The synthetic peptide SVVYGLR promotes cell motility of myogenic cells and facilitates differentiation in skeletal muscle regeneration

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The present study was designed to evaluate the effects of the osteopontin-derived multifunctional short peptide, SVVYGLR (SV) peptide on the biological properties of skeletal muscle-specific myogenic cells. We employed human-derived satellite cells (HSKMSC) and skeletal muscle myoblasts (HSMM) and performed a series of biochemical experiments. The synthetic SV peptide showed no influence on the proliferation and adhesion properties of HSKMSC and HSMM, while it showed a significant increase in cell motility, including migration activities upon treatment with the SV peptide. In a rat model with volumetric loss of masticatory muscle, immunohistochemical staining of regenerating muscle tissue immediately after injury demonstrated an increase of the number of both MyoD- and myogenin-positive cells in SV peptide-treated group. These results suggest that SV peptide plays a potent role in facilitating skeletal muscle regeneration by promoting the migration, and differentiation of myogenic precursor and progenitor cells.

Keywords: SVVYGLR, Osteopontin, Skeletal muscle, Myogenic cells, Regeneration

INTRODUCTION

Skeletal muscle in the oral and maxillofacial regions play a key role in producing rhythmically coordinated functional activities, such as chewing, sucking, and swallowing. It is well known that skeletal muscle has an ability to adapt and regenerate, and is thus different from the myocardium, which has a comparatively reduced self-renewal ability. Damage to the skeletal muscle caused by exercise or minor injury can, therefore, be healed without issue, whereas severe damage, such as that caused by an accident or surgical resection, can lead to lasting functional impairment1,2).

In the regenerative process that follows muscle injury, myogenic cellular populations are critically involved. Satellite cells are precursor cells with self-renewal capabilities which become activated and differentiate into myoblasts, embryonic progenitor cells, that undergo terminal differentiation and form matured myotube. Perivascular stem cells play critical role in neovascularization3-4. In addition, inflammatory cell populations are recruited and subsequently produce several cytokines and growth factors1,3). Recently, cell-based therapies employing the local injection of a mixture containing muscle-specific miRNAs or the delivery of growth factors have been reported to accelerate muscle regeneration in animal models5-7). These treatment strategies, however, have not yet been established in the clinic due to limitations, such as an undesirable transdifferentiation, spontaneous malignant transformation of stem cells8,9), inadequate or unstable effects due to the rapid depletion of growth factors6), and off-target effects of therapeutic miRNAs10).

Osteopontin (OPN), which is a constituent of the ECM in a wide variety of cells including skeletal muscle cells, is expressed by myoblasts and plays a potent role in controlling myogenesis through modulation of cultured mouse myoblasts11). In addition, the N-terminal fragment produced upon the proteolytic cleavage of OPN by thrombin has been shown to express the SVVYGLR motif on the C-terminus close to the RGD domain12). Previous studies have demonstrated that synthetized SVVYGLR motifs (SV peptide) can induce angiogenesis, potentially equivalent to that induced by VEGF, increase the synthesis of collagen type III, and lead to myofibroblast differentiation by binding to the TGF-β receptor, resulting in a functional improvement of the myocardium in animal models of myocardial infarction13-19). This short peptide was easily degraded by peptidase and showed less adverse effects, indicating high biocompatibility13,14). Furthermore, our recent study revealed that SV peptide has long-term benefits...
for wound healing in severe skeletal muscle injury; it facilitates muscle regeneration composed of matured myofibers with a greater diameter and inhibits fibrosis, thereby conferring favorable effects on the functional recovery of injured muscles\(^{(20)}\).

Therefore, we hypothesized that SV peptide could influence the biological properties of myogenic precursor and/or progenitor cells from skeletal muscle and contribute to tissue regeneration after muscle injury. In the present study, we used human-derived skeletal muscle-specific satellite cells and myoblasts and evaluated the effects of SV peptide on their biological properties. We further investigated the immunohistochemical expression of myogenic markers using a model of volumetric masticatory muscle loss.

