Effect of Mesenchymal Cells on Myoblast Sheets Embedded in Collagen Gel

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Abstract

The objective of this study was to investigate the effects of mesenchymal cells on myoblasts in long-term cultivation of myoblast cell sheets. Sheets of myoblasts and mesenchymal cells from Japanese rabbit oral mucosa were generated and analyzed by histochemistry, Western blot, and reverse transcription-polymerase chain reaction. The presence of desmin and type IV collagen, which is seen in normal muscle tissue, was also confirmed in all the sheets produced. Expression of desmin and type IV collagen showed a decrease under co-culture conditions. In addition, expression of genes important in maintaining the undifferentiated state (Pax7, CD34, myogenin, MyoD) in myoblasts was observed throughout the long cultivation period. Insulin-like growth factor was expressed only when the mesenchymal cells were co-cultured with myoblasts. These data suggest that the presence of mesenchymal cells in a long-term co-culture system influences myoblast differentiation.

Key words: Cell sheet — Myoblast — Mesenchymal cells — Long-term culture — IGF

Introduction

Recent years have seen progress in the field of cell sheet engineering, and much research has been done with regard to this technology, which has been employed for a variety of clinical applications, including regeneration of heart muscle and corneal tissue, leading to the establishment of new treatment methodologies.¹,¹³,¹⁵,²²,²⁵

Treating cancer arising in the buccal mucosa often entails extensive removal of mucous membrane, necessitating reconstruction of the underlying muscle layers for recovery of mastication and swallowing functions. Therefore, this team has been attempting to
develop laminated cell sheets with a 3-layer structure resembling that of normal oral mucosa. They are made up of a layer of epithelium, a middle layer of interstitial tissue containing mesenchymal cells, and a final layer of inner muscular tissue.

The potential to facilitate regeneration of muscular tissue is an essential requirement for such sheets. Yamane et al. reported that 3-dimensional rabbit oral epithelial-mesenchymal-muscular hybrid sheets underwent multi-stratification in the myoblast layers in a time-dependent manner\(^\text{28}\). Mesenchymal cells are needed to produce stratified epithelium through direct cell-to-cell interaction or indirect interaction through growth factors or other factors\(^\text{12,16}\). Myoblasts do not stratify under the usual methods of culture in general use, and it is possible that their stratification is influenced by mesenchymal cells. Umezawa et al. reported that the proliferative activity and thickness of rabbit skeletal myoblast sheets was enhanced during the early stages of co-culture with rabbit mesenchymal cells\(^\text{26}\). Short-term culture systems are limited in that they allow for only a small window of opportunity for transplantation of the hybrid sheet.

To our knowledge, no studies to date have investigated muscle layer cell-sheet dynamics under long-term cultivation. A culture model employing rabbit oral mucosal epithelial sheets and isolated rabbit oral mesenchymal cells instead of human cells has already been established\(^\text{21}\). Therefore, the purpose of the present study was to investigate the effect of mesenchymal cells on rabbit myoblasts in cell sheets undergoing long-term cultivation, focusing on the interaction of the myoblasts and mesenchymal cells during regeneration of the muscle layer.

Materials and Methods

1. Preparation of rabbit oral mucosa tissue

This study was approved by the Animal Care and Use Committee of Tokyo Dental College (Approval number: 270101). Two Japanese rabbits were first euthanized by intravenous (i.v.) administration of 50 mg/ml (total, 4 ml) pentobarbital (Kyoritsu Seiyaku Co., Tokyo, Japan) and 4 ml 1M potassium chloride solution (Wako, Osaka, Japan). Oral mucosa tissue was then resected.

2. Isolation of oral mucosal mesenchymal cells

Skeletal muscle tissue underlying the oral mucosa was removed, followed by separation of oral mucosa epithelium and subepithelial tissue by enzymatic digestion with 1.2 U/ml dispase II overnight at 37°C\(^\text{29}\). The tissues were then placed under a microscope and subepithelial connective tissue removed. The subepithelial connective tissue was incubated with 2 mg/ml collagenase at 37°C overnight and oral mucosal subepithelial cells isolated\(^\text{10}\). Proliferated oral mucosal subepithelial cells were treated with 0.25% trypsin at 37°C overnight and mesenchymal cells isolated in 0.8% methyl cellulose single-cell culture\(^\text{9}\). The isolated mesenchymal cells were cultured in mesenchymal stem cell growth medium (Lonza, Walkersville, MD, USA) supplemented with mesenchymal cell growth supplement, L-glutamine, and GA-1000. The cells were sub-cultured at a 1-week interval.

