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ORIGINAL ARTICLE

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The function of platelet-derived growth factor in the differentiation of mouse tongue striated muscle

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Structured Abstract

Objective – to determine the function of platelet-derived growth factor (PDGF) in the final differentiation phase of tongue striated muscle cells.

Materials and Methods – We analyzed the expressions of PDGF-A, -B, platelet-derived growth factor receptor (PDGFR)-α, and PDGFR-β in mouse tongues between embryonic days (E) 11 and 15. Furthermore, we examined the effects of human recombinant PDGF-AB and the peptide antagonist for PDGFRs using an organ culture system of mouse embryonic tongue. Mouse tongues at E12 were cultured in BGJb medium containing human recombinant PDGF-AB for 4 days or the peptide antagonist for PDGF receptors for 8 days.

Results – PDGF-A, -B, PDGFR-α, and -β were expressed in the differentiating muscle cells between E11 and 15. The human recombinant PDGF-AB induced increases in the mRNA expressions of myogenin and muscle creatine kinase (MCK) and the number of fast myosin heavy chain (fMHC)-positive cells, markers for the differentiation of muscle cells. On the other hand, the peptide antagonist for PDGFRs induced suppressions in the mRNA expressions of myogenin and MCK, and the number of fMHC-positive cells. Both the PDGF-AB and the antagonist failed to affect the expressions of cell proliferation markers.

Conclusion – These results suggest that PDGF functions as a positive regulator in the final differentiation phase of tongue muscle cells in mouse embryos.

Key words: muscle differentiation; organ culture; platelet-derived growth factor; tongue

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Introduction

Platelet-derived growth factor (PDGF) was first identified in a search for serum factors that stimulate the proliferation of arterial smooth muscle cells (1). Since then, mammalian PDGFs have been extensively characterized in culture-based assays, where they have been shown to drive cellular responses including proliferation, survival, migration, and the deposition of extracellular matrix (ECM) and tissue remodeling factors. The signaling of PDGFs is known to function in the cell through two receptors, PDGF receptor α and β (2). PDGFR-α binds the A or B chain of PDGF dimer with high affinities, whereas PDGFR-β only binds the B chain. PDGFR dimerization occurs as a result of PDGF binding and is a prerequisite for signal transduction. Consequently, the receptor dimer specificity for PDGF-AA is $\alpha\alpha$, for PDGF-AB is $\alpha\alpha$ and $\alpha\beta$, and for PDGF-BB is $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ (2).

Recently, studies using null mutation mice have demonstrated that PDGFs are essential for various kinds of biological activities during mouse development (3–9). PDGF-B and PDGFR-β have been shown to be essential for the development of support cells in the vasculature, whereas PDGF-A and PDGFR-α are more broadly required during embryogenesis and play essential roles in the development of numerous structures including in the central nervous system, neural crest, and organs (2). Malformation of myotomes, a source of skeletal muscle precursor cells, was observed in PDGFR-α null mice (9).

The roles of PDGFs in the development of skeletal muscle have been studied in cell culture-based assays using C2C12, L6, satellite cells, and primary cultured myogenic cells from various kinds of skeletal muscles, but they still remain elusive. PDGF-AA and PDGF-BB stimulate the proliferation of L6 and C2C12 myogenic cells, respectively (10–12). PDGF-BB inhibits the differentiation of C2C12 myogenic cells (11), and PDGF-AA and PDGF-AB have little or no effect on the proliferation and differentiation (10, 11). PDGF-BB stimulates migration of limb myogenic cells (13). PDGF-AB and BB enhance the proliferation of satellite cells of turkey and porcine skeletal muscles (14), whereas PDGF-AA has no effect (15).

The tongue is a complex muscular organ comprised of several intrinsic and extrinsic muscles and is involved in several important physiological tasks such as suckling, swallowing, mastication, respiration, and vocalization. Tongue striated muscles have several unique characteristics compared with other skeletal muscles such as limb and trunk muscles. For example, tongue muscles are capable of moving in three dimensions. The embryonic origin of connective tissue cells in tongue striated muscle is the neural crest, whereas that in trunk and limb skeletal muscle is the mesoderm (16–19). Fast myosin heavy chain (fMHC) is expressed not only in the myotubes and myofibers of tongue muscles but also in the myoblasts of tongue muscles, but is not expressed in the myoblasts of trunk and limb muscles (20). Tongue myogenesis and synaptogenesis are almost complete at birth, which is earlier than in other skeletal muscles (21, 22).

Although tongue striated muscles have several unique characteristics, to our knowledge, there is no study on the roles of PDGFs in the development of these muscles. The present study attempted to define the role of PDGF in the final differentiation stage of tongue muscle cells. We analyzed the changes in the mRNA expression levels and immunolocalization of PDGFs and their receptors during the embryonic development of tongue striated muscle. Furthermore, we analyzed the effects of recombinant PDGF-AB and the peptide antagonist of PDGF receptor on the proliferation and differentiation of tongue striated muscle cells using an organ culture system of embryonic mouse tongue.

