Selective Inhibition of a Step of Myotube Formation with Wheat Germ Agglutinin in a Murine Myoblast Cell Line, C2C12

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ABSTRACT. Myoblast cells, C2C12, which is an established cell line from satellite cells of skeletal muscle of C3H mouse, start to fuse and form multinucleated cells (myotubes) and begin to express creatine phosphokinase and myosin, when culture medium is changed from the growth medium to the differentiation medium. Among the 12 lectins that we tested, wheat germ agglutinin apparently suppressed the myotube development judged by phase-contrast microscopy, but did not affect the induction of creatine phosphokinase activity. The addition of N-acetylglucosamine or N,N',N''-triacetylchitotriose, which is a specific ligand for wheat germ agglutinin, to the differentiation medium, recovered this apparently suppressive effect of wheat germ agglutinin on the myotube development. Tachypleus tridentatus (Japanese horseshoe crab) lectin that specifically recognizes N-acetyleneuraminic acid, one of the sialic acids, showed no effect on the myotube development. It was suggested that wheat germ agglutinin suppressed the process through recognizing N-acetylglucosamine containing sugar. Surprisingly, even in the presence of wheat germ agglutinin, the ratio of mononucleated cell numbers to the genomic DNA content, which represents the fusion level, decreased after incubation in the differentiation medium, indicating that even when wheat germ agglutinin was present in the medium, cell fusion, which is the initial step of the myotube formation, occurred. Immunostaining with anti-skeletal muscle myosin antiserum confirmed that the myosin expressing cells actually fused and formed multinucleated cells. Their shape, however, was thin compared to that in the absence of wheat germ agglutinin. We propose that the membrane fusion step to form myotubes is composed of two distinct steps in C2C12; one fusion step is to form long and thin myotubes from mononucleated cells and the other one is to develop fat myotubes. Wheat germ agglutinin specifically inhibits the latter fusion step.

During the terminal differentiation of myogenesis, myoblasts fuse to form multinucleated myotubes. To date, several proteins that are involved in the process of myoblast fusion have been reported. Neural cell adhesion molecule (N-CAM) and N-cadherin are expected to function in the initial recognition step for the fusion of embryonic chicken myoblasts (1–4). However, the specific antibodies against the proteins do not inhibit the myoblast fusion efficiently (1, 3–5). Both VLA-4 (integrin α4β1) and vascular cell adhesion molecule 1 (VCAM-1) play important roles in the cell to cell recognition at the stage of secondary myoblast fusion to form mature myotubes in vivo (5). The monoclonal antibody raised against either VLA-4 or VCAM-1 effectively inhibits the myotube formation of C2C12 cells (5). Besides these four proteins, many other proteins are reported to be involved in the fusion of myoblasts (6–11).

Myoblast fusion is expected to be controlled by proteins localized on the cell surface. Many of them are glycoproteins. Tunicamycin, an inhibitor for protein glycosylation, inhibits myotube formation (12, 13). Actually, almost all the proteins that are expected to play roles in the myoblast fusion described above are glycoproteins. Therefore, the lectins that recognize specific structure of the sugar chain are useful tools to examine the myoblast fusion. At present, concanavalin A (ConA) and wheat germ agglutinin (WGA) are known to inhibit the fusion of primary culture of chicken myoblasts (14). The inhibitory effect of ConA was not specific on the myoblast fusion but on the entire myogenic process of the cells (15, 16).

We tested the effect of 12 lectins on the myotube for-
mation in C2C12 cells, and found that one of the tested lectins, WGA, specifically suppressed the development of the myotubes without affecting the initial myoblast fusion.

**MATERIALS AND METHODS**

**Materials.**

Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Nissui Seiyaku (Tokyo, Japan), and Cosmedium was from CosmoBio (Tokyo, Japan). Fetal bovine serum (FBS) and G418 were obtained from Gibco BRL Japan (Tokyo). Trypsin was from DIFCO (Detroit, Michigan), and gelatin was from Iwaki Glass (Tokyo, Japan). ConA, Dolichos biflorus lectin (DBA), Lens culinaris agglutinin (LCA), Lotus tetragonolobus lectin (lotus lectin), Macelisia amurensis lectin (MAM), peanut lectin (PNA), Ricinus communis lectin 120 (RCA-120), soybean lectin (SBA), Sambucus sieboldiana lectin (SSA), Ulex europaeus lectin-I (UEA-I), and WGA were purchased from Honen Corp. (Tokyo, Japan). Aleuria aurantia lectin (AAL) and Japanese horseshoe crab lectin (TTA) were from Nichirei (Tokyo, Japan) and Seikagakukogyo Corp. (Tokyo, Japan), respectively. N,N’,N”-triacetylchitotriose (TAC), penicillin, and streptomycin were obtained from Sigma (St. Louis, Missouri). Hoechst 33258 and goat anti-rabbit antibody-peroxidase were obtained from Wako Pure Chemicals (Osaka, Japan). The other chemicals used were of the highest quality available.