**MATERIALS AND METHODS**

**Primary culture of the 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) and incubated at 37°C in humidified air enriched with 5% CO\(_2\).

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

**Cell proliferation assay**

The WST-1 assay was employed to evaluate the effect of the SV peptide (SV) on the proliferation of skeletal muscle-derived cells. SV was synthesized using Fmoc (Fluorenylmethyloxycarbonyl) through a high efficacy solid phase method, as previously reported\(^{(4)}\). HSkMSC or HSMM were placed into 96-well plates at a density of 1.0×10\(^4\) cells/well and cultured in a medium containing SV (20 ng/mL), rSV (20 ng/mL), or PBS as a chemoattractant. The WST-1 solution (Takara Kurabou, Osaka, Japan), and serum-free medium was placed in the lower chamber along with SV (20 ng/mL), rSV (20 ng/mL), or PBS as a chemoattractant. 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, and the cells were counted with an optical microscope.

A standard scratch wound healing assay was adapted to evaluate the motility of HSkMSC or HSMM. The cells were seeded in φ 57×16 mm Petri dish plates (Iwaki, Tokyo, Japan) and cultured to confluence as a monolayer. After a cell-free gap was made in the center of the monolayer by scratching using a 900 µm disposable micropipette tip, the well was replenished with fresh medium containing SV (20 ng/mL), rSV (20 ng/mL), or PBS. Images of the scratched cell monolayers were taken at regular time points of 0, 6, 12 h for the HSkMSC sample and at 0, 12, 24, 36, 48 h for the HSMM sample, under a light microscope with 200× magnification for monitoring cell migration. For the quantitative analyses, each image was captured and used to measure the rate of reduction in the gap relative to the total scratched area in order to estimate the rate of cell motility, using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

**Expression of myogenic markers in regenerated muscle tissue**

The histological assessment of the rats used in this study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, updated 2011) and approved by the Animal Care and Use Committee of Osaka University Graduate School of Dentistry. Adult male Sprague-Dawley rats weighing 350–400 g (Oriental Yeast, Tokyo, Japan) were anesthetized by intraperitoneal injection with a mixture of midazolam (2 mg/kg), medetomidine (0.375 mg/kg), and butorphanol (2.5 mg/kg). The volumetric muscle loss in the bilateral masseter muscle was performed by excision of the full-thickness of the belly of muscle, approximately 5–7 mm in width, between the external canthus and the angle of the mandible as previously reported\(^{(20)}\). The cutting was performed using electric...
cauterization, and SV (20 ng/mL) or PBS was injected into the vicinity of the rostral and caudal cut ends in the left side masseter muscle at a total volume of 1 mL, while the right side muscle was left uninjected. The animals were anesthetized, and the muscular tissue was resected from both sides 1 week after the injection; the specimens were then fixed with 10% formalin buffer and embedded in paraffin. Immunohistochemical staining with primary antibodies against MyoD (Abcam, Cambridge, UK) and myogenin (Abcam) was performed and the sections were then incubated with biotinylated secondary antibody (Dako, Glostrup, Hovedstaden, Denmark) and peroxidase-conjugated streptavidin (GE Healthcare, Little Chalfont, England). Visualization was carried out using a biphenyl-3,3′,4,4′-tetramine solution (Sigma, St. Louis, MO, USA). The ratio of the myoD- or myogenin-positive cells in the treated side vs. the non-treated side was calculated for quantitative evaluation.

Statistical analyses
All the data are expressed as the mean±standard deviation. Statistical differences were determined using one-way analysis of variance with post-hoc test, or Student’s t-test for comparison between the groups. Values of *p*<0.05 were considered to be statistically significant.