Materials and methods

Mouse embryos and organ culture

Pregnant ICR mice were purchased from Nippon Clea, Ltd. (Tokyo, Japan) and killed by cervical dislocation under ether anesthesia. Embryos at embryonic days (E) 11, E12, E13, E15, and E17 were isolated from uterine decidua and were removed from their membranes under a dissection

microscope. Neonatal mice were also killed by cervical dislocation under ether anesthesia. The tongues of the embryos and neonatal mice were carefully microdissected. Three tongues at each time point for histological analysis were fixed in 4% paraformaldehyde for 1 h. Six tongues at each time point for PCR analysis were immediately frozen and stored at −80°C until use.

The tongues of embryos at E12 were explanted on membrane filters having a 0.8-μm pore size (type AABP, Millipore Corp., Bedford, MA, USA) on steel rafts and were cultured in BGJb medium (Life Technologies, Rockville, MD, USA). Recombinant human PDGF-AB (RELIATech GmbH, Braunschweig, Germany) was supplemented to the cultured medium at a final concentration of 0, 25, 50, and 100 ng/ml. A peptide antagonist of PDGFRs (N-Ala-Asn-Phe-Leu-Val-Trp-Glu-Ile-Val-Arg-Lys-Lys-Pro-OH) was supplemented to the cultured medium at a final concentration of 0, 200, and 400 ng/ml. The 13-amino acid peptide derived from the primary sequence of the B chain of PDGF was found to be an antagonist for PDGFR-α and -β (23). Cultures were maintained at 37°C in an atmosphere of 5% carbon dioxide and 95% air with medium changes every 2 days. Three cultured tongues of each concentration of recombinant PDGF-AB or a peptide antagonist for histological analysis were fixed in 4% paraformaldehyde for 1 h. Six cultured tongues of each concentration of recombinant PDGF-AB or a peptide antagonist for PCR analysis were immediately frozen and stored at −80°C until use. Experimental protocols concerning animal handling were reviewed and approved by the Institutional Animal Care Committee of Tsurumi University School of Dental Medicine.

RNA extraction, reverse transcription, and real-time polymerase chain reaction (real-time PCR) amplification

Total RNA extraction, reverse transcription, and PCR amplification were performed according to the manufacturer's specifications (Trizol, Life Technologies, Gaithersburg, MD, USA). The RNA was treated with two units of ribonuclease-free deoxyribonuclease I (Life Technologies, Gaithersburg, MD, USA) and was then reverse-transcribed with 200 units of reverse transcriptase (SuperScript II, Life Technologies, Gaithersburg, MD, USA).

SYBR Green real-time PCR was performed on the ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA, USA) using the following cycle parameters for all genes studied: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec for denaturation, and 55°C for 15 sec for annealing and extension. The nucleotide sequences of primer pairs for PDGFs, muscle creatine kinase (MCK), myogenin, cyclin-dependent kinase 4 (CDK4), cyclin D1, glyceraldehyde phosphate dehydrogenase (GAPDH), and S16 were identical to those used in previous studies (21, 24–26). The quantity of each target mRNA was normalized by the quantity of GAPDH or S16. The resulting ratio value was expressed as a percent value relative to the mean value of each target gene at 0 ng/ml of human recombinant PDGF-AB or PDGF antagonist.

Immunohistochemistry

Sagittal sections of tongues were prepared at a 10-μm thickness with a cryostat. Immunoenzyme and immunofluorescent stainings were performed as previously described (27, 28). Briefly, the following primary antibodies were used in the present study: rabbit polyclonal antibodies against PDGF-A, PDGF-B, PDGFR-α, PDGFR-β (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); mouse monoclonal antibody against fast myosin heavy chain (fMHC) (Sigma-Aldrich, Inc., St. Louis, MO, USA); and proliferating cell nuclear antigen (PCNA) (Progen Biotechnik GMBH, Heidelberg, Germany). The dilution for antibodies against PDGF-A, PDGF-B, PDGFR-α and PDGFR-β was 50 times, that for fMHC 500 times, and that for PCNA 10 times. For immunoenzyme staining, biotinylated secondary antibodies against rabbit or mouse IgG were used for immunoenzyme staining (Vectastain Universal Elite ABC kit PK-6200 or MOM kit PK-2200; Vector Laboratories, Inc., Burlingame, CA, USA). The site of the immunoenzyme reaction was made visible by incubating the sections with horseradish peroxidase-conjugated streptoavidin and then with 3-amino-9-ethylcarbazole (AEC) and hydrogen peroxide. For

immunofluorescent staining, TRITC-conjugated secondary donkey antibody against mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) was used. For control staining, the primary antibodies were replaced with phosphate buffered saline or heat-denatured primary antibodies.