**Methods.**

**Cells and Cell Culture.** Mouse skeletal muscle myoblast cell line, C2C12, and its transformant, MTR4C (17), were maintained as monolayers in a humidified incubator (5% CO₂) at 37°C in plastic dishes that were coated with 1% gelatin, containing DMEM supplemented with 10% FBS, 100 u/ml of penicillin, and 100 μg/ml of streptomycin. As for maintaining MTR4C, 4–5 mg/ml of G418 in 0.1 M Hepes-NaOH, pH 7.4, was added at 0.2 mg/ml to the culture medium. Two days prior to the experiment, samples of 1 × 10⁵ cells of C2C12 or 2 × 10⁵ cells of MTR4C were seeded into 60-mm plastic dishes containing 3 ml of growth medium. On day 2 of cell growth, the growth medium was replaced by 3 ml of Cosmedium, the differentiation inducing medium (18). Every 24 h, the differentiation medium with or without appropriate concentration of lectin was changed for the fresh one. Each lectin was added to the differentiation medium at various concentrations (0.2–10 μg/ml) after 12 or 6 h incubation, and the incubation was continued for 72 or 48 h for C2C12 or MTR4C, respectively (Fig. 1). The conditions were used for all the studies described hereafter unless otherwise stated.

**Measurement of muscle creatine phosphokinase (MCK) activity.** The cells cultured in 60-mm plastic dishes were washed twice with calcium-magnesium free Dulbecco’s phosphate buffered saline (PBS (−)) and lysed with 1 ml of ice
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cold cell lysis buffer (1 M NaCl, 1 mM EGTA, 1% Triton-X100, 10 mM Tris-HCl, pH 7.2) (19). The lysate, after freezing and thawing, was briefly (about 5 seconds) sonicated at 50 W in a Branson Sonifier equipped with a micro tip, and then centrifuged at 20,000 x g at 4°C for 15 min. The supernatant fraction served for the measurement of MCK activity (20, 21). The protein concentration of the cell extract was determined by BCA protein assay kit (Pierce) using bovine serum albumin as a standard.

Determination of the mononucleated cell numbers and the

Fig. 2. Effects of WGA on the myotube development. C2C12 cells (A) and MTR4C (B) were cultured in the growth medium. The medium was then changed to the differentiation medium and the culture was continued for 72 h for C2C12 (C, E), and 48 h for MTR4C (D, F), with (E, F) or without (C, D) WGA (2 μg/ml). The bar indicates 100 μm.
total DNA content. The C2C12 cells in 60-mm dish were washed with PBS (−), and dissociated with 1 ml of 0.25% trypsin and 0.02% EDTA at 37°C for 15 min. The cells were suspended and the cell numbers were counted in a hemocytometer. A small portion of the cell suspension was fixed with 3.7% formaldehyde on coverglass coated with poly-L-lysine (Sigma) for 10 min. The fixed cells were stained with Mayer-haematoxylin (MERCK), and the numbers of nuclei per cell were counted. More than 99% of the counted cells contained one nucleus. From the rest of cell suspension, the genomic DNA was isolated as described previously (22). The genomic DNA thus prepared was dissolved in 0.5 ml of 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) and was briefly sonicated. An aliquot of this fraction was added to 200 µg/ml of Hoechst 33258 to a final concentration of 1 µg/ml (23). The reaction mixture was excited at 356 nm and an emission at 456 nm was measured. Salmon sperm DNA was used as a standard.