RESULTS

**Effects of SV peptide on the proliferation and adhesion of HSkMSC and HSMM**

The WST-1 test showed no significant difference in the pattern of proliferation and the absorbance measured at 430 nm for HSkMSC or HSMM treated with SV compared to control groups treated with rSV or PBS (Figs. 1A and B). In the quantification of cell adhesion to fibronectin, SV also caused no significant changes in the value of absorbance at 550 nm in either cultured HSkMSC or HSMM, compared to the rSV, PBS, and BSA groups, which were used as negative controls (Figs. 1C and D).

**Effects of SV peptide on the motility of HSkMSC and HSMM**

In the Boyden chamber assay, SV significantly increased the number of migrated cells in both HSkMSC and HSMM compared to rSV and PBS treated conditions (Figs. 2A and B). In addition, both cell groups cultured with SV showed accelerated wound healing with significantly enhanced effects on the rate of cell motility after scratching compared to the rSV and PBS treatment controls (Figs. 2C and D).

**Effects of SV peptide on the expression of myogenic markers in regenerated muscle tissue**

In regenerative tissue, 1 week after muscle injury, the myoD-positive nuclei were identified as small mononuclear cells, and the myogenin-positive nuclei were identified as multinuclear cells, distributed throughout the immature skeletal muscle tissue (Figs. 3A and B). The degree of immunoreactivity of myogenic cells was assessed using the mean value form three fields of view, and SV induced a significantly higher expression of both myo-D- and myogenin compared to the control group.
Fig. 2  Effects of SV peptide on the motility of human-derived myogenic cells. (A) and (B) Boyden chamber assay for cell migration using independent cultured HSkMSC (A) and HSMM (B) in three different treatment conditions (PBS, rSV, SV, n=5–9/group). (C) and (D) Calculated rate of cell motility obtained by scratch wound healing assay using HSkMSC (C) and HSMM (D) represented by % wound closure area. Treatment with SV peptide was compared with control (PBS) and rSV (n=4). Data is presented as the mean±SD. *p<0.05, **p<0.01.

Fig. 3  Effects of SV peptide on the expression of myogenic markers in regenerated muscle tissue after injury. (A) and (B) Representative immunohistochemical staining with anti-MyoD antibody (A) and anti-myogenin antibody (B) in the wound specimens 1 week after volumetric loss of masseter muscle. Black arrow heads show immunopositive cells and the white arrow head shows a myotube. (C) and (D) Average ratio of immunoreactive cells expressing Myo-D (C) and myogenin (D), calculated by numbers obtained from left (injection side)/numbers obtained from right (non-injection side) masseter muscles pretreated with PBS or SV peptide, respectively (n=4). Scale bar, 50 µm. Data is presented as the mean±SD. *p<0.05.
treated with PBS (Figs. 3C and D).

**DISCUSSION**

In the early phase of skeletal muscle regeneration, OPN is known to mediate diverse biological functions, such as cell proliferation, adhesion, and migration of myogenic cells\(^{11,23}\). The present study demonstrated, for the first time, the role of the thrombin-cleaved N-terminal fragment of OPN, the SV peptide, in skeletal muscle regeneration through the facilitation of the migration of both human-derived satellite cells and myoblasts, and prominent expression of myogenic markers in the early stage of healing process after injury. These results support our previous study, in which local injection of SV-peptide into the volumetric muscle loss animal model significantly recovered electromyogram activities accompanied with facilitated regeneration of muscles composed of matured myofibers compared to control condition\(^{20}\).

In this study, the SV peptide did not have any effect on cell proliferation ability and cell adhesion to fibronectin in either of the skeletal cell populations, unlike in endothelial cells where SV increased adhesion and migration, but not proliferation\(^{14}\). With respect to the cell motility and migration, SV peptide substantially potentiated these properties in both satellite cells and myoblasts. These data identify a novel function of SV peptide in enhancing cell motility potential of those myogenic cells and facilitating skeletal muscle regeneration, during which activated satellite cells migrate along the basal lamina and differentiated myoblasts must migrate to the required position within the wound and align and fuse to form multinucleated myotubes\(^{22}\).