Statistical analyses

We compared the mean value of 0 ng/ml of human recombinant PDGF-AB or peptide antagonist for PDGFR with that of each concentration of human recombinant PDGF-AB or peptide antagonist for PDGFR with Tukey's method after the one-way analysis of variance (ANOVA). Tukey's method is a standard method to compare the mean values of two groups among multiple groups.

Results

The expression of PDGFs and their cognate receptors in the mouse tongues between E11 and E15

Figure 1 shows changes in the expression level of PDGF-A and B mRNAs at E11 ∼ birth (A), immunolocalization of PDGF-A and -B (B ∼ G) and immunolocalization of PDGFR-α and β (H ∼M) at E11 ∼ 15 in the mouse tongue. PDGF-A and -B showed a similar expression profile during the development of mouse tongue, although the expression level of PDGF-B mRNA was higher than that of PDGF-A mRNA (Fig. 1A). The expression levels of PDGF-A and -B mRNAs were high at E11 and E13, and then decreased gradually until birth. Intensive immunostaining for PDGF-A was observed in the striated muscle tissue of

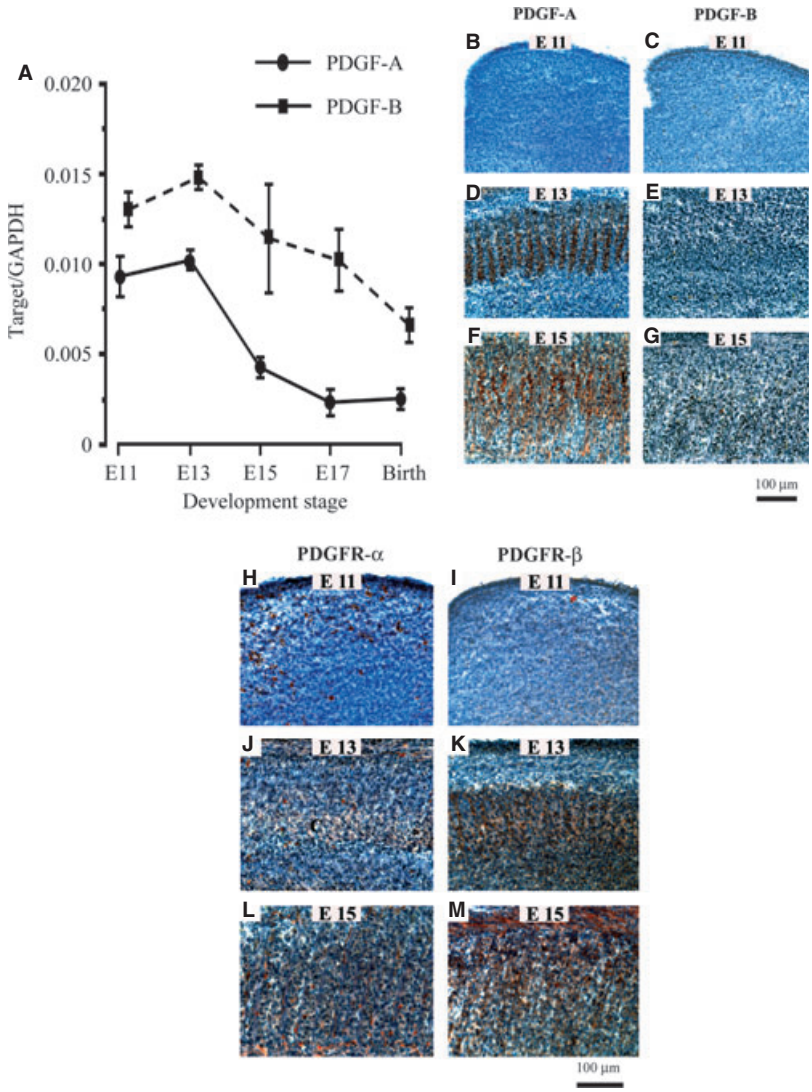

Fig. 1. Changes in the expression levels of PDGF-A and -B mRNAs in the mouse tongue at E11, 13, 15, 17, and birth (A). The longitudinal axis represents the ratio of PDGF-A or PDGF-B mRNA relative to GAPDH mRNA. Each point and its vertical bar represent the mean ± SD of six samples. Immunostaining images for PDGF-A (B, D, F) and PDGF-B (C, E, G) in the whole portion of tongue at E11 (B, C), and in the middle portion of tongue at E13 (D, E) and at E15 (F, G). Brown color indicates immunostaining images for PDGF-A and PDGF-B. Immunostaining images for PDGFR-α (H, J, L) and PDGFR-β (I, K, M) in the whole portion of tongue at E11 (H, I), and in the middle portion of tongue at E13 (J, K) and at E15 (L, M). Brown color indicates immunostaining images for PDGFR-α and PDGFR-β.

tongue from E13 and 15 (Fig. 1D, F), whereas that for PDGF-B was first observed in the E15 tongue (Fig. 1G).

