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Effects of type IV collagen on myogenic characteristics of IGF-I gene-engineered myoblasts

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Skeletal muscle regeneration requires migration, proliferation and fusion of myoblasts to form multinucleated myotubes. In our previous study, we showed that insulin-like growth factor (IGF)-I gene delivery stimulates the proliferation and differentiation of mouse myoblast C2C12 cells and promotes the contractile force generated by tissue-engineered skeletal muscles. The aim of this study was to investigate the effects of the extracellular matrix on IGF-I gene-engineered C2C12 cells *in vitro*. Retroviral vectors for doxycycline (Dox)-inducible expression of the IGF-I gene were transduced into C2C12 cells. When cultured on a type IV collagen-coated surface, we observed significant increases in the migration speed and number of IGF-I gene-engineered C2C12 cells with Dox addition, designated as C2C12/IGF (+) cells. Co-culture of C2C12/IGF (+) cells and parental C2C12 cells, which had been cultured in differentiation medium for 3 days, greatly enhanced myotube formation. Moreover, type IV collagen supplementation promoted the fusion of C2C12/IGF (+) cells with differentiated C2C12 cells and increased the number of myotubes with striations. Myotubes formed by C2C12/IGF (+) cells cultured on type IV collagen showed a dynamic contractile activity in response to electrical pulse stimulation. These findings indicate that type IV collagen promotes skeletal muscle regeneration mediated by IGF-I-expressing myoblasts, which may have important clinical implications in the design of myoblast-based therapies.

[**Key words:** Skeletal muscle; Myogenic differentiation; Myoblast; IGF-I; Type IV collagen]

Skeletal muscle is composed of highly dense and oriented contractile myofibers. These myofibers are surrounded by a basal lamina, which includes laminin and type IV collagen, and are further sheathed by the endomysium containing fibronectin and type I, III and V collagen (1). In response to tissue regeneration stimuli arising from damaged muscle tissues, quiescent muscle satellite cells are activated to differentiate into myoblasts and subsequently enter the tissue regeneration process involving migration, proliferation and fusion of myoblasts to form multinucleated myotubes (2,3). Although skeletal muscle is one of the most highly regenerative tissues in the body, muscle degeneration due to aging, severe injuries or myopathies may lead to long-term loss of muscle mass and functional deficiency. A possible strategy for skeletal muscle regeneration is cell therapy in which myogenic progenitor cells including myoblasts are injected into muscle tissues (4,5). However, the therapeutic efficacy is limited by insufficiencies of migratory capacity, proliferation rate and differentiation rate of the transplanted cells (4,5).

Genetically engineered cells have great potential for cell-based therapies, and may be important for the next generation of regenerative medicine (6). Insulin-like growth factor (IGF)-I, a peptide growth hormone, plays a key role in muscle growth and regeneration as an autocrine/paracrine mediator (7). Upon binding to its receptor, IGF-I activates intracellular signaling pathways, such as mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K) and calcium/calmodulin-dependent protein kinase pathways, leading to myoblast proliferation, myogenic differentiation, myotube hypertrophy, and increased protein synthesis and cell survival (8–10). In our previous study, we showed that IGF-I gene delivery to mouse myoblast C2C12 cells stimulates their proliferation, differentiation and hypertrophy, and promotes the contractile force generation of tissue-engineered skeletal muscles *in vitro* (11). These results indicate that IGF-I gene-engineered myoblasts are a promising cell source in genetically engineered cell therapy for skeletal muscle regeneration.

During skeletal muscle regeneration, the extracellular matrix (ECM) of the basal lamina and endomysium plays an important role in the migration, proliferation and differentiation of myoblasts (12,13). Therefore, the study of ECM regulation in myoblast behaviors is important to construct functional skeletal muscle tissues using myoblasts *in vitro*. However, the effects of the ECM on IGF-I-expressing myoblasts are still unknown. *In vivo* analysis of myoblast migration, proliferation and differentiation during skeletal muscle regeneration is difficult. On the other hand, *in vitro* experiments in adherent tissue culture enable easy observations of cell behaviors using a microscope and allow manipulation of culture conditions

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by supplementing the medium with exogenous factors including ECM materials or coating the culture surface with ECM proteins. In addition to exogenous factors, assessing the role of endogenous factors is possible, including overexpression of factors of interest via gene transfer into myoblasts. For *in vitro* study, immortalized myoblast cell lines are convenient and consistent. Mouse myoblast C2C12 cells are the best-characterized cell line derived from muscle satellite cells (14), and the most commonly used cells in migration (15), proliferation (16) and differentiation (15–17) studies. In the present study, we used *in vitro* systems to analyze the behaviors of IGF-I gene-engineered C2C12 cells cultured on ECM proteins.