Immunostaining of Myotube Using Anti-myosin Antiserum. The cells induced to differentiate were washed with ice cold PBS (−) and fixed with methanol at −20°C for 5 min. The cells were then washed with PBS (−), and soaked with 3% H2O2 for 5 min to inactivate endogenous peroxidase. The cells were washed again with PBS (−) and incubated with rabbit anti-myosin antiserum in the antibody buffer (1% bovine serum albumin and 0.02% sodium azide in PBS (−)) at room temperature for 90 min. The antiserum used was raised against mouse skeletal muscle myosin heavy chain. After the incubation, the cells were washed with PBS (−). Next, the cells were incubated with anti-rabbit antibodies conjugated to peroxidase in PBS (−) at room temperature for 30 min, and then washed with PBS (−). The bound second antibodies were visualized using a color reaction buffer containing 0.4 mg/ml 3-amino-9-ethylcarbazole, 0.03% H2O2 in 50 mM sodium acetate, pH 5.3. Nuclei DNA was stained with Mayer-haematoxylin according to the manufacturer’s protocol (Daiichi Pure Chemicals; Tokyo, Japan).

RESULTS

WGA apparently suppressed the myotube development. When cultured myoblasts are induced to differentiate, the cells start to fuse and form multinucleated cells. We tested the effect of 12 lectins on the myotube formation of C2C12 and MTR4C according to the time table shown in Fig. 1. MTR4C is established by transfecting the mouse DNA methyltransferase cDNA to C2C12 (17), of which myotube formation including the induction of MCK and myosin is about 24 h earlier than that of the parent C2C12. After 72 or 48 h culture in

![Diagram](image-url)

**Fig. 3.** The induction of MCK activities in C2C12 cells after the medium was changed to the differentiation medium, in the presence or absence of WGA (2 µg/ml) and/or GlcNAc (20 mM). The closed arrow indicates the time when WGA and/or GlcNAc were added. The cells cultured in Cosmedium were harvested at the indicated time periods, and the MCK activities and the amount of proteins were determined as described in the "MATERIALS AND METHODS". The activities were demonstrated as specific activities (units/µg protein). The open arrow indicates the time point at which photographs shown in Fig. 4A–D were taken.
Cosmedium, C2C12 or MTR4C cells clearly formed multinucleated myotubes (compare Fig. 2 panels A and B with C and D). In contrast, apparently, no typical myotube was observed when WGA (2 μg/ml), AAL (10 μg/ml), LCA (10 μg/ml), RCA-120 (0.2 μg/ml), SSA (2 μg/ml), or MAM (10 μg/ml) was added to Cosmedium. ConA (5 μg/ml), 10 μg/ml each of DBA, SBA, PNA, lotus lectin, or UEA-I showed no effect on the myotube formation (summarized in Table I). When 10 μg/ml of Con A was added to the medium, the cells were apt to dissociate from the dish. Typical shapes of the cells in the presence of WGA (2 μg/ml) are shown in Fig. 2E and F. At the concentration of 10 or 0.4 μg/ml, WGA suppressed the cell proliferation or showed no effect, respectively (data not shown).

MCK, a skeletal muscle specific enzyme, is induced to express by changing the medium to the differentiation medium. All of the lectins, ConA, DBA, SBA, PNA, lotus lectin, and UEA-I, that did not inhibit the myotube formation, also had no effect on the induction of MCK activity (Table I, group II). The three lectins, RCA-120, SSA, and MAM apparently inhibited both the myotube formation and the MCK activity (Table I, group III). RCA-120 and MAM suppressed the cell growth. SSA inhibited the MCK activity, but had no effect on the cell growth. The effect of the lectins on the myotube formation in the group III may be complicated or indirect. Interestingly, WGA, AAL, and LCA, which apparently suppressed the myotube development, showed little effect on the MCK activity (Table I, group I). The lectins in group I seemed to inhibit specifically the multinucleation step of the myotube formation without affecting the biochemical differentiation. However, AAL and LCA made the cells aggregate. Therefore, we next exam-
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ined the action of WGA.

The MCK activity started to increase after 24 h incubation in the differentiation medium and rapidly increased thereafter (Fig. 3). The increasing profile of the MCK activity in the presence of WGA was almost identical to that in the absence of WGA. It was confirmed that WGA, which apparently suppressed the myotube development, showed little effect on the MCK activity.