Immunostainings for both PDGFR-α and -β were first observed in the striated muscle of tongue at E13, but the immunostaining for PDGFR-α was weak (Fig. 1J, K) (PDGFR-α and -β positive cells observed in the tongue at E11 appeared to be blood cells based on their location and morphology). Intensive immunostainings for both PDGFR-α and -β were observed in the striated muscle of tongue at E15 (Fig. 1L, M).

The effects of human recombinant PDGF on the differentiation and proliferation of tongue striated muscle cells

To study the function of PDGFs in the development of tongue striated muscle cells, we analyzed the effects of human recombinant PDGF-AB on the differentiation and proliferation of tongue striated muscle cells using an organ culture system of mouse embryonic tongue.

Figure 2A shows the gross morphology of E12 tongues cultured for 4 days in the BGJb medium containing 0, 25, 50, or 100 ng/ml of PDGF-AB. There was no marked difference in the shape or size of the cultured tongue between the control and PDGF-treated tongues. To estimate the whole tissue volume of the cultured tongues, we measured the expression level of GAPDH mRNA, a house keeping gene (Fig. 2B). The PDGF-AB treatment induced no statistically significant difference in the expression level of GAPDH mRNA. Taken together, these findings suggest that PDGF-AB did not have harmful effects on the cultured E12 tongues.

To evaluate the effects of PDGF-AB on the differentiation of tongue myoblasts, we analyzed the expression levels of MCK and myogenin mRNAs (Fig. 3), and the immunolocalization of fMHC (Fig. 4), which are markers for the differentiation of skeletal muscle cells, in the cultured E12 tongue. The treatment of 100 ng/ml PDGF-AB induced 180% and 100% increases in the expression levels of MCK and myogenin mRNAs, respectively ($p < 0.01$) (Fig. 3). The number of fMHC-positive cells in the posterior portion of tongue treated with 100 ng/ml of PDGF-AB (Fig. 4F) appeared to be greater than that treated with 0 ng/ml of PDGF-AB (Fig. 4B).

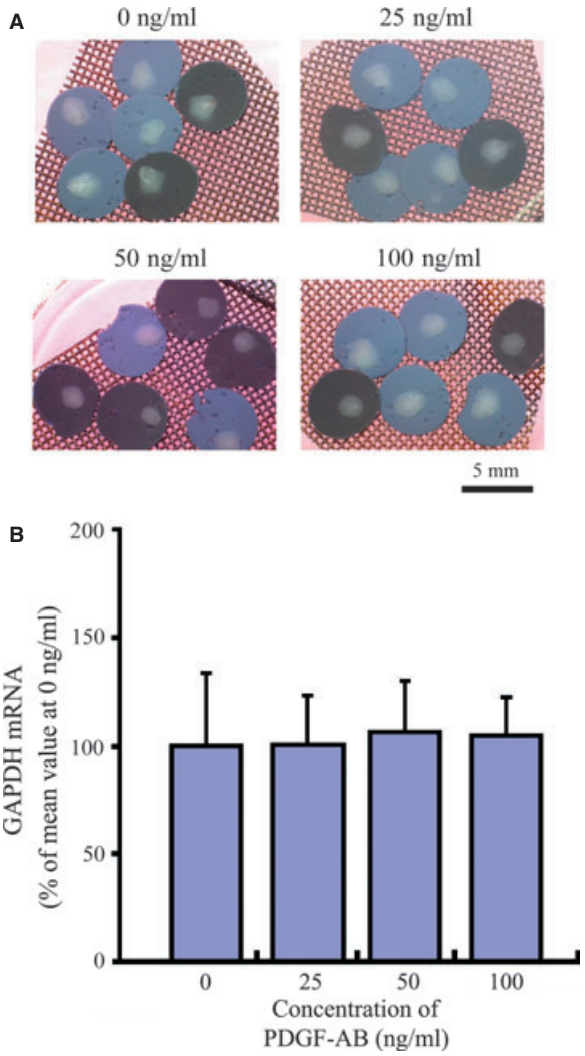

Fig. 2. Gross morphology of E12 tongues cultured for 4 days in BGJb medium containing 0 ~ 100 ng/ml of human recombinant PDGF-AB (A). Expression level of GAPDH mRNA in one E12 tongue cultured for 4 days in BGJb medium containing 0 ~ 100 ng/ml of human recombinant PDGF-AB (B). The longitudinal axis represents the percent value relative to the mean value at 0 ng/ml of human recombinant PDGF-AB set at 100. Each column and vertical bar represents the mean + one SD of six cultured tongues.