MyoD and myogenin are members of a family of myogenic transcription factors, and the expression levels of these proteins are known to increase not only during embryonic and neonatal myogenesis, but also in denervated or regenerating skeletal muscles in aged animals\(^{20}\). These factors are recognized as playing a key role in the determination and fusion of myoblasts\(^{24}\). Our immunohistological findings support the idea that SV peptide can modulate the biological properties of myogenic cells by increasing the expression of myogenic transcription factors.

In terms of the molecular mechanisms that regulate myoblast fusion and subsequent differentiation, integrin receptors, which are composed of \(\alpha\) and \(\beta\) subunits, participate as receptors for ECM ligands\(^{25}\). Previous studies have provided evidence showing that \(\beta 1\) integrins regulate myoblast fusion and the assembly of the muscle fiber cytoskeleton\(^{26}\). Other studies have revealed that \(\alpha 9\beta 1\) also contributes to cell migration and proliferation\(^{27}\) and that the thrombin cleaved N-terminal OPN fragment, but not full-length OPN, can bind to \(\alpha 9\beta 1\) by recognition through the exposed SVVYGLR motif\(^{22}\). Further investigation should be undertaken to verify the molecular mechanisms underlying the effects of SV peptide shown in this study. One possible explanation is that an increased production of thrombin during the early stage of the regeneration process post injury, through coagulation or inflammation, could cleave OPN and lead to enhanced expression of N-OPN with an exposed SVVYGLR motif, which can then activate the myogenic response in precursor cells. Application of the SV peptide in earlier phases of injury might further accelerate these responses, facilitating the regeneration of muscle tissue. Severe muscle damage also tends to prevent revascularization and cause ischemic conditions, which are favorable for fibroblast proliferation and fibrosis and lead to functional impairment of the muscle\(^{28}\). OPN is known to be strongly expressed in fibrotic lesions and induces myofibroblast differentiation by TGF-\(\beta 1\)\(^{29}\). The SV peptide has the potential to promote angiogenesis and fibroblast differentiation into myofibroblasts, and the production of collagen III in cardiac fibrosis by influencing TGF-\(\beta\)/Smad signaling in rat dermal fibroblasts\(^{14,15,19}\). Although not investigated in detail in this study, these pathways may also be involved in the facilitation of muscle regeneration.

SVVYGLR, the functional peptide used in the present study, is a small peptide comprising of 7 amino acids and its use as a therapeutic agent has the advantages of fast metabolism, low risk of immune response due to its lower molecular weight, and low cost of synthesis as previously reported\(^{13,14}\), unlike cell based or molecular signaling therapies. Another potent functional peptide for muscle regeneration, the pigment epithelium-derived factor (PEDF)-derived short peptide (PSP), has recently been reported\(^{20}\). PEDF is known to be a potent inhibitor of angiogenesis in the eye and is expressed in a variety of tissues, including skeletal and smooth muscle\(^{30}\). Although a single injection of PSP possessed the ability to enhance the proliferation of satellite cells and myoblasts during the early regeneration stage, it has a higher molecular weight and length than SV, being composed of 20 amino acids. In addition, while SV could induce myogenic effects by the injection of a single bolus without a biological scaffold, PSP required co-administration with a biodegradable alginate gel\(^{20}\).

**CONCLUSION**

In conclusion, the SV peptide, which is formed through the cleavage of OPN by thrombin, can potentially facilitates muscle regeneration after injury by improving its self-regeneration ability. This effect was also seen in an animal model for volumetric muscle loss, in which the expression of myogenic transcription factors was significantly increased. These results could provide the usefulness of the clinical application of the SV peptide in skeletal muscle dysfunctions involving serious damage caused by injury or surgery and in congenital diseases causing deformed or poorly developed muscular tissue, such as a cleft palate. Further *in vitro* studies aimed at identifying the signaling pathway in the SV peptide-induced migration of myogenic cells will be informative and helpful in establishing new strategies for regenerative therapy.
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