WGA specifically recognizes and binds to N-acetylglucosamine (GlcNAc), oligo-saccharides containing GlcNAc (24, 25), and sialic acids (26). As shown in Fig. 3, GlcNAc did not affect the MCK induction. If the inhibitory effect of WGA was due to the specific binding to the cell surface, GlcNAc and its trisaccharide, TAC, which are the specific ligands for WGA with dissociation constants of 0.76 mM and 12 μM, respectively (24), should restore the suppression of myotube development. In fact, three days after the medium was changed to Cosmedium, C2C12 cells showed normal myotubes when WGA was added with 20 mM GlcNAc or 200 μM TAC (Fig. 4). WGA is also known to bind to sialic acids. We tested whether or not Japanese horseshoe crab lectin (TTA), which is known to recognize N-acetylneuraminic acid (one of the sialic acids), also suppressed the myotube development. WGA (2 μg/ml) clearly inhibited the myotube development of MTR4C, while TTA (10 μg/ml) had no effect on that step (Fig. 5). Therefore, it was strongly suggested that WGA suppressed the myotube development by recognizing GlcNAc containing sugar.

WGA did not actually inhibit the myoblast fusion. Membrane fusion is the initial step for the myotube formation. However, it was difficult to accurately quantify the step. To evaluate the extent of the fusion of C2C12 cells, the ratio of the numbers of mononucleated cells to the total DNA content (= amount of nuclei) was measured. When the cells start to fuse, the parameter is expected to drop. The mononucleated cells were dissociated with trypsin and EDTA and counted in a hemocytometer. After the medium was changed to the different-

![Graphs showing量化 of cell fusion in the process of myotube formation](image)

Fig. 6. Quantification of cell fusion in the process of myotube formation. The changes in the numbers of the mononucleated cells (A), the amount of DNA (B), and the ratio of the numbers of mononucleated cells to the amount of the DNA (C), during the cell differentiation in the presence (●) or absence (○) of WGA (2 μg/ml) were demonstrated. C2C12 cells were trypsinized at the indicated time periods, the numbers of mononucleated cells counted, and the content of DNA determined as described in the "MATERIALS AND METHODS".
tiation medium, in the presence or absence of WGA, the numbers of the mononucleated cells and the amount of DNA per dish were measured. After 36 h incubation, the cell numbers reached twice that of time 0 and decreased thereafter (Fig. 6A). The DNA content increased as well, but did not drop after 36 h incubation (Fig. 6B). As a result, the ratio of cell numbers to DNA content dropped after 36 h incubation (Fig. 6C). Multinucleation (=cell fusion) occurred actively at this period. This was supported by morphological observations. This, surprisingly, was also observed for the cells added to which WGA was added (Fig. 6). The result clearly indicates that the addition of WGA to the differentiation medium did not inhibit the cell fusion, the initial step of the myotube formation.

We next examined the expression of skeletal muscle myosin in the cells cultured in the presence of WGA. If the myoblasts in the presence of WGA were really fused and formed multinucleated cells, visualization of the skeletal muscle myosin by immunostaining would make it clear. Immunostained C2C12 and MTR4C cells with anti-myosin antiserum are shown in Fig. 7. Both C2C12

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Fig. 7. Effect of WGA on the myotube development and the myosin expression. The cell samples were incubated in the presence (C, D, F) or absence (A, B, E) of 2 μg/ml of WGA in Cosmedium. C2C12 (A, C) and MTR4C (B, D–F) cells were stained with anti-myosin antiserum raised against mouse skeletal muscle myosin heavy chain, after 72 h (A, C) and 48 h (B, D–F) incubations, respectively. MTR4C (E, F) cells were also stained with Mayer-haematoxylin after 48 h incubation. The bar indicates 100 μm.
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Fig. 8. Schematic illustrations of the process of the myotube development in C2C12 cells. After the induction of differentiation, initially, myoblasts fuse each other to form long and thin myotubes. The thin myotubes then fuse to each other or with myoblasts to form flat and extended shape. WGA inhibits the second step of the myotube formation.

and MTR4C cells expressed myosin in the presence of 2 μg/ml of WGA (Fig. 7C and D). The expressed skeletal muscle myosin was found in the myotubes that were very thin but their length was as long as those formed in the absence of WGA. Thus, it was confirmed that WGA did not inhibit the myoblast fusion. The cells stained with anti-myosin antibody and haematoxylin showed that the very thin myotubes found in the presence of WGA actually contained multiple nuclei (Fig. 7F). Interestingly, the numbers of non-fused cells that did not express myosin in the presence or absence of WGA were similar (Fig. 7E and F, and refer to Fig. 6A). The shapes of the myotubes in the presence or absence of WGA were similar to each other until 21 h incubation in Cosmedium (data not shown), and the inhibitory effect of WGA became clear only after the 48 h incubation (Fig. 7E and F).

