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Original Article

Proliferative activity of skeletal myoblast sheet by paracrine effects of mesenchymal stem cells

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ABSTRACT

Objectives: The purpose of this study was to examine the proliferative activity enhancement of skeletal myoblasts in the presence and absence of mesenchymal stem cells (MSC).
Methods: We artificially fabricated two types of cell sheets by co-culturing rabbit skeletal myoblast sheets with rabbit MSCs using type IA collagen gel (MC+), and cell sheets without rabbit MSCs (MC−). *Results:* The results of hematoxylin and eosin staining revealed that MC+ was thicker than MC− on day 7. Immunohistochemical staining revealed a low level of desmin expression in both sheets on day 2. Desmin expression increased at days 7 and 12, and desmin localization was consistent with the stratified area. Reverse transcription PCR revealed the presence of MyoD and PAX7 in both sheets on days 2, 7, and 12. The presence of myogenin was confirmed in both sheets on days 7 and 12. Hepatocyte growth factor expression was evident in MC+ on day 2, and in both sheets on day 7. Measurement of cell proliferative activity based on DNA cell cycle analysis indicated that MC+ had significantly higher cell proliferative activity than MC− on day 7. There were no significant differences in cell proliferative activity between MC+ and MC− on day 12.
Conclusions: This study demonstrated that the presence of MSCs could transiently enhance the proliferative activity of myoblasts, but that this enhancement is ultimately diminished due to contact inhibition.

1. Introduction

Regeneration of muscle tissue typically consists of directly injecting or grafting muscle progenitor cells into the damaged muscle tissue [1]. One challenge of injecting or grafting of these muscle progenitor cells is an inadequate therapeutic response, due to the low survival and retention rates of the injected/grafted cells in the muscle tissue [2]. The recent clinical application of "cell sheet technology", in which cells are cultured on a sheet and then grafted to the intended site, improves muscle tissue function more effectively than muscle progenitor cell injection or grafting [3]. Cell sheet technology was developed using cultured epidermal allografts to treat skin ulcers [4] and severe burns by Phillips et al. [5]. Since then, various cell sheet types have been developed, including cell sheets intended for kidney [6], eye [7], and heart [8] grafts. Sawa et al. [9] reported improved cardiac function after grafting a skeletal myoblast sheet in a patient with dilated cardiomyopathy. The effects of skeletal myoblast sheet grafting are attributed primarily to paracrine effects, in which various cytokines secreted by the grafted skeletal myoblast sheet promote neovascularization of the graft bed and regulation of inflammatory responses [10]. However, it was reported that an insufficient blood supply from the graft bed to the grafted skeletal myoblast sheet was restricting the cell survival rate within the sheet and the therapeutic response due to paracrine effects [11]. Meanwhile, Shudo et al. [12] described that grafting conventional skeletal myoblast sheets was inadequate for improving severely damaged muscle tissue due to their low cell survival rate, and that skeletal

Abbreviations: ALP, alkaline phosphatase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GA, gentamicin/amphotericin-B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HE, hematoxylin and eosin; HGF, hepatocyte growth factor; hOMNEC, human oral mucosal non-epithelial cells; MC+, rabbit skeletal myoblast sheets with rabbit MSCs; MC−, rabbit skeletal myoblast sheets without rabbit MSCs; MCGS, mesenchymal cell growth supplement; MSC, mesenchymal stem cells; PFA, paraformaldehyde; PBS, phosphate buffered saline; RT, room temperature; TGF, transforming growth factor

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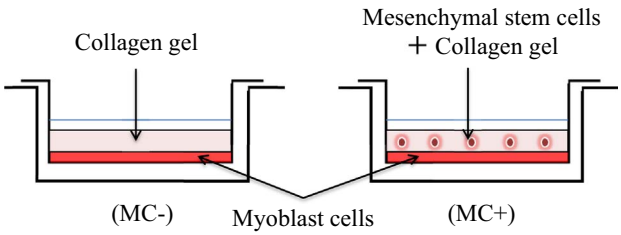

Fig. 1. *Cultivation of rabbit skeletal myoblast sheets.* Myoblasts were collected and seeded on culture inserts. After three days in culture, collagen gel without MSCs (MC−) or with MSCs (MC+) were laminated on myoblasts and cultured for up to 12 days.

myoblast sheets with a higher post-graft survival rate were needed to achieve better treatment outcomes.

Mesenchymal stem cells (MSC) were first isolated from bone marrow, and in recent years they have been found in various tissues, including the bone marrow, umbilical cord, dental pulp, periosteum, skeletal muscle, fat, pancreas, placenta, and endometrium [13–19]. Gnecchi et al. [20] demonstrated that MSCs secrete hepatocyte growth factor (HGF), while Zarnegar et al. [21] discovered that HGF possesses a myoblast proliferative action and plays a key role in intramuscular regeneration. Based on these studies, it is conceivable that the presence of MSCs could enhance the proliferative activity of the myoblast sheet.

