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CD36 is required for myoblast fusion during myogenic differentiation

Seung-Yoon Park [a], Youngeun Yun [b], In-San Kim [b,c,*]

[a] Department of Biochemistry, College of Medicine, Dongguk University and Medical Institute of Dongguk University, Gyeongju 780-714, Republic of Korea
[b] Department of Biochemistry and Cell Biology, Cell and Matrix Research Institute, School of Medicine, Kyungpook National University, Daegu 700-422, Republic of Korea
[c] Biomedical Research Institute, Korea Institute Science and Technology, Seoul, Republic of Korea

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ABSTRACT

Recently, CD36 has been found to be involved in the cytokine-induced fusion of macrophage. Myoblast fusion to form multinucleated myotubes is required for myogenesis and muscle regeneration. Because a search of gene expression database revealed the attenuation of CD36 expression in the muscles of muscular dystrophy patients, the possibility that CD36 could be required for myoblast fusion was investigated. CD36 expression was markedly up-regulated during myoblast differentiation and localized in multinucleated myotubes. Knockdown of endogenous CD36 significantly decreased the expression of myogenic markers as well as myotube formation. These results support the notion that CD36 plays an important role in cell fusion during myogenic differentiation. Our finding will aid the elucidation of the common mechanism governing cell-to-cell fusion in various fusion models.

1. Introduction

Myoblast fusion is required for skeletal muscle formation during regeneration and growth after muscle injury and during myogenesis. Skeletal muscle consists of multinucleated myofibers formed by the fusion of mononucleated myoblasts in a process involving cell migration, alignment, recognition, adhesion, and membrane fusion [1,2]. Many molecules, such as cytokines, soluble proteins, transcription factors, and membrane receptors, have been implicated in myoblast fusion during myogenic differentiation [3,4]. However, the molecular mechanisms governing myoblast fusion remain to be investigated.

CD36 is a multifunctional protein that is involved in metabolism, immunity, angiogenesis, and behavior [5,6], and is also known to mediate apoptotic cell clearance via the recognition of phosphatidylserine (PS) on apoptotic cell surface [7,8]. Although the externalization of PS is known as a marker of apoptotic cells and critical for cell corpse clearance, accumulating data show that PS is also important for cell-to-cell fusion [9–13], which suggests that PS-binding proteins play a crucial role during muscle cell fusion. Recently, the exposure of PS on cell surfaces and lipid recognition by CD36 were found to be required for the cytokine-induced fusion of macrophages, but not for osteoclast fusion [14], which suggests that CD36 is selectively involved in cell-to-cell fusion of a specific cell type. Interestingly, a search of the GEO Profiles database revealed that CD36 expression is attenuated in the muscles of muscular dystrophy patients (GEO accession numbers GDS262 and GDS265) [15]. These observations led us to speculate that CD36 might be required for muscle cell fusion during myogenic differentiation. Here, we present evidence that CD36 is involved in myoblast fusion during myogenic differentiation.

2. Materials and methods

2.1. Antibodies

Goat polyclonal anti-CD36 antibody was purchased from R&D system, anti-myogenin antibody from Santa Cruz Biotechnology, monoclonal anti-MyHC antibody (clone MF20) from the Developmental Studies Hybridoma Bank, anti-actin antibody from Sigma Aldrich, and Alexa Fluor 488-conjugated anti-goat IgG and Alexa Fluor 647-conjugated anti-mouse IgG were purchased from Molecular Probes.

2.2. Cell culture

C2C12 myocytes were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM (high glucose) medium containing 10% (v/v) fetal bovine serum (FBS) and antibiotics. To induce myogenic differentiation, cells were grown to 100% confluence in maintenance medium and then switched to differentiation medium (DMEM supplemented with 2% horse serum).

* Corresponding author. Address: Department of Biochemistry and Cell Biology, Cell and Matrix Research Institute, School of Medicine, Kyungpook National University, 101 Dongin-dong, Jung-gu, Daegu 700-422, Republic of Korea. Fax: +82 53 422 1466.
E-mail address: iskim@knu.ac.kr (I.-S. Kim).

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2.3. Western blotting

C2C12 cells were lysed in cold lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, and proteinase inhibitors] for 30 min on ice. Identical amounts (20 μg of protein) of total cell lysates were resolved by 6–10% SDS–PAGE and then transferred to nitrocellulose membranes, which were incubated in blocking solution consisting of 5% skim milk in TBS-T [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20] for 1 h at room temperature and then immunoblotted with anti-CD36 (R&D system), anti-MHC (Hybridoma bank), anti-myogenin (Santa Cruz), or anti-actin antibody (Sigma). Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham).

2.4. Real-time quantitative PCR

Total RNA was purified from C2C12 cells using Trizol reagent, in accordance with the manufacturer's instructions (Invitrogen). Reverse transcription was performed with M-MLV reverse transcriptase (Promega) using 2 μg of total RNA for 50 min at 42 °C followed by 3 min at 95 °C. Real time PCR amplification was carried out in a LightCycler 480 (Roche Applied Science) using SYBR green master mix (Roche Applied Science) as follows: initial denaturation at 95 °C for 5 min followed by 45 amplification cycles (denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s). The primers used in this study are detained in Table 1. qRT-PCR results were analyzed as previously described [16,17]. Briefly, the comparative cycle threshold (C_T) method was used to analyze data by generating relative values for the amounts of target cDNA. Relative quantitations for given genes (expressed as fold induction over control) were calculated after determining the difference between the C_T of the target gene A and that of the reference gene B (β-actin) in C2C12/shCont cells ($\Delta C_{T1} = \Delta C_{T1A} - \Delta C_{T1B}$) and C2C12/shCD36 cells ($\Delta C_{T0} = \Delta C_{T0A} - \Delta C_{T0B}$) using the formula $2^{-\Delta\Delta C_{T(1-0)}}$.

