INTRODUCTION

Skeletal muscle has high self-renewal potency and undergoes a series of healing processes that consist of degeneration, inflammation, regeneration, and fibrosis after the injury\(^1\). In general, the regeneration process begins promptly and heals without any functional impairment in case of exercise or minor injury, whereas severe injury often causes insufficient and hindered muscle regeneration due to the formation of scar tissue. Excessive fibrosis might cause a variety of functional impairment depending on the severity and type of muscle injury\(^1\). Thus, it is believed that the enhancement of the muscle regeneration and muscle fibrosis prevention through the use of biological approaches should contribute to the improvement of muscle healing as well as to obtaining a complete functional recovery\(^1,2\).

Skeletal muscles also participate in the production of rhythmic oral motor activities such as chewing, sucking, swallowing, and articulation. Above all, feeding behavior is characterized by coordinated activities of bilateral masticatory muscles which contribute to balanced mandibular movement. Complex soft tissue injury or defects including skeletal muscle is one of the most popular problems in surgery or trauma in the oral and maxillofacial region. Particularly, large volumes of muscle loss may lead to muscle necrosis and intramuscular scar tissue formation, which results in decreased musculoskeletal health and persisting oral functional impairment. Therefore, interventional treatments using biological approaches are expected to achieve more favorable muscle repair and functional regeneration.

As possible treatment options aim at the regeneration of muscle tissues, cell therapy using iPS-derived cells, growth factor therapy, including fibroblast growth factors, insulin-like growth factors, and nerve growth factors, as well as gene therapy using microRNA have been reported to be useful treatment options\(^3-6\). However, several other treatment solutions have not yet reached clinical application due to emerging problems including the spontaneous malignant transformation of stem cells, the restriction of the route of administration, the instability of effective concentration in vivo, the immune response, the off-target effect by therapeutic miRNA, as well as the high-cost issues in manufacturing and quality control.

Upon the injury of the muscle, osteopontin participates in the activation, adhesion, and migration of myogenic cells and in the process of fibrosis following muscle degeneration and inflammation, similarly to other compounds in the extracellular matrix, such as ...
collagen, fibronectin, and laminin\(^7\). As a supporting fact, the delay of the onset of muscle regeneration was reported in osteopontin-deficient mice previously\(^8\). Besides, the N-terminal fragment of the osteopontin reveals the SVVYGLR motif at the C-terminus near the RGD domain during the event of thrombin cleavage. This synthetic peptide (SV peptide) that is composed of seven amino acids has angiogenic potential and promotes the secretion of the type III collagen as well as the differentiation of myocardial-derived fibroblasts into myofibroblasts\(^9\)–\(^16\), in which the involvement of the activation of the TGF-\(\beta\) receptor is revealed to be critically important\(^12\). This short peptide also promotes the secretion of the type III collagen as well as differentiating myofibroblasts\(^8\)–\(^16\), in which the involvement of the activation of the TGF-\(\beta\) receptor is revealed to be critically important\(^12\). This short peptide also promotes skeletal muscle regeneration with scarless tissue healing after injury, increasing EMG activities\(^17\). Furthermore, a recent study using human-derived myogenic cells demonstrated that the SV peptide can facilitate cell motility and the migration of both the myogenic precursor cells (satellite cells) and the progenitor cells (myoblasts), as well as the expression of myogenic markers, myoD and myogenin, which are consistently increased in regenerated muscle tissue in vivo, suggesting that the cell differentiation is accelerated by the SV peptide during the regeneration process\(^18\). Although synthetic SVVYGLR has been revealed to be bound to the TGF-\(\beta\) receptor\(^11\), it needs to be further elucidated whether the mechanism of the SV peptide inducing an increase in the ability associated with the migration of skeletal myogenic cells also includes TGF-\(\beta\) signals like myocardial fibroblasts.

Therefore, in this study, the hypothesis was examined whether the TGF-\(\beta\) signaling pathway could be involved in the pathway of that the SV peptide induced facilitation of the migratory activities of myogenic cells. Also, we investigated whether the cell differentiation during the process where myoblasts fuse to form long, multinucleated myotubes in vitro can be accelerated by the SV peptide.

