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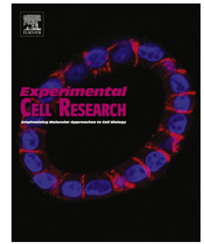

Research Article

Intercellular adhesion molecule-1 expression by skeletal muscle cells augments myogenesis

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ABSTRACT

We previously demonstrated that the expression of intercellular adhesion molecule-1 (ICAM-1) by skeletal muscle cells after muscle overload contributes to ensuing regenerative and hypertrophic processes in skeletal muscle. The objective of the present study is to reveal mechanisms through which skeletal muscle cell expression of ICAM-1 augments regenerative and hypertrophic processes of myogenesis. This was accomplished by genetically engineering C2C12 myoblasts to stably express ICAM-1, and by inhibiting the adhesive and signaling functions of ICAM-1 through the use of a neutralizing antibody or cell penetrating peptide, respectively. Expression of ICAM-1 by cultured skeletal muscle cells augmented myoblast–myoblast adhesion, myotube formation, myonuclear number, myotube alignment, myotube–myotube fusion, and myotube size without influencing the ability of myoblasts to proliferate or differentiate. ICAM-1 augmented myotube formation, myonuclear accretion, and myotube alignment through a mechanism involving adhesion-induced activation of ICAM-1 signaling, as these dependent measures were reduced *via* antibody and peptide inhibition of ICAM-1. The adhesive and signaling functions of ICAM-1 also facilitated myotube hypertrophy through a mechanism involving myotube–myotube fusion, protein synthesis, and Akt/p70s6k signaling. Our findings demonstrate that ICAM-1 expression by skeletal muscle cells augments myogenesis, and establish a novel mechanism through which the inflammatory response facilitates growth processes in skeletal muscle.

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Introduction

Cellular and molecular processes associated with the development of muscular tissue (myogenesis) serve to restore structure and function to skeletal muscle injured by exercise, trauma, or disease, as well as facilitate muscle hypertrophy after mechanical loading (*e.g.*, muscle overload). Myogenesis is preceded by the proliferation of a specialized population of myogenic stem cells known as satellite cells, which normally reside in a quiescent state between the sarcolemma and the basal lamina of myofibers, leading to an increased number of muscle

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precursor cells (myoblasts) in skeletal muscle [1]. In the initial stage of myogenesis, myoblasts differentiate, adhere to each other, and fuse to form multinucleated myotubes [2–6]. Nascent myotubes add nuclei and hypertrophy through the fusion of myoblasts with myotubes, myotube–myotube fusion, and increased protein synthesis [2–7]. In adult muscle, muscle injury recapitulates some aspects of developmental myogenesis as indicated by the formation and maturation of centrally nucleated (regenerating) myofibers, which reflect myotube formation and their subsequent hypertrophy into normal myofibers with peripherally located nuclei [2]. These newly formed myofibers restore muscle structure and function by replacing necrotic myofibers within injured muscle. Satellite cell/myoblast proliferation and regenerating myofibers have also been observed in hypertrophying muscles after muscle overload [8–12]. The extent to which satellite cells and regenerating myofibers contribute to overload-induced hypertrophy remains controversial [11,12].

We previously reported that adhesion molecules of the inflammatory response, such as leukocyte specific β2 integrins and intercellular adhesion molecule-1 (ICAM-1;CD54), contribute to the regulation of regenerative and hypertrophic processes in skeletal muscle [9,10,13]. ICAM-1 is a membrane glycoprotein consisting of an extracellular domain, a transmembrane segment, and a short cytoplasmic domain. The extracellular domain of ICAM-1 functions as a ligand for α-subunits of β2 integrins (CD11a and CD11b) and fibrinogen, and ligation of ICAM-1 transduces an intracellular signal through the interaction of its cytoplasmic domain with adapter and other cytoskeletal proteins [14,15]. Prior work has established that ICAM-1 is not constitutively expressed by skeletal muscle cells *in vitro* or *in vivo* [10,16,17]. In contrast, we found ICAM-1 on the membrane of satellite cells/myoblasts, regenerating myofibers, and normal myofibers after muscle overload [10]. Expression of ICAM-1 by skeletal muscle cells and other cell types (*e.g.*, endothelial cells and leukocytes) contributed to regenerative and hypertrophic processes in skeletal muscle, as indicated by an attenuation in regenerating myofiber formation, protein synthesis, and hypertrophy in overloaded muscles of ICAM-1−/− compared to wild type mice [10]. As the extracellular domain of ICAM-1 facilitates cell-to-cell adhesion, and the cytoplasmic domain of ICAM-1 can activate signaling pathways (*e.g.*, p38 MAPK and Akt) [14,15] that are pertinent to muscle growth, we speculate that the expression of ICAM-1 by skeletal muscle cells augments myogenic processes critical to muscle regeneration and hypertrophy.