DISCUSSION

In the present study, we tested the effect of 12 lectins on the myotube formation of C2C12 cells. Among the lectins tested, WGA suppressed the myotube development, but apparently had no effect on the MCK induction. Although WGA seemed to suppress the myotube development, the index for the fusion (Fig. 6C) and the immunostaining of the cells with anti-skeletal muscle myosin antiserum (Fig. 7) revealed that WGA did not inhibit the initial step of the process. The inhibitory action of WGA was on the development of the fused myotubes into large and extended ones. The myotubes formed in the presence of WGA contained less nuclei per length than those in the absence of WGA (Fig. 7E and F). We propose that the cell fusion process to form myotubes is composed of two distinct steps; one is to form long and thin myotubes and the other is to form fat and extended myotubes, which is schematically illustrated in Fig. 8. At present, we do not have direct evidence that the cell fusion along the long axis to form the thin myotube precedes that of the fusion along the short axis, as shown in Fig. 8. However, significant amounts of the long and thin myotubes were found in the immunostained MTR4C cells after 48 h incubation even in the absence of WGA (Fig. 7B). It seems likely that the myoblasts that are in contact with the end of the long axis fuse first to form long and thin myotubes, and that the myoblasts or the other thin myotubes then fuse to the primary myotubes to form the fat and ex-
Fig. 9. Effect of anti-VLA4 antibody on the myotube development in MTR4C. The cells were incubated without (A), or with 2 μg/ml of WGA (B), 25 μg/ml (C), or 50 μg/ml (D) of PS/2 in Cosmedium. The antibody was added after 6 h incubation in Cosmedium. The micrographs were taken after 48 h incubation. The bar indicates 100 μm.

As to be molecule targeted by WGA to inhibit the secondary fusion of the myoblasts, GlcNAc (24) and sialic acids (26) are reported to be the specific ligands for WGA. Actually, GlcNAc and its trisaccharide suppressed the inhibition of WGA (Fig. 4), and Japanese horseshoe crab lectin that specifically binds to N-acetyleneuraminic acid (27), one of the sialic acids, showed no effect on the myotube formation. Therefore, it was suggested that WGA inhibited the myotube development through recognizing GlcNAc containing sugar.

At present, four membrane glycoproteins, N-CAM (1, 2, 4), N-cadherin (3), VLA-4 (integrin α4β1), and VCAM-1 (5), are reported to be involved in the myogenesis. N-CAM utilizes muscle specific exons when the cells differentiate into myotubes (28–30), and the introduction of the cDNA containing this muscle specific domain into the myoblasts promotes the myotube formation (3, 4). The specific antibody against the molecule produced from the introduced cDNA partially suppresses the myogenesis (3, 4). However, peanut lectin (PNA), which specifically binds to the O-linked glycosylation site of the muscle specific domain (31), did not inhibit the myotube formation of C2C12 (Table I). N-CAM might not be the target of WGA. On the other hand, 100 μg/ml of anti-VLA-4 (integrin α4β1) monoclonal antibody, PS/2, or anti-VCAM-1 monoclonal antibody, MK/1, inhibits the myoblast fusion of C2C12 very efficiently (5). In another study 100 μg/ml of the monoclonal antibody PS/2 (generously provided by Dr. Miyake at Saga Medical School) (32) inhibited not only the myoblast fusion, but also the expression of myosin (data not shown). Less than 10 μg/ml of PS/2 showed no effect on the myotube formation, but 25 or 50 μg/ml of PS/2 partially inhibited the development of the myotube formation, similar to that of 2 μg/ml of WGA (Fig. 9). The myosin expression was induced in the presence of 25 or 50 μg/ml of PS/2 (data not shown). In addition, the receptor for fibronectin, known as a member of integrin family, is reported to
bind to WGA (33, 34). It is reasonable to speculate that VLA-4 is the best candidate of the target of WGA. A discrepancy exists however, since in C2C12, α4 subunit of VLA-4 is not expressed in myoblasts but in the cells that are induced to differentiate (5), while, in the present study, WGA had to be present at very beginning of the induction of the differentiation for the inhibition. When WGA was added to C2C12 cells after 24 h incubation in Cosmedium, the added WGA could not suppress the myotube development anymore (data not shown). Further experiments are needed to determine which molecule is the target of WGA.

REFERENCES


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