To elucidate the effects of PDGF-AB on the proliferation of tongue myoblasts, we analyzed the expression levels of CDK4 and cyclin D1 mRNAs (Fig. 5A, B) and immunolocalization for PCNA, markers for cell proliferation (Fig. 5C, D). We observed no effect of PDGF-AB on mRNA expression levels and immunolocalization of PCNA in the cultured E12 tongue.

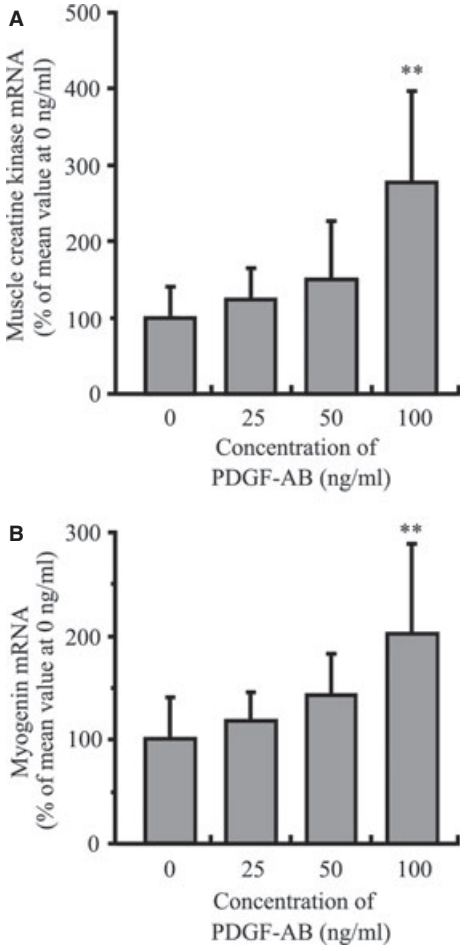

Fig. 3. Expression levels of muscle creatine kinase (MCK) (A) and myogenin (B) mRNAs in E12 tongues cultured for 4 days in BGJb medium containing 0 ~ 100 ng/ml of human recombinant PDGF-AB. The longitudinal axis represents the percent value relative to the mean value of each target gene at 0 ng/ml of human recombinant PDGF-AB set at 100. Each column and vertical bar represents the mean + one SD of six cultured tongues. Significant difference from 0 ng/ml of human recombinant PDGF-AB, $^{**}p < 0.01$.

The effects of the peptide antagonist for PDGFR on the differentiation and proliferation of tongue striated muscle cells

To further study the function of PDGFs in the development of tongue striated muscle cells, we analyzed the effects of the peptide antagonist for PDGFR on proliferation and differentiation processes using an organ culture system of mouse embryonic tongue. Figure 6A shows the gross morphology of E12 tongues cultured for 8 days in the BGJb medium containing 0, 200, or 400 ng/ml of the antagonist. There was no marked difference in the shape or size of the cultured tongue between the control and antagonist-treated tongues. To estimate the whole tissue volume of the cultured tongues, the expression level of GAPDH mRNA, which is a housekeeping gene, was also measured (Fig. 6B). The antagonist treatment induced no statistically significant difference in the expression level of GAPDH mRNA in one cultured tongue. Taken together, these findings suggest that the treatment did not have harmful effects on the cultured E12 tongues.

To evaluate the effects of the peptide antagonist for PDGFR on the differentiation of tongue myoblasts, we analyzed the expression level of myogenin and MCK mRNAs (Fig. 7A, B) and the immunolocalization of fMHC (Fig. 7C, D) in the cultured E12 tongue. Treatment with 400 ng/ml antagonist induced 87% ($p < 0.05$) and 50% ($p < 0.05$) decreases in the expression levels of myogenin (Fig. 7A) and MCK (Fig. 7B) mRNAs, respectively, compared with exposure to 0 ng/ml antagonist. The number of fMHC-positive cells in the tongues treated with 400 ng/ml antagonist appeared to be less than that in the E12 tongues treated with 0 ng/ml antagonist (Fig. 7C, D).

To elucidate the effects of the peptide antagonist for PDGFR on the proliferation of tongue myoblasts, we analyzed the expression levels of CDK4 and cyclin D1 mRNAs (Fig. 8A, B) and the immunolocalization of PCNA (Fig. 8C, D) in the cultured E12 tongue. Treatment with 200 and 400 ng/ml antagonist did not affect the expression levels of CDK4 and cyclin D1 mRNAs (Fig. 8A, B) or the number of PCNA-positive cells (Fig. 8C, D) compared with the treatment with 0 ng/ml antagonist.