**MATERIALS AND METHODS**

**Primary culture of skeletal muscle-derived cells**

Human skeletal muscle satellite cells (HSkMSC; Sciencell, Carlsbad, CA, USA) were grown in skeletal muscle cell medium (SkMCM; Sciencell) supplemented with 5% fetal bovine serum (Fetal bovine serum (FBS); Sciencell), skeletal muscle cell growth supplement (Sciencell), and penicillin/streptomycin solution (Sciencell) as well as incubated at 37°C in humidified air enriched with 5% CO\(_2\).

Furthermore, human skeletal muscle myoblasts (HSMM; Lonza, CA, USA) were cultured in skeletal muscle growth media-2 (Lonza) supplemented with a SingleQuots™ Kit (human epidermal growth factor, Dexamethasone, L-glutamine, FBS, and Gentamicin/Amphotericin-B; Lonza) as well as incubated under 5% CO\(_2\) at 37°C.

**Cell migration assay**

The Boyden chamber principle was selected to be employed as a cell migration assay. A total of 2.0×10^4 cells of HSkMSC or HSMM seeded in the upper chamber were allowed to migrate through a fibronectin (10 \(\mu\)g/mL)-coated polycarbonate membrane with a pore size of 8 \(\mu\)m (Chemotaxicell; Kurabou, Osaka, Japan), as well as serum-free medium was placed in the lower chamber along with SV (20 ng/mL), nonfunctional SV (GYRVLSV; rSV, 20 ng/mL), or PBS as a chemoattractant. Herein, rSV was used as a control, which had the same molecular weight but different sequence from SV and had been proved to be inactive\(^9\)–\(^16\)–\(^18\). With respect to the applied dosage of SV or rSV, a previous study reveals that the effect of SV on endothelial cell adhesion is not dose dependent and the minimum effective concentration is 20 ng/mL\(^10\), which is also effectively employed to examine the effect of SV on other cell lines, including the myogenic cells used in this study\(^11\)–\(^13\),\(^15\)–\(^18\). Moreover, TGF-\(\beta\)1 (5 ng/mL; Pepro Tech, NJ, USA) was applied as a chemoattractant to examine the effects of the activation of the TGF-\(\beta\) receptor on the induction of the migratory activity. Subsequently, one of the TGF-\(\beta\)1 receptor-specific inhibitors, SB431542 (10 \(\mu\)M; Selleck Chemicals, TX, USA) or SB505124 (10 \(\mu\)M; Selleck Chemicals), were added concomitantly with the SV peptide as another condition. The chamber was incubated in 5% CO\(_2\) at 37°C for 12 h, then cells from the lower chamber were fixed with 10% neutral buffered formalin solution (Wako, Osaka, Japan), stained with hematoxylin, as well as the cells were counted with an optical microscope.

**Western blotting**

HSkMSC and HSMM were incubated with a medium supplemented with the PBS, the rSV peptide, or the SV peptide for 60 min. Then, the cells were lysed with RIPA buffer subsequently collected with a scraper, and the lysate was collected after centrifugation. The supernatants were resolved using SDS-PAGE and eventually transferred to a polyvinylidene difluoride transfer membrane (Millipore, Billerica, MA, USA). Afterwards, the membranes were probed with primary antibodies against the rabbit monoclonal anti-total-Smad, the rabbit polyclonal anti-phosphorylated Smad3, and the rabbit polyclonal anti-\(\beta\)-actin. The reaction was carried out with the use of a Horseradish peroxidase-labeled anti-mouse IgG antibody and an HRP-labeled anti-rabbit IgG antibody as secondary antibodies. Finally, the band was detected by Super signal West Femto (Thermo Scientific, MA, USA). Densitometric analysis of Western blots was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Immunofluorescence staining**