The ultimate goal for muscle regeneration is recovery of the contractile properties of muscle tissues. The assembly of sarcomeres, the contractile units in striated muscle, is required for contractile activity. It has been reported that electrical pulse stimulation accelerates sarcomere assembly in C2C12 myotubes and induces contractile activity *in vitro* (18,19). In this study, the effects of ECM proteins on migration, proliferation, fusion and contractile activity of IGF-I gene-engineered C2C12 cells were investigated *in vitro*.

MATERIALS AND METHODS

Cells and culture IGF-I gene-engineered C2C12 cells (C2C12/IGF cells) were established as described previously (11). Briefly, the Tet-On system (Clontech, Mountain View, CA, USA) was incorporated into retroviral vectors for inducible expression of the IGF-I gene. For retroviral vector production, we used two plasmids, pQMSCV/EGFP-CMV-rtTA-WPRE and pQMSCV/EGFP-TRE-IGF-WPRE. pQMSCV/EGFP-CMV-rtTA-WPRE encoded a constitutive expression cassette for a transactivator (rtTA) that was activated by addition of doxycycline (Dox), whereas pQMSCV/EGFP-TRE-IGF-WPRE encoded an expression cassette for the IGF-I gene including a tet-responsive element to promote IGF-I gene expression by activation of rtTA. These plasmids included an enhanced green fluorescent protein (EGFP) gene under the control of a viral long terminal repeat promoter. Retroviral vectors pseudotyped with vesicular stomatitis G protein (VSV-G) were produced by transient transfection of 293FT cells with a retroviral vector plasmid (pQMSCV/EGFP-CMV-rtTA-WPRE or pQMSCV/EGFP-TRE-IGF-WPRE), pcDNA4-gag/pol and pLP/VSV-G using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). For retroviral infection, C2C12 cells were cultured for 24 h and then the medium was replaced with a retroviral solution containing the retroviral vectors carrying the rtTA gene and inducible IGF-I gene expression cassette. The cells were then cultured in the presence of polybrene for 6 h, resulting in the generation of C2C12 cells capable of Dox-inducible IGF-I gene expression (C2C12/IGF cells). The viral titers against C2C12 cells were determined by flow cytometry using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Viral titers were in the range of $0.7–1.2 \times 10^7$ IU/ml. The secretion level of IGF-I from C2C12/IGF cells in the absence [C2C12/IGF (−) cells] or presence [C2C12/IGF (+) cells] of Dox (1 μg/ml; Sigma–Aldrich, St Louis, MO, USA) was 1.0 and 27.2 fg/(cell day), respectively (11).

C2C12 and C2C12/IGF cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G potassium and 0.1 mg/ml streptomycin sulfate (growth medium). To induce myogenic differentiation, the medium was changed to DMEM supplemented with 2% calf serum, 100 U/ml penicillin G potassium and 0.1 mg/ml streptomycin sulfate (differentiation medium). Cells were cultured at 37°C in a 5% CO_2 incubator.

Cell migration assay Cells (1.5×10^3) were seeded in 35-mm tissue culture dishes (control; Greiner Bio-one, Frickenhausen, Germany) or ECM (fibronectin, type I collagen, laminin or type IV collagen) pre-coated dishes (BD Biosciences), and cultured in growth medium for 24 h. Subsequently, the cells were observed under a BZ-9000 fluorescence microscope (Keyence, Tokyo, Japan) equipped with a 5% CO_2 incubation chamber (Keyence) and BZ-II multidimensional time-lapse software (Keyence). The cells were cultured in growth medium for 24 h in the 5% CO_2 incubation chamber at 37°C, and time-lapse images of cell movements were automatically captured every 10 min at three positions on the bottom surface of three separate dishes. A single cell in the captured images was tracked, and the migration speed was analyzed using motion analyzer software (Keyence).

Cell proliferation assay Cells (3.5×10^4) were seeded in 35-mm tissue culture dishes (control) or ECM pre-coated dishes and cultured in growth medium for 48 h. Subsequently, cell samples were collected, and the numbers of viable cells were measured by the trypan blue dye exclusion method. The cells were cultured at 37°C in a 5% CO_2 incubator.