The objective of the present study was to test the hypothesis that the expression of ICAM-1 by skeletal muscle cells augments regenerative and hypertrophic processes of myogenesis. We report that ICAM-1 expression by cultured skeletal muscle cells (C2C12 cells) augmented events of myogenesis in which myotubes are forming, adding nuclei, aligning, fusing, synthesizing proteins, and hypertrophying. We also explored the involvement of the extracellular and cytoplasmic domains of ICAM-1, as well as p38 MAPK and Akt/p70s6k signaling, as mechanisms through which ICAM-1 expression by skeletal muscle cells augmented events of myogenesis.

Materials and methods

Stable transfections

C2C12 myoblasts (ATCC) were stably transfected with an expression vector containing murine ICAM-1 under transcriptional regulation of the human β-actin promoter (pHβA-ICAM1; kindly provided by Dr. Stephen Hedrick at The University of California, San Diego; ICAM-1+ cells) [18]. Another population of C2C12 myoblasts were stably transfected with an empty pHβAPr-1 vector (generously provided by Dr. Peter Gunning at the University of New South Wales; EV cells) [19]. Transfection quality plasmid DNA was prepared using a commercially available kit (Qiagen) and transfected using Lipofectamine™ 2000 according to the manufacturer's protocol (Life Technologies). Cells transfected with the ICAM-1 plasmid or empty vector were placed under G418 (800 μg/ml) selection for a total of 24 d to create a population of stably transfected cells. Non-transfected C2C12 myoblasts served as control cells.

Transfection efficiency was assessed *via* flow cytometry, western blotting, and immunofluorescence. For flow cytometry, cells were detached from tissue culture dishes using enzyme free cell disassociation buffer (Life Technologies), treated with Fc Block™ (BD Biosciences), and incubated for 30 min with a phycoerythrin (PE)-conjugated anti-ICAM-1 antibody (clone YN1/7.4) or an equivalent amount of a isotype control antibody (eBiosciences). Cells were analyzed using FACSCalibur (BD Biosciences) at the University of Toledo Flow Cytometry Core Facility using standard procedures. Western blot and immunofluorescence detection of ICAM-1 were performed as described below.

Cell cultures

Myoblasts (control, EV, or ICAM-1+) were seeded in 24 well plates for proliferating cultures (2500 cells/cm^2), and in 6 or 12 well plates or 100 mm dishes for differentiating cultures (5000 cells/cm^2). Cells proliferated in Dulbecco's modified eagle medium (DMEM; Thermo Scientific) containing 10% fetal bovine serum (Sigma-Aldrich; growth medium) for up to 4 d. Upon reaching 90% confluence, cells were treated with DMEM containing 2% horse serum (Sigma-Aldrich; differentiation medium) for up to 6 d. To explore the possibility that components of horse serum influenced ICAM-1 mediated myogenesis, we also differentiated ICAM-1+ cells in DMEM supplemented with insulin (5 μg/ml), transferrin (5 μg/ml) and selenium (5 ng/ml) (ITS medium; Sigma-Aldrich). All media contained antibiotic–antimycotic reagents (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml of amphotericin B; Sigma-Aldrich) and were changed either daily (ITS medium) or every 2 d (growth and differentiation medium).

Antibody neutralization of ICAM-1

The involvement of the extracellular domain of ICAM-1 in myogenesis was tested using an antibody neutralization approach. ICAM-1+ cells were washed three times in phosphate buffered saline (PBS) and then treated with differentiation medium supplemented with vehicle (water), an ICAM-1 neutralizing antibody (YN1/1.7.4; eBioscience; 100 μg/ml) or an isotype control antibody (eBioscience; 100 μg/ml) for 2 or 24 h. The antibody concentration was chosen based on findings from experiments that examined the influence of varying concentrations of the ICAM-1 neutralizing antibody on myotube indices. For the myoblast adhesion assay (described below), ICAM-1+ cells were suspended in differentiation medium containing vehicle, isotype

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