HSMM seeded on Matrigel-coated coverslips (Matsunami Glass Ind, Osaka, Japan) was co-incubated with the SV (20 ng/mL), the SV random peptide (GYRVLSV; 10 ng/mL), the PBS, or 2% Horse Serum (HS; Lifé Technologies Japan, Tokyo, Japan) for 48 or 72 h. The cultured cells were fixed with 4% paraformaldehyde, and permeabilization buffer (0.2% triton-x) was added,
followed by blockage with 5% BSA. The cells were subsequently incubated with a mouse anti-Myogenin primary antibody overnight at 4°C, followed by the incubation with a secondary FITC anti-mouse IgG antibody (Dako, Glostrup, Denmark) for 30 min. The nuclei were stained with 4,6-diamino-2-phenylindole (DAPI; Invitrogen Life Technologies, Grand Island, NY, USA), and fluorescent images were captured via fluorescence microscopy (ECLISPE E600, Nikon, Tokyo, Japan). Eventually, the ratio of myogenic marker-positive cells to cells counterstained with DAPI was used to perform the quantification.

**Immunohistochemical analysis**

HSMMs were seeded and cultured at 5.0×10⁴ cells/well on the slides of a 4-well chamber (WATSON, Tokyo, Japan). When the 50% confluent state was reached, the medium was changed to 2% horse serum-containing medium (Dulbecco's Modified Eagle Medium: F12, Lonza) supplemented with the PBS, the rSV peptide (20 ng/mL), or the SV peptide (20 ng/mL), and each sample was subsequently cultured for 5 days. Activation of the TGF-β1 and its inhibitor on the migration activity of HSkMSC and HSMM were investigated. The addition of the TGF-β1 as a chemoattractant substantially increased the migratory activities of both cell populations in comparison with

**Fig. 1 Involvement of TGF-β1 receptor activation in SV peptide-induced cell migratory activities of HSkMSC and HSMM**

The migration assay revealed that the migratory activities of the HSkMSC and HSMM were significantly increased by the SV peptide in comparison with the rSV and the PBS groups, as it was recently reported¹⁸) (Figs. 1A and B). Previous studies demonstrate the possible involvement of the activation and subsequent phosphorylation of the TGF-β1 receptor, including the Smad2 and the Smad3, in the SV peptide-induced migration of cardiac and mucosal fibroblasts¹⁵,¹⁶). Therefore, the effect of the TGF-β1 and its inhibitor on the migration activity of HSkMSC and HSMM were investigated. The addition of the TGF-β1 as a chemoattractant substantially increased the migratory activities of both cell populations in comparison with

**RESULTS**

The involvement of the TGF-β1/Smad signaling pathway in the SV peptide-induced cell migratory activities of HSkMSC and HSMM

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**Fig. 1** Involvement of TGF-β1 receptor activation in SV peptide-induced cell migration of HSkMSC and HSMM

A: B: Migratory activity assessed by the Boyden chamber assay using an independently cultured HSkMSC (A) and HSMM (B) under different treatment conditions (n=5). The TGF-β1 receptor-specific inhibitor, the SB431542 or the SB505124, suppressed the SV peptide-induced migration activities. Data are presented as means±SD.

**Statistical analyses**

All the data are expressed as the mean±standard deviation. Statistical differences were determined using a one-way analysis of variance with post-hoc tests, or the Student's t-test for comparison between the groups. Values of p<0.05 were considered to be statistically significant.
HSkMSC | HSMM
---|---
PBS | PBS
SV | SV
rSV | rSV
pSmad | 1 | 1.7 | 1
| 1 | 1.7 | 1
| PBS | SV | rSV
| β-actin | 2.5 | 1 | 1
| 2.5 | 1 | 1

**Fig. 2** Analysis of TGF-β1/Smad signaling in HSkMSC and HSMM exposed to the SV peptide
Assessment of the TGF-β1/Smad signaling by Western blotting in HSkMSC and HSMM exposed to the SV. The expression of phospho-Smad3 was evaluated 1 h after the addition of the SV peptide. β-actin was used as a loading control. The ratios of PBS to SV or rSV were calculated by densitometric analysis.

**Fig. 3** Effects of the SV peptide on the cellular differentiation of human-derived myoblasts.
A and B: Representative fluorescent images from the immunostaining of the HSMM for the detection of myogenic markers at 48 h (A) and 72 h (B) after incubation.
C and D: Fluorescence micrographs of the immunostaining for myogenic markers in the HSMM at 48 h (C) and 72 h (D) after incubation (n=3). The percentage of the positively stained cells from cultured myogenic cells treated by the SV peptide was compared with the control (PBS) and the rSV. The immunoreactivity in horse serum (HS) was used as a positive control. Scale bar, 50 µm. Data are presented as the mean±SD. *p<0.05.