Discussion

In the present study, we observed that both the ligands and receptors of PDGF-A and -B were localized to the striated muscle tissues in the mouse tongue at E13 ~ E15 during which the differentiation of tongue muscle cells actively occurred, suggesting that PDGFs are involved in the differentiation process of tongue muscle cells. Recombinant PDGF-AB stimulated the differenti-

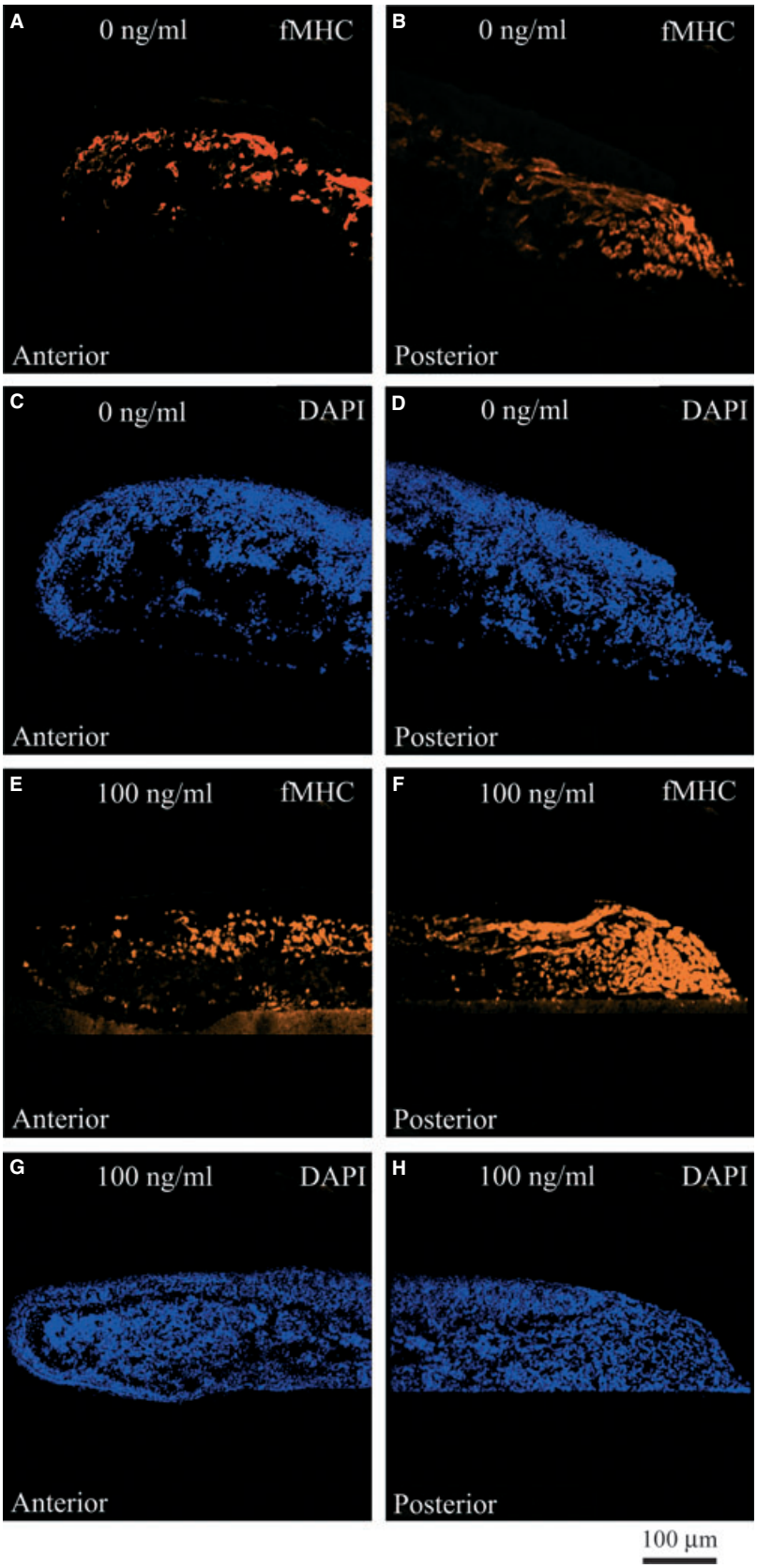

Fig. 4. Immunostaining images for fast myosin heavy chain (fMHC) (A, B, E, F) and DNA staining images with DAPI (C, D, G, H) in the anterior portion (A, C, E, G) and the posterior portion (B, D, F, H) of E12 tongues cultured for 4 days in BGJb medium containing 0 (A ~ D) and 100 (E ~ H) ng/ml of human recombinant PDGF-AB.

