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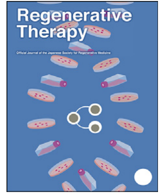

Original Article

Technical advantage of recombinant collagenase for isolation of muscle stem cells

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ABSTRACT

Background: Muscle satellite cells are resident skeletal muscle stem cells responsible for muscle regeneration. Isolation of satellite cells is a critical process for clinical application such as drug screening and cell transplantation. Fluorescence-activated cell sorting (FACS) enables the direct isolation of satellite cells from muscle tissue. During the process used to isolate satellite cells from skeletal muscle, enzymatic digestion is the first step. Therefore, the evaluation and standardization of enzymes is important not only for reproducibility of cellular yield and viability, but also for traceability of material used in protocols.
Methods: The comparison of muscle digestion was performed either by a mixture of recombinant collagenase G (ColG) and collagenase H (ColH) or by a conventional collagenase II. The degree of cell damage and surface antigen expression upon collagenase treatment were analyzed by FACS. To investigate whether satellite cells isolated using recombinant collagenase can regenerate injured muscle, satellite cells were cultured, transplanted into injured muscles, and analyzed by immunostaining.
Results: We show that ColG and ColH were efficient to isolate satellite cells from mouse skeletal muscle tissue. Digestion with a combination of ColG and ColH enriched satellite cells with intact surface antigens such as $\alpha7$ and $\beta1$ integrins. Furthermore, satellite cells isolated using ColG and ColH dramatically proliferated and remained undifferentiated *in vitro*. When transplanted, satellite cells isolated using ColG and ColH enhanced the therapeutic efficacy *in vivo*.
Conclusions: Our results provide an efficient method of satellite cell preparation using recombinant collagenases with a high cell yield, viability of cells, and regeneration potency to fit the biological raw material criteria.
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1. Introduction

Skeletal muscle is crucial for physical support and motion [1]. However, loss of skeletal muscle function unable the maintenance of skeletal muscle tissues, which may cause severe muscular disorders, such as muscular dystrophy and age-associated muscle atrophy [2]. Although several curative treatments for those severe muscular diseases are still under development, screening of molecules that enhances the muscle regeneration or cell transplantation approach has been expected to be the promising treatment as to promote the regeneration of muscle cells. Muscle regeneration is mediated by satellite cells, which are located in the sarcolemma and beneath the basement membrane of muscle fibers [3,4], composed of collagens, laminins, and proteoglycans [5]. The function and survival of satellite cells are maintained by multiple adhesion receptors, including integrins, which are responsible for cell adhesion to the extracellular matrix (ECM) [6–8]. Signals are transmitted into cells upon binding to the ECM, and integrins are important for regulation of physiological functions, such as survival, proliferation, and differentiation [9,10]. The paired box transcription factor

Abbreviations: ColG, collagenase G; ColH, collagenase H; Ct, cycle threshold; CTX, cardiotoxin; ECM, extracellular matrix; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; PE, phycoerythrin; PI, propidium iodide; TA, tibialis anterior.

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Pax7 is critical for maintenance of satellite cell function, and its inactivation causes severe depletion of satellite cells in skeletal muscle [4,11,12]. Pax7, together with the myogenic regulator MyoD, determines the fate of satellite cells [13–15]. In adult skeletal muscle, satellite cells remain in the Pax7+MyoD− quiescent phase [16]. However, once the muscle tissue is damaged, satellite cells converts into the Pax7+MyoD+ activated state and proliferate, followed by differentiation into mature myofibers [17]. After tissue repair, a small number of activated satellite cells self-renew and return to the quiescent state [3,7,18].

To date, methods have been established to isolate satellite cells from skeletal muscle tissue using fluorescence-activated cell sorting (FACS). In mice, isolated satellite cells were transplanted into the damaged muscle, and engraftment, regeneration, and self-renewal were observed [19,20]. During the process of isolating mononuclear cells from skeletal muscle, enzymatic digestion using collagenase is needed for efficient isolation [21,22]. Conventionally used collagenase II is prepared from *Clostridium histolyticum* extracts, which contains multiple enzymes such as collagenases, neutral proteases, and others in various ratios depending on the company and the product batch [23,24]. Since most of these enzymes are not defined and free of unknown derivatives, therefore, using conventional collagenase II does not necessarily fit to the biological raw material criteria. Also, isolating stem cells with intact surface antigens is another important point for analysis and clinical applications.

In this study, we compared the effects of purified recombinant collagenases (collagenase G, ColG and collagenase H, ColH) and conventional collagenase II to isolate skeletal muscle satellite cells. We showed an efficient method of satellite cell preparation using ColG and ColH with a high cell yield, viability of cells, and regeneration potency to fit the biological raw material criteria. This approach can be applicable to isolate somatic stem cells, such as mesenchymal stem cells and pancreatic islet cells.