To investigate enhancement of proliferative activity, we co-cultured rabbit MSCs and rabbit skeletal myoblast sheets, and measured cell proliferative activity. Additionally, we performed hematoxylin and eosin (HE) staining, immunohistochemistry of desmin, and reverse transcription PCR (RT-PCR) of phenotype markers on the myoblast sheets.

2. Material and methods

2.1. Preparation of rabbit oral mucosa tissues

All experimental procedures and protocols were approved by the Animal Care and Use committee of Tokyo Dental College (approval number: 270106) and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Japanese white rabbits (female, 2.5 kg body weight, Shiraishi Experimental Animal Breeding Farm, Tokyo, Japan) were anesthetized with 100 mg/kg pentobarbital sodium (Kyoritsu Seiyaku CO., Tokyo, Japan) and sacrificed using 1 M potassium chloride (Wako, Osaka, Japan). Rabbit oral mucosal tissues were prepared from oral cavities.

2.2. Isolation of MSCs

MSCs were isolated from oral mucosal connective tissues. Rabbit mucosal specimens were dissected, and submucosal connective tissues, such as adipose and muscle tissues, were removed with scissors. The tissues were cut into small pieces, and connective tissues were separated from the epithelium using 1.2 U/mL dispase II (Roche, Mannheim, Germany) at 4 °C overnight [22]. Connective tissues were treated with 2 mg/mL collagenase at 37 °C overnight. To prepare rabbit MSCs from individual cells, cells were cultured at 8.0×10^3 cells/mL, to minimize cellular aggregation, from amplified rabbit MSCs with 0.8% methylcellulose in advanced-Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) on low adhesive plates (HydroCell; CellSeed, Tokyo, Japan) to avoid attachment of cells to the plate bottom [23]. After two weeks at 37 °C in humidified air with 5% CO_2, clusters were formed from single cells. Clusters were replated onto adhesive plates and amplified by explant adhesive culture.

Table 1
Primer sequences and product size of RT-PCR.

Primer	Sequence (5′→3′)	Product size (bp)
MyoD	GCTCGCGAGGATGAGCATGT ATGGCGTTGCGCAGGATCTC	239
Myogenin	TACCCAAGGTGGAGATCCT GCATATGGTCTCCTGGGTT	219
Pax7	ATCCGGCCCT GTGTCATCTC CACGCGGCTA ATCGAACTCA	278
HGF	CAGACCAATGTGCCAACAG GTCACAGACTTCGTAGCG	407
GAPDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	452

2.3. Isolation of oral mucosal myoblasts

Oral mucosal myoblasts were also isolated from the oral mucosa immediately beneath muscle tissues. The muscle tissue, separated from rabbit mucosal specimens, was cut into small pieces with scissors and treated with 2.5% trypsin at 37 °C for 2 h. Isolated cells from dissociated tissue were cultured in advanced-DMEM with 10% FCS at 37 °C for 30 min, and non-adhesive cells were transferred to a fresh 1% gelatin-coated flask (Becton, Dickinson and Company, Sparks, MD, USA) using differential adhesion rates to remove non-myoblastic cells such as fibroblasts [24]. These cells were capable of being subcultured over 20 times using differential adhesion rates on 1% gelatin-coated flasks.

2.4. In vitro differentiation

When cells became semi-confluent, they were washed in phosphate buffered saline (PBS) and incubated with TrypLE (Invitrogen, Carlsbad, CA, USA) at 37 °C for 5 min. The collected cells were seeded at a density of 5.0×10^3 cells/cm^2 in 4-well chamber slides (Nalge Nunc, Rochester, NY, USA). Isolated cells were cultured in DMEM with 10% FCS until they reached semi-confluence. For osteogenic induction, the cultures were further grown in osteogenic induction medium (Lonza, Walkersville, MD, USA), containing dexamethasone, ascorbate, mesenchymal cell growth supplement (MCGS), L-glutamine, β-glycerophosphate, and gentamicin/amphotericin-B (GA)−1000 (Lonza), for three weeks. Control groups were grown in DMEM with 10% FCS for three weeks. For adipogenic induction, the cultures were further grown in adipogenic induction medium (Lonza), containing human recombinant insulin, L-glutamine, MCGS, dexamethasone, indomethacin, 3-isobutyl-methyl-xanthine, and GA-1000. Control groups were grown in adipogenic maintenance medium (Lonza), containing human recombinant insulin, L-glutamine, MCGS, and GA-1000, for three weeks. For chondrogenic induction, the cultures were further grown in complete chondrogenic induction medium (Lonza), containing dexamethasone, ascorbate, insulin-transferrin-selenium (Lonza) supplement, GA-1000, sodium pyruvate, proline, L-glutamine, and transforming growth factor (TGF)-β3 (Lonza). Control groups were grown in incomplete chondrogenic induction medium without TGF-β3. The medium was changed three times a week and cultures were analyzed after three weeks.

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