Myogenic differentiation assay Cells (5×10^4) were seeded into the wells of 6-well tissue culture plates (control) or ECM pre-coated dishes at 3 days before induction of differentiation (day −3). After 3 days of culture in growth medium, the medium was replaced with differentiation medium (day 0), and the cells were cultured for a further 4 days. The cells were subjected to a standard immunofluorescence staining procedure as described previously (11). Briefly, the cells on day 7 were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 min. The cells were then permeabilized in PBS containing 0.2% Triton X-100 for 15 min, washed three times with PBS, and blocked in PBS containing 1% (w/v) bovine serum albumin (BSA) for 30 min. The cells were incubated with a primary antibody against α-actinin (monoclonal anti-α-actinin EA-53; Sigma–Aldrich) for 45 min, washed three times with PBS and then incubated in PBS containing an Alexa546-conjugated secondary antibody (Life Technologies), 4′,6-diamidino-2-phenylindole (DAPI), and 1% BSA for 45 min. After washing three times with PBS, the cells were observed under a BZ-9000 fluorescence microscope. Microscopic images of five fields in each of three separate wells per sample were captured. The width of α-actinin-positive myotubes and the number of DAPI-positive nuclei were measured using BZ-II measurement module software (Keyence) and hybrid cell count software (Keyence), respectively. The differentiation rate was calculated by the following equation: Differentiation rate = (the number of DAPI-positive nuclei in α-actinin-positive myotubes in the field)/(the number of DAPI-positive nuclei in the field). To estimate the mean value of myotube widths, the five largest myotubes of five fields in each of three separate wells per sample were measured.

Myogenic cell fusion assay C2C12 cells (5×10^4) were seeded into the wells of 6-well tissue culture plates (Greiner Bio-one) on day −3, and the medium was replaced with differentiation medium on day 0. In some experiments, C2C12 cells were pre-stained with (5-(and-6)-(((4-chloromethyl)benzoyl)amino))tetramethylrhodamine (25 μM, CellTracker; Molecular Probes, Eugene, OR, USA). Subsequently, C2C12/IGF cells (5×10^4) with or without 10 μg/ml type IV collagen (from human placenta, Sigma–Aldrich) were added to the C2C12 cells on day 3 of differentiation. For the cell adhesion assay, after 2 h of co-culture, the cells were washed three times with PBS, and the number of EGFP-positive cells (C2C12/IGF cells) attached to the culture surface was counted under a BZ-9000 fluorescence microscope. For the cell fusion assay, the cells were cultured for a further 4 days, and immunofluorescence staining for α-actinin was performed on day 7. Microscopic images of five fields in each of three separate wells per sample were captured under a BZ-9000 fluorescence microscope, and the number of EGFP-positive myotubes was counted.

Myotube contractile activity assay C2C12 cells (5×10^4) were seeded in 35-mm tissue culture dishes on day −3. In some experiments, the cells were seeded in 35-mm glass bottom dishes (Code 3970-035, Asahi Glass, Tokyo, Japan) to visualize striation patterns in myotubes after α-actinin staining. The medium was replaced with differentiation medium on day 0. C2C12/IGF cells (5×10^4) with or without 10 μg/ml type IV collagen were added to the C2C12 cells on day 3 of differentiation. The cells were cultured for a further 4 days. On day 7, electrical pulse stimulation was applied to the cells. Carbon electrodes were placed 18 mm apart at opposite sides of a tissue culture dish. The generation of electric pulses was controlled by a personal computer with specially designed LabView software (National Instruments, Austin, TX, USA). The cells were stimulated with an electric pulse for 30 min with the following properties: voltage, 0.3 V/mm; width, 4 ms; frequency, 1 Hz. After 30 min of electrical pulse stimulation, the electric pulses were applied again and myotube movement was recorded at a rate of 15 frames/s for 25 s at three positions on the bottom surface in each of three separate dishes using a BZ-9000 fluorescence microscope. A single cell within myotubes in the captured images was tracked during the electrical pulse stimulation, and its displacement was measured using motion analyzer software. To estimate the range of displacement, three myotubes displaying the highest contractile activity in each of three fields in three separate dishes were measured using motion analyzer software. To visualize striation patterns in myotubes, α-actinin staining was performed. Microscopic images of five fields in each of three separate wells per sample were captured under a BZ-9000 fluorescence microscope, and the number of myotubes possessing sarcomeric α-actinin striations was counted.

Statistical analysis Statistical comparisons were performed using the Mann–Whitney rank sum test. Values of $P < 0.05$ were considered to indicate significant differences.

RESULTS

Effects of ECM proteins on migration, proliferation and differentiation of C2C12/IGF (+) cells To investigate the effects of various ECM substrata on migration, proliferation and myogenic differentiation of C2C12/IGF (+) cells, the cells were cultured on fibronectin-, type I collagen-, laminin- or type IV collagen-coated culture surfaces in the presence of Dox. Time-lapse analysis of cell locomotion revealed that C2C12/IGF (+) cells on type IV collagen migrated the longest distance (Fig. S1) and showed a significantly higher migration speed than that of cells on the control tissue culture surface ($P < 0.05$) (Fig. 1A). Next, to assess which ECM protein enhanced the proliferation of C2C12/IGF (+)