2. Methods

2.1. Animals

C57BL/6 wild-type mice and C57BL/6-Tg (CAG-EGFP) mice were purchased from CLEA Japan, Inc and Japan SLC, Inc., respectively. Eight to twelve-week-old male mice were analyzed. All procedures for animal experiments were approved by the Tokyo Medical and Dental University Animal Care and Use Committee (Protocol number: 0170282C).

2.2. Satellite cell isolation

Mouse skeletal muscles from the fore- and hind-limbs were dissected and digested with collagenases. In terms of enzyme concentrations, we measured enzymatic activities of ColG (Meiji Seika Pharma) and collagenase type II (Worthington Biochemical) using a substrate, Azcoll (Sigma). Also, enzymatic activities of ColH (Meiji Seika Pharma) and collagenase type II using a substrate, N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (Sigma), were measured as well. From the measurements, the appropriate concentrations of ColG (57.456 μg/ml) and ColH (12.125 μg/ml) that exert equal activities to that of collagenase type II (1.4 mg/ml) was determined and used for the experiments. Since collagenase type II is crude and possesses neutral protease activity, Dispase II (Godo shusei) was used as a supplementation of neutral protease into the ColG/ColH solution. The neutral protease activities of Dispase II and collagenase type II were measured using a substrate, FA-Gly-Leu-NH2 (Bachem). According to the measurement, 155.4 μg/ml of Dispase II was expected to have an equal activity to that of collagenase type II. As a result of an optimization for the satellite cell isolation, 2-fold the concentration (310.8 μg/ml) of Dispase II was suitable and used as a supplementation of neutral protease to ColG and ColH in this study. Collagenases were used for digestion at 37 °C for 1 h. Then, the digested tissue was filtered through 100 μm- and 40 μm-cell strainers (BD Biosciences). The filtered mononuclear cells were stained with phycoerythrin (PE)-conjugated anti-CD31 (BD Biosciences), PE-conjugated anti-CD45 (BD Biosciences), PE-conjugated anti-Sca1 (BD Biosciences), and biotinylated anti-SM/C-2.6 antibodies [26], and streptavidin–allophycocyanin (Becton, Dickinson and Company), on ice for 30 min. To analyze expression of integrins, a fluorescein isothiocyanate-conjugated anti-integrin α7 antibody (3C12; Novus Biologicals) and a PE-conjugated hamster anti-rat CD29 antibody (BD Bioscience) were also added. All the cells were resuspended in HBSS and propidium iodide (PI). Cell sorting was performed using a MoFlo flow cytometer (Beckman), and $CD31^-$, $CD45^-$, $Sca\text{-}1^-$, and $SM/C\text{-}2.6^+$ cells were collected as mouse satellite cells.

2.3. Cell culture

Isolated mouse satellite cells were plated on glass chamber slides coated with Matrigel (BD Biosciences). For proliferative conditions, satellite cells were cultured in Dulbecco's modified Eagle's medium with GlutaMAX (Life Technologies) containing 20% fetal bovine serum (Sigma–Aldrich), 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 5 ng/ml basic fibroblast growth factor (ReproCell) in 5% CO_2 at 37 °C.

2.4. RT-PCR

Total RNA was isolated from sorted cells using the TRI reagent (Sigma–Aldrich). cDNA was generated from 0.5 μg of total RNA using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). The RNA was previously treated with the DNase (Invitrogen). RT-PCR was performed using the Applied Biosystems StepOne Real-Time PCR System. PCR was performed in duplicate with reaction volumes of 10 μl containing Fast SYBR Green Master Mix (Applied Biosystems), forward and reverse primers, and the cDNA template. Data were analyzed using a comparative critical threshold (Ct) method, where the amount of the target normalized to the amount of the endogenous control relative to the control value is given by $2^{-\Delta\Delta Ct}$. The primers were as follows: HPRT: 5′-tcagtcaacggggacataaa-3′ (forward), 5′-ggggctgtactgcyyaaccag-3′ (reverse), Pax7: 5′-ctcagtgagttcgattagccg-3′ (forward), 5′-agacggttccctttgt-3′ (reverse).

2.5. Muscle injury and cell transplantation

To induce skeletal muscle regeneration, mice were anesthetized with isoflurane and their hind-limbs were shaved. 100 μl of cardiotoxin (CTX; 10 μM in 0.9% NaCl; Sigma–Aldrich) was injected into the tibialis anterior (TA) muscle using a 29-gauge needle. For transplantation experiments, 8–10-week-old WT mice were used as recipients and were injured with CTX 24 h before transplantation of cells into TA muscles. $SM/C\text{-}2.6^+$ satellite cells were sorted by flow cytometry from EGFP-expressing mice and were cultured for 5 or 6 days, and were transplanted into injured muscle. At 14 days after injury, mice were euthanized and frozen sections were prepared.

2.6. Cryosections

Mouse TA muscles were dissected and frozen in isopentane (Wako) that was cooled with liquid nitrogen. Using a cryostat

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