimulation. Furthermore, our results show that type I collagen and decorin mRNA expression is the highest after VV treatment at frequencies of 8 and 10 Hz. Moreover, the most effective frequency of VV treatment for the expression of myoD and myogenin was 10 Hz. The greatest effect of VV stimulation on myotube formation was also seen at frequencies of 8 to 10 Hz. Therefore, the optimal frequency of VV treatment for inducing myogenesis of myoblasts should be between 8 and 10 Hz.

The ECM maintains cell structure and regulates cell behavior through mechanotransduction. Type I collagen is the main structural element of skeletal muscle that enhances myoblast differentiation and myotube formation (21). Type I collagens are commonly used scaffolds for skeletal muscle tissue engineering (11). Collagen scaffolds can increase the transmitted forces of mechanical load on embedded muscle cells during myotube formation (36). In our study, type I collagen synthesis increased after VV treatment, which may increase the transmitted forces of VV on myoblast cells and subsequently enhance myogenesis. Decorin, which is associated with type I collagen, can govern the rate of collagen fibrillogenesis (43). Decorin also promotes myogenesis by enhancing the proliferation and differentiation of myoblasts (29) and accelerates muscle regeneration and repair (24). Our results support this role for decorin in myogenesis and show that decorin expression increases after VV treatment, which can subsequently promote myotube formation of the myoblasts.

MyoD and myogenin, important regulators of myogenic transcription, can activate muscular differentiation of myoblasts into myotubes (10), and regulate the proliferation of muscle satellite cells (54). MyoD and myogenin have been regarded as important regulators through their role in the adaptation of muscle cells to mechanical load (50). The induction of MyoD expression in the context of electrical and mechanical stimulation is sensitive to stimulation at high frequency (23). Consistent with these data, our study shows that VV treatment results in pronounced increases in MyoD and myogenin expression at a frequency of 10 Hz, compared with 5 or 8 Hz, which can subsequently enhance myogenesis of the myoblast.

Myotube formation plays an important role in restoring muscle function. The effects of myotube length, size, and number are related to muscle contraction during gait performance (12, 16). In this study, we demonstrate that VV stimulation positively increases the total number of myotubes that are formed as well as the length and area per myotube. Most notably, the number of myotubes (>100 μm in length) and myotube hypertrophy were increased substantially in the VV groups treated at higher frequencies (i.e., 8 to 10 Hz). Therefore, VV stimulation has positive effects on these myotube characteristics and may promote higher myogenic contractile ability. Recent studies indicate that PI3-kinase-Akt signaling pathway is a crucial regulator of skeletal muscle hypertrophy and myotube formation (6, 22, 26). Our results support this role for PI3-kinase signaling in myogenesis and show that VV-induced myotube formation was suppressed by using the PI3-kinase inhibitor (LY294002). It is tempting to speculate which members of PI3-kinase downstream signaling pathways mediate VV-regulated hypertrophy and myotube formation in myoblasts. Experiments are now underway to investigate the role of Akt in the downstream signaling pathway of PI3-kinase.

In summary, our investigation of the effects of VV on C2C12 myoblasts has shown that the procedure is both safe and effective in stimulating myogenesis. Our results demonstrate that a low frequency of VV can increase myotube formation, expression of the MRFs MyoD and myogenin, and expression of the ECM components type I collagen and decorin. While many clinical trials have already demonstrated positive results from VV stimulation in various populations, this study provides some insight into how these clinically observed increases in muscle strength and balance occur. Nevertheless, the in vitro model of C2C12 satellite myoblasts that we used may not completely reflect the true physiology of regenerating muscle. Further research using primary satellite cells or an in vivo animal model is required to determine the extent of these molecular effects over a greater range of VV frequencies and their implications for clinical use.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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