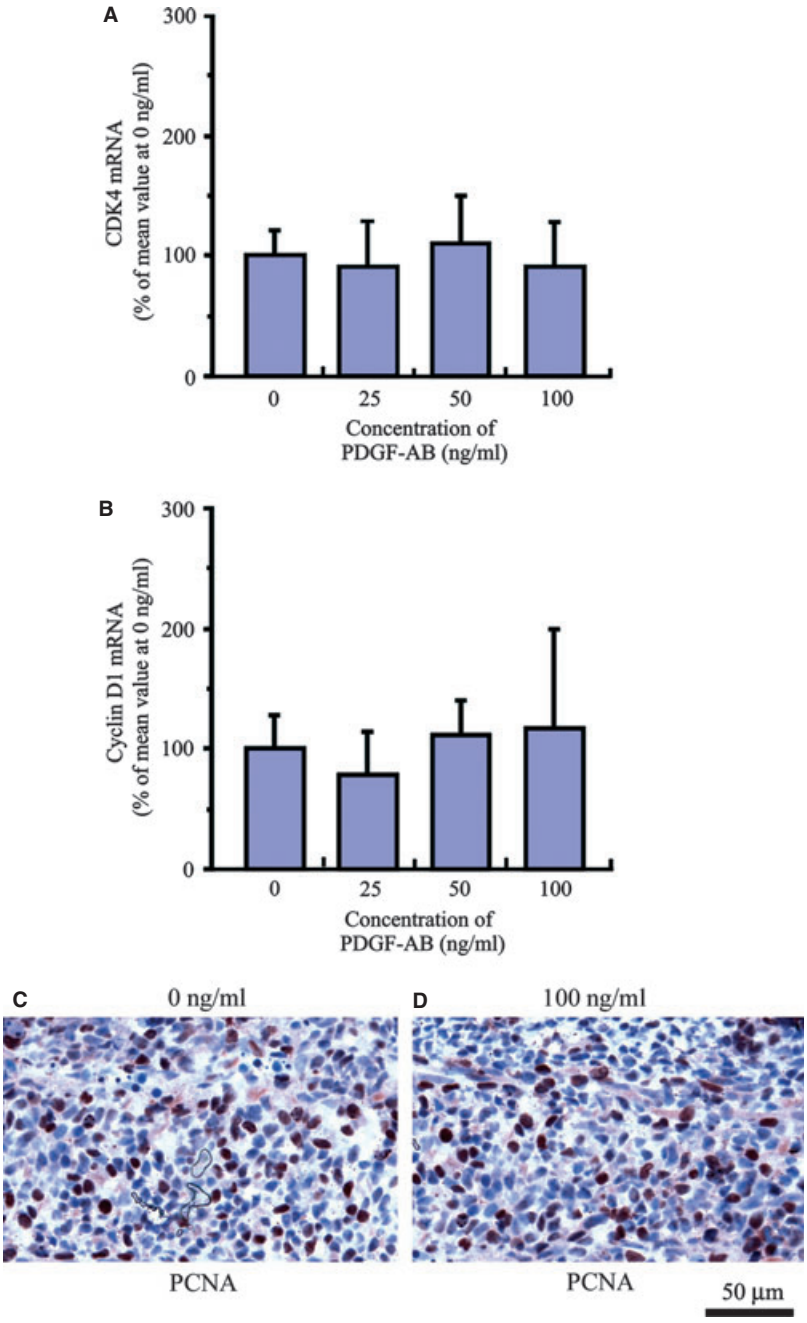

Fig. 5. Expression levels of cyclin-dependent kinase 4 (CDK4) (A) and cyclin D1 (B) mRNAs in E12 tongues cultured for 4 days in BGJb medium containing 0 ~100 ng/ml of human recombinant PDGF-AB. The longitudinal axis represents the percent value relative to the mean value of each target gene at 0 ng/ml of human recombinant PDGF-AB set at 100. Each column and vertical bar represents the mean + one SD of six cultured tongues. Immunostaining image for proliferating cell nuclear antibody (PCNA) in the middle portion of E12 tongues cultured for 8 days in BGJb medium containing 0 (C) and 400 (D) ng/ml of PDGF antagonist. Brown color indicates immunostaining image for PCNA.

ation of tongue muscle cells, whereas the peptide antagonist for PDGFR-α and -β inhibited it. However, neither the recombinant PDGF-AB nor the peptide antagonist for PDGFRs affected the expressions of markers for cell proliferation such as cyclin D1, CDK4, and PCNA. These results suggest that PDGFs function as a positive regulator in the differentiation of tongue muscles cells but have no effect on the proliferation.