3. Isolation of oral mucosal myoblasts

Skeletal muscle tissue, which was separated from the oral mucosal tissue, was treated with 0.125% trypsin at 37°C for 2 hr to achieve cell dissociation\(^\text{14,15,17}\). Initially, the cells contained high amounts of fibroblasts. Myoblasts were isolated using a selective adhesion method based on their differential adhesion properties\(^\text{14,15,17}\). The harvested myoblasts were cultured in A-DMEM and 10% fetal bovine serum (FBS) and sub-cultured at a 1-week interval.

4. Preparation of cell sheets and culture conditions

First, a well-stirred mixture of collagen gel (Cellmatrix\(^\text{8}\); Nitta-gelatin Co., Osaka, Japan) and isolated myoblasts was placed in the cell culture inserts of 6-well plates (Transwell; Corning, NY, USA). Two different cultures
were then prepared and placed in the inserts to investigate the effects of co-culture with mesenchymal cells on the myoblast sheets: 1) a layer of collagen gel on top of myoblasts embedded in the same (Gel); and 2) a well-stirred mixture of collagen gel and isolated mesenchymal cells overlaid on myoblasts embedded in collagen gel (OS) (Fig. 1). Myoblasts and mesenchymal cells were seeded at a density of $1.0 \times 10^4$ cells/0.8 ml collagen gel/well and $0.25 \times 10^5$ cells/0.8 ml collagen gel/well, respectively. Cell sheets were cultivated in 2 ml A-DMEM culture media containing 10% FBS supplemented with 5 μl Aprotinin (666 KIU/ml, Wako) to prevent dissolution of the collagen gel. The media volume above and below the culture layer was 0.5 ml and 1.5 ml, respectively. The cell sheets were harvested after 2, 4, or 6 weeks of cultivation to determine the progress of cell sheet development.

5. Histochemical analysis

To prepare samples for histochemical staining, two pieces (10 mm × 0.5 mm) were excised from the harvested cell sheets and embedded in Tissue-Tek compound. Frozen 5-μm sections were prepared and subjected to hematoxylin-eosin (H-E) staining and immunohistochemical (IHC) staining for histological observation. For the IHC staining, the section was fixed with 2% paraformaldehyde for 5 min and incubated with blocking solution (10% Normal Donkey Serum and 1% BSA in 0.001M PBS) for 60 min. The primary antibodies comprised anti-desmin antibody (1/300, D9, LSBio and LS-B7175) and anti-collagen type IV (Coll IV) antibody (1/300, Southern Biotec, 1340-01). The sections were incubated with one or other of the primary antibodies at room temperature for 90 min. For fluorescence labeling, the sections were incubated with a secondary antibody, comprising either Cy3-Donkey anti-Mouse IgG (CHEMICON, AP192C) or Rho-Donkey anti-Goat IgG (Jackson, 705-025-147) at room temperature (RT) for 30 min. Cell nuclei were stained with 0.5 mg/ml 4',6-diamino-2-phenylindole (DAPI; Dojindo Laboratories, Tokyo, Japan) for 5 min. The stained sections were viewed using a fluorescence microscope (Axioplan2 imaging; Carl Zeiss Inc., NY, USA).

6. Reverse transcription-polymerase chain reaction

The remaining cell sheets were collected for RNA purification. RNA was extracted using the SV Total RNA Isolation System (Promega, WI, USA) and cDNA synthesized with
avian myeloblastosis virus reverse transcriptase (Takara Bio Inc., Shiga, Japan). To investigate mRNA expression for genes related to myoblasts, a reverse transcription-polymerase chain reaction (RT-PCR) analysis was conducted for growth factor genes (hepatocyte growth factor, \textit{HGF}; and insulin-like growth factor, \textit{IGF}), genes associated with maintenance of the undifferentiated state in myoblasts (\textit{Pax7}, myogenin, \textit{MyoD}, and \textit{CD34}), and \textit{IGF}-related factor genes (\textit{IGF-1} receptor, \textit{IGF-1R}; and myoferlin), in addition to the genes encoding desmin and collagen IV used in the IHC staining. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. RT-PCR cycling conditions include 30 cycles consisting of thermal denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 20 sec, followed by the final extension at 72°C for 5 min. Gel electrophoresis was carried out at 100 V for 30 min. The primer sequences used in this study are listed in Table 1.