**Fig. 4** Effects of the SV peptide on cell differentiation and myotube formation in HSMM.
A: Immunostaining with antibodies against the MHC in cultured HSMM with the PBS, the rSV, and the SV peptides. Each arrowhead shows a nascent myotube immunostained with the MHC as well as the formed syncytia. Scale bar, 5 µm.
B: Quantitation of the average number of nascent myotubes per field (n=4, *p<0.05, Repeated-Measures ANOVA, Turkey-Kramer post).

Effects of the SV peptide on the expression of myogenin in cultured HSMM
As shown in Figs. 3A and B, the myogenin-positive

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nuclei were predominantly seen in the HSMM treated with the SV, but not in samples treated with the rSV or the PBS in both periods of incubation. The number of nuclei that were positive for myogenin was significantly increased in the SV group in comparison with the PBS or the rSV groups and was comparable to the immunoreactivity seen in the HS at 72 h after incubation (Figs. 3C and D).

Effects of the SV peptide on cell differentiation and myotube formation in HSMM

Figure 4A showed a series of representative photographs of immunostaining with antibodies against the MHC at day 1 to day 5 treated with the PBS, the rSV, and the SV, respectively. As shown, fewer nascent myotubes with MHC expression and formed syncytia were observed in the PBS, the rSV, and the SV groups at 1 day to 3 days, whereas the average number of nascent myotubes was heavily increased in the SV group after day 4, and a significant difference was observed in comparison with the PBS and the rSV groups at day 5 (F=3.86, p=0.00041, Repeated-Measures ANOVA, Fig. 4B).

DISCUSSION

The present study demonstrated the facilitative effects of synthetic SV peptide on the migration ability of myogenic cells through TGF-β/Smad pathway and myogenic differentiation, which provided additional evidence of the benefits for skeletal muscle regeneration and clinical application of the SV peptide as a candidate for the treatment of severe muscle injury accompanied with functional impairment including oral and maxillofacial region, as we recently reported.[17,18]

Upon the injury of the skeletal muscle, the migration of both types of satellite cells released to the basal membrane as well as the subsequently activated myoblasts to the injured region is critically involved in the formation of mature muscle fibers.[1,7] In this study, osteopontin-derived SV peptide could facilitate the migration of those cells through signal transduction from the TGF-β receptor activity. Besides, the SV peptide increased the expression of myogenin in myoblasts and facilitated the fusion of myoblasts and the formation of myotubes in vitro, that is consistent with our previous findings.[17,18]

Previous studies have demonstrated the interaction (binding ability) of the SV peptide associated with the TGF-β type II receptor as well as its potential to induce the differentiation of cardiac fibroblasts into active myofibroblasts via the TGF-β/Smad pathway.[11,12] TGF-β is believed to interact with other growth factors and other environmental factors that are induced by an inflammatory response upon injuries, and regulate the proliferation and differentiation of myogenic cells, thus influencing the process of skeletal muscle regeneration.[19-21] Also, it has been reported that the SB431542, a selective inhibitor of activin receptor-like kinase (ALK5), that is a type I receptor, is responsible for the inhibition of the action of the specific transcription factor Myostatin, resulting in the hypertrophy of the myotube.[22] Based on these findings, it was considered that the activation of the TGF-β receptor and its related pathway may be involved in the expression of migratory activities of skeletal muscle precursor and progenitor cells by the SV peptide.