2.5. Immunofluorescent staining

C2C12 cells were seeded onto 8-well chamber slides (Nunc), incubated in differentiation medium for the indicated times, fixed in 4% paraformaldehyde for 10 min at room temperature, and then permeabilized with 0.03% Triton X-100 for 5 min. Non-specific binding was then minimized by incubating the cells in PBS containing 10% goat serum for 2 h. After three washes with PBS, slides were incubated for 1 h at room temperature with a polyclonal anti-CD36 antibody and/or a monoclonal anti-MHC antibody. The slides were washed three times for 5 min each with PBS at room temperature and then incubated with Alexa Fluor 488-conjugated anti-goat IgG and Alexa Fluor 647-conjugated anti-mouse IgG (Molecular Probes) for 1 h at room temperature, followed by staining DAPI. The slides were washed five times with PBS for 5 min each and then treated with a solution of SlowFade (Molecular Probes). Slides were viewed under a fluorescence microscope (Leica).

Table 1
DNA sequences of the primers used for RT-PCR and quantitative real-time PCR.

Identity	Nucleotide sequences	Product size
CD36	Forward: 5′-GGTCTATCTACGCTGTGTTCG-3′ Reverse: 5′-ATCTAAGTATGTCCTATGCTC-3′	299
Myogenin	Forward: 5′-GTAAGGTGTGTAAGAGGAAG-3′ Reverse: 5′-TGTGGGAGTTGCATTCACTG-3′	288
MyoD	Forward: 5′-ATGGCATGATGGATTACAGCG-3′ Reverse: 5′-TCCCTGTTCTGTGTCGCTTAG-3′	260
Myf5	Forward: 5′-ATGCCATCCGCTACATTGAG -3′ Reverse: 5′-GGGTAGCAGGCTGTGAGTTG-3′	353
MRF4	Forward: 5′-CTACATTGAGCGTCTACAGG-3′ Reverse: 5′-CTTAGCAGTTATCACGAGGC-3′	190
GAPDH	Forward: 5′-AACATCAAATGGGGTGAGGCC-3′ Reverse: 5′-GTTGTCATGGATGACCTTGGC-3′	252
β-Actin	Forward: 5′-TCACCCACACTGTGCCCATCTACGA-3′ Reverse: 5′-GGATGCCACAGGATTCCATACCCA-3′	348

2.6. Knockdown of CD36 in C2C12 cells

A shRNA target sequence for mouse CD36 (accession number: NM_007643), 5′-GCU CAA GAA UGU CCG CAU AGA-3′, was selected using the Invitrogen shRNA design tool. For control shRNA, a scrambled sequence was used. The following forward and reverse oligonucleotides (sense-loop-antisense) were synthesized: shCD36 (sense), 5′-gatcccc GCTCAAGAATGTCCGCATAGA cgaa TCTATGCG GACATTCTTGAGC tttttggaaa-3′; shCD36 (antisense), 5′-agcttttc-caaaaa GCTCAAGAATGTCCGCATAGA ttcg TCTATGCGGACATTCTT-GAGC ggg-3′; scrambled shCont (sense), 5′-gatcccc GTACGATA CGACCCTTAATGT cgaa ACATTAAGGGTCGTATCGTAC tttttggaaa-3′; and scrambled shCont (antisense), 5′-agcttttccaaaaa GTACGATAC-GACCCTTAATGT ttcg ACATTAAGGGTCGTATCGTAC ggg-3′. Oligonucleotides were annealed, and cloned into the BglII/HindIII site of pSuper/neo vector (OligoEngine). C2C12 cells were then transfected with pSuper/shCD36 or pSuper/shCont vector using Lipofectamine 2000 (Invitrogen). At 24 post-transfection, cells were selected in the medium containing G418 (600 μg/ml). Individual G418-resistant colonies were isolated after 2 weeks of culture and designated as C2C12/shCD36. Negative control clones were randomly selected following transfection with pSuper/shCont vector (C2C12/shCont). The down-regulation of CD36 expression was verified by quantitative real-time PCR and Western blotting.

2.7. Fusion assays

After 3 and 5 days in differentiation medium (DM3 and 5), C2C12 cells were fixed and immunostained with antibody against myosin heavy chain (MF20, Developmental Studies Hybridoma Bank). The fusion index was calculated by dividing the number of nuclei in myotubes (⩾2 nuclei) by the total number of nuclei analyzed (1000–2000), as previously described [18]. At DM5, myoblast fusion was analyzed by calculating numbers of nuclei in MyHC+ cells, as previously described [19] with slightly modification. The number of nuclei in individual myotubes was counted for 100–200 myotubes in 10 random fields. MyHC+ cells were then grouped into: those with one nuclei, those with 2–4 nuclei, and those with ⩾5 nuclei. The percentages of nuclei in these three groups were expressed as percentages of total nuclei in MyHC+ cells.

2.8. Cell proliferation assay

Cell proliferation was analyzed using a CellTiter 96 AQueous One Solution cell proliferation assay kit (Promega), as previously described [20]. In brief, C2C12/shCD36 and C2C12/shCont cells were seeded at a density of 1000 cells per well into 96-well plates and incubated for 4 days under standard culture conditions. At the indicated time points, 20 μl of the AQueous One Solution reagent were added to each well and incubated for 1 h. Absorbance at 490 nm was then measured using a plate reader (BioRad).

2.9. Statistical analysis

Statistical significances were assessed using the student's *t*-test. *P* values of <0.05 were considered statistically significant.