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

| Primer | Sequence (5’ → 3’) | Product size (bp) |
|--------|-------------------|------------------|
| \textit{HGF} | CAGACCAATGTGGCCAACAGGTCACAGACTTCGTAGCG | 407 |
| \textit{IGF} | ACTACCTGACGGCCGCGTTTTCGTAGCG | 1,417 |
| Desmin | TGCAGGAGCTCAATGACC | 337 |
| Coll IV | ATACCTGGAAGGCGAACAGAGAT | 484 |
| \textit{Pax7} | ATCCCGCCCTGTGTGTCATCTCA | 278 |
| \textit{CD34} | AATCTAGCCCACTGTCAGAGGTCTTGCGGAATAGCTTGGT | 174 |
| \textit{MyoD} | GCTCGCGAGGATGACAGCATGATGGCGTTGCGCAGGATCTT | 239 |
| Myogenin | TACCAAGGTTGGAGACCCCTGCATAGGTGTCGCTGTTT | 219 |
| \textit{IGF-1R} | TCGTCCACAGAGACCTGTGGTATAGCTGCGCCAAGCAT | 367 |
| Myoferlin | GTCTCCTTCTGCATCGAAGAATCAGGATCACAGCGAAAG721 |
| GAPDH | ACCACATGGTCCATTGCATCGAC | 452 |

7. Western blot analysis

The cell sheet specimens were dissociated with lysis buffer comprising 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Nonidet P-40 (Calbiochem, Darmstadt, Germany) and homogenized. Each sample was incubated for 40 min at 4°C and then centrifuged at 15,000 rpm for 30 min at 4°C. Protein concentrations in the supernatants were determined using the DC protein assay (Bio-Rad Laboratory,
Hercules, CA, USA). All samples were then diluted in modified 2× sample buffer comprising 4× NuPAGE LDS sample buffer (Invitrogen), 2-mercaptoethanol (Wako), and lysis buffer. The samples were then boiled. Thirty micrograms each sample was loaded on a 12% Bis-Tris gel (Novex NuPAGE; Invitrogen) and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with diluted normal serum (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA, USA) for 60 min at RT. The membranes were incubated with antibodies for desmin (1:100), collagen type IV (1:250), or β-actin (1:1,000, mAbcam 8226; Abcam, Cambridge, UK) for 90 min at RT. After the membranes were washed 3 times in PBS, biotinylated secondary antibodies (Vector Laboratories) were added for 30 min at RT. Protein bands were visualized (Vectastain ABC Elite Kit; Vector Laboratories) with DAB (Vector Laboratories) as the substrate. The plot profiles of the bands were analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA). Statistical significance was evaluated using t-tests.

**Results**

1. **Histological analysis**

Regardless of the culture conditions, micrographs of H-E staining showed that myoblast numbers in the cell sheets continued to increase, even after a long cultivation period (Fig. 2). The IHC micrographs revealed that desmin was present in all myoblast layers tested, confirming the presence of myoblasts (Fig. 3A). The difference in desmin expression was demonstrated by Western blot analysis at 6 weeks (Fig. 3B, C). The IHC micrographs with collagen IV showed little difference between the samples tested. Expression of collagen IV, however, showed a decrease at 6 weeks in the mesenchymal cell co-cultured sample according to the results of Western blot analysis (Fig. 4).

2. **RT-PCR**

The results of RT-PCR revealed that genes associated with maintenance of the undifferentiated state in myoblasts such as Pax7, myogenin, MyoD, and CD34, in addition to genes encoding desmin and collagen IV, were expressed throughout the 6-week cultivation period (Fig. 5). An RT-PCR analysis was also conducted for genes encoding HGF and IGF, which are known regulatory factors important in proliferation and motility in a wide variety of cells, including myoblasts. The results showed that the HGF gene was expressed under all tested culture conditions. Expression of the IGF gene, however, was only observed at weeks 4 and 6 in the mesenchymal cell co-cultured samples (Fig. 5). In order to examine the effects of IGF on the myoblasts at the mRNA expression level, RT-PCR was conducted for genes encoding IGF-1R and myotrofin. The results showed the expression of both genes under all tested culture conditions (Fig. 6). No clear difference was observed.
in their expression levels between samples with or without co-culture with mesenchymal cells.