The TGF-β signaling is produced by the TGF-β ligand and the activation of the receptor complex consists of a type II and a type I receptor. The TGF-β signaling mainly causes the phosphorylation of the signal transduction substances Smad2 and Smad3, as well as it translocates into the nucleus and activates the transcription of the target gene. In this study, either the TGF-β or the SV peptide substantially increased the migration ability of both human-derived satellite cells and myoblasts, and the migratory activities induced by the SV peptide were significantly decreased in the presence of the SB431542 or the SB505124, suggesting a possible involvement of the TGF-β/Smad signaling pathway as previously discussed in cardiac fibroblasts.[12]

In case of excessive inflammatory reactions caused by an infection or tissue necrosis by injury, fibrosis is caused as a result of insufficient regeneration of the muscle fibers that impairs muscle functions via the reduction of the motile and contractile function.[21] TGF-β1 is also thought to participate in skeletal muscle fibrosis in the later period of the muscle regeneration by promoting extracellular matrix preservation. Furthermore, the phosphorylation and activation of the Smad2/3 proteins are also believed to be involved.[21] The stimulation of the TGF-β1 can induce the production of fibrosis-related proteins in myoblasts, and the TGF-β1 gene-transfected myoblasts can differentiate into myofibroblasts after an intramuscular transplantation.[28] Interestingly, the TGF-β1 levels in skeletal muscle cells increase by age and are believed to contribute to aging-associated fibrosis.[31] If the activation of the TGF-β/Smad pathway and the increase of related reactions become persistent even in the later phase of the muscle regeneration process, it might cause excessive fibrosis or the emergence of hypertrophic scars. However, our previous study has demonstrated that the local administration of the SV peptide can facilitate the regeneration of the skeletal muscle with scarless healing in a volumetric muscle loss model.[17] The SV peptide could rapidly be degenerated due to its associated short-length peptide, which might lose its potency to activate the TGF-β receptor and contribute to less scar formation as discussed for the effects of the SV peptide in association with the healing of mucosal wounds.[16]

It is well-known that some specific transcription factors are expressed in myogenic cells during the regeneration process of injured skeletal muscle, depending on the current phase of cellular differentiation. Myogenin, one of the transcription factors, is known to be expressed during the fusion of myoblasts and their differentiation into myotubes.[25,26] Our recent study reveals enhanced expression of myogenin during the local administration of the SV peptide into the injured skeletal muscle tissue.[30] In line with these findings, the
SV peptide also promotes the expression of myogenin in cultured myoblasts in this study. To identify whether the SV peptide could facilitate the differentiation of the myoblasts into myotubes, an immunohistochemical study was conducted using a MHC that is expressed in myotube formation and mature fibers in the later step of myogenesis. As shown, the formation of MHC-positive multinucleated cells was promoted under the various culture condition created by the application of differentiation mediums supplemented with the SV peptide. Taken together, our findings support the concept that the migration ability of progenitor cells is increased by the SV peptide, which preferably increase the efficiency of the later phase of the myogenic differentiation: the formation of the myotube in the regenerative tissue.

Osteopontin has multifunctional domains and two integrin binding sites, namely the RGD and the SVVYGLR, in the molecule. Moreover, previous studies clarified that the SVVYGLR amino acid sequence has a binding site with integrin α4β1, α9β1, α4β727-29. Integrin αvβ3 is expressed in human-derived myoblasts and was proven to regulate the proliferation and the differentiation of mouse-derived muscle satellite cells30. Integrin α7β1 is involved in the proliferation and the differentiation of myoblasts, and integrin α4β1 is involved in the fusion of myoblasts31,32. However, these findings could not rule out the possibility that it is not only the TGF-β signal that is involved in the promotion of the migration and the differentiation of myogenic cells by the SV peptides but integrins are also involved, therefore further investigations will be necessary to establish a new therapeutic strategy to address the skeletal muscle regeneration after injury.

CONCLUSION

In conclusion, the synthetic SVVYGLR peptide potentially facilitates the migratory activities of myogenic satellite cells and myoblasts via the TGF-β/Smad signaling pathway. In the muscle regeneration process, the upregulated migration of myoblasts accelerates the expression of a myogenic transcription factor, myogenin, which promotes the later phase of the myogenic differentiation process: the fusion of the myoblasts into multinucleate syncytia, myotubes. These effects might contribute to the facilitation of muscle regeneration upon injury and may provide a new strategy for designing an efficient treatment option for the dysfunctions of the skeletal muscle that involve serious damage caused by injury, surgery, as well as congenital diseases in the oral and maxillofacial region.

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