**Discussion**

Muscle organization takes place over a sequence of stages as the tissues involved develop. Satellite cells, first presenting as myogenic precursor cells at the beginning of the process, subsequently differentiate into myoblasts or myotubes\(^3\). Many myotubes then fuse together to form myofibers. In the present study, mainly 4 kinds of cell were observed in the H-E stained micrographs at high magnification: those with a large nucleus; those with a large cytoplasm; those with no nucleus; and those forming myotube-like structures. It is not certain from the micrographs whether these myotube-like structures were actually myotubes originating in myoblast differentiation, however. Culture condition-dependent quantitative differences in these cells was not
investigated here. However, the proportion of such cells tended to vary according to the length of culture.

Desmin is a specific marker for skeletal muscle cells and a protein expressed in early differentiation of muscle cells\textsuperscript{10}. In the present study, desmin expression showed a statistically significant decrease under co-culture with mesenchymal cells compared to under the other conditions set, suggesting that myoblast differentiation was suppressed.

Satellite cells reside in the basal lamina of the basement membrane, which contains collagen IV. An adequate concentration of collagen IV is important to the microenvironment for satellite cells in terms of both regulation of muscle regeneration and satellite cell homeostasis\textsuperscript{2,8,19,20}. In the present study, collagen expression differed between the Gel and OS conditions (Fig. 4), suggesting that this was due to the presence of immature myoblasts.

A wide variety of growth factors has been shown to affect the differentiation, proliferation, and motility of various types of cell, including myoblasts\textsuperscript{27}. Muscle satellite cells, or myoblast precursor cells, play crucial roles in the proliferation of myoblasts. It has been reported that Pax7 and CD34, satellite cell-specific genes, were expressed even during the quiescent state of the myogenic program prior to activation\textsuperscript{29}. In addition, expression of genes encoding myogenin and MyoD, myogenic differentiation regulatory factors, has been detected upon activation of satellite cells, which then proceed to the cell differentiation stage to form myoblasts\textsuperscript{29}. In the present study, gene expression was performed for genes associated with the undifferentiated state in satellite cells. It was observed that such genes were expressed throughout the cell-sheet cultivation period, even after 6 weeks, indicating that muscle satellite cells are maintained in cell sheets undergoing long-term cultivation.

Several studies have shown that HGF and IGF protein play roles in promoting the proliferation and differentiation of myoblasts\textsuperscript{4,23,24}. In the present study, the expression patterns
of genes encoding HGF and IGF, myoblast growth factors, were examined. The results showed expression of the HGF gene under all tested culture conditions, whereas the IGF gene was only expressed at 4 and 6 weeks in the samples co-cultured with mesenchymal cells. This suggests that expression of the IGF gene is triggered by some unknown interaction with mesenchymal cells during long periods of cultivation. It has been reported that IGF-1R, an IGF receptor, is located at the myoblast membrane. In IGF-1R knockout mouse, myofiber repair and myoblast differentiation were impaired upon muscle regeneration induced by muscle tissue damage. In addition, myoferlin is a membrane-associated protein and has been known as a critical factor in IGF response and muscle growth. In the present study, RT-PCR analysis was performed for genes encoding IGF-1R and myoferlin to clarify the effect of IGF on myoblasts at the mRNA expression level. The results showed expression of both genes under all tested culture conditions. Moreover, no differences were observed in their expression levels between samples with or without co-culture with mesenchymal cells.

While further studies are needed to explore the effects of IGF on myoblasts, the results of the present study demonstrated that expression of genes encoding cell growth factors, as well as factors associated with maintenance of undifferentiated cells in myoblasts, was maintained throughout the long-term cultivation period. Expression of the IGF gene was only detected in samples co-cultured with mesenchymal cells. Furthermore, the results suggest that myoblast differentiation is suppressed under co-culture with mesenchymal cells. Consequently, it is possible that the presence of mesenchymal cells within a system influences differentiation of myoblasts when myoblast cell sheets are cultured over a long period of time.

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Conflict of Interest

The authors declare no conflict of interest.

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