PDGFRs are receptor tyrosine kinases. Upon ligand binding, PDGFRs dimerize, activating the tyrosine kinase domains, which then autophosphorylate several tyrosine residues in the receptor cytoplasmic domains. This creates docking sites for signaling proteins and adaptors that initiate signal transduction upon PDGF binding. PDGFRs can activate various signal transduction pathways, including mitogen-activated protein kinase (MAPK), the phosphatidylinositol 3-kinase (PI3K), and Akt/PKB signaling pathways (2). These signaling pathways are known to play essential roles in the development

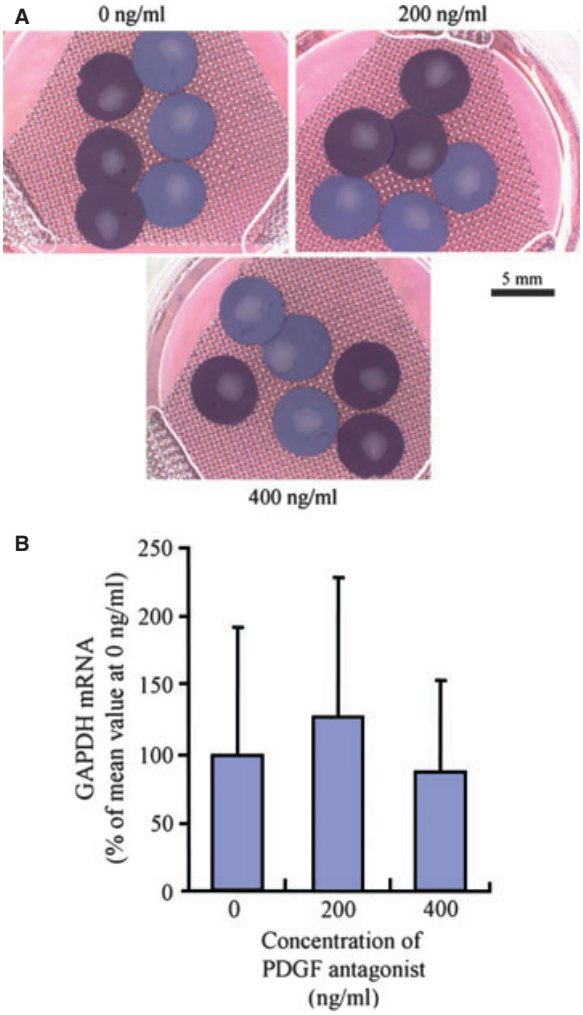

Fig. 6. Gross morphology of E12 tongues cultured for 8 days in BGJb medium containing 0 ∼ 400 ng/ml of PDGF antagonist (A). Expression level of GAPDH mRNA in one E12 tongue cultured for 8 days in BGJb medium containing 0 ∼ 400 ng/ml of PDGF antagonist (B). The longitudinal axis represents the percent value relative to the mean value at 0 ng/ml of PDGF antagonist set at 100. Each column and vertical bar represents the mean + one SD of six cultured tongues.

and maintenance of skeletal muscle cells (29–31). Thus, PDGFs may stimulate the differentiation of tongue muscle cells via these signaling pathways.

There are several reports on the effects of PDGFs on the proliferation and differentiation of cultured myogenic cell lines; PDGF-AA or PDGF-BB stimulate the proliferation of L6 and C2C12 myogenic cells, respectively (10–12); PDGF-BB inhibits the differentiation of C2C12 myogenic cells (11), and PDGF-AA and PDGF-AB have little or no effect on the proliferation and differentiation (10, 11). The present findings are inconsistent with the previous results from cultured myogenic cell lines.

We previously reported on the different functions of peptide growth factors in the development of tongue muscle cells from limb and cultured myogenic cell lines as follows. TGF-α promotes early differentiation of mouse tongue myoblasts (24, 32, 33), while it does not affect the differentiation of the C2 myoblast (34). The signal of TGFβ3, not of TGFβ1 or TGFβ3, plays a role in the early stages of differentiation of mouse tongue muscle cells through TGFβRI, TGFβRII, and smad2/3 (35), but TGFβ1 inhibits the differentiation of limb skeletal muscle cells (36, 37). The present findings also support our notion that the program governing tongue myogenesis differs from those for limb and cultured myogenic cell lines.

Disorders of the development of skeletal muscle were observed only in PDGFR-α null mice (9), implying that the PDGF signal primarily functions through the receptor dimer containing at least one α receptor such as PDGFR-$\alpha\alpha$ and/or -$\alpha\beta$ in the tongue muscle cells. In the present study, we used the PDGF-AB because it has specific binding ability for PDGFR-$\alpha\alpha$ and/or -$\alpha\beta$. We observed that PDGF-AB stimulated the differentiation of tongue muscle cells, suggesting that the PDGF signal primarily functions through the receptor dimer containing at least one α receptor in the tongue muscle cells.

Conclusion

Insulin-like growth factors (IGFs) play critical roles in the normal development and maintenance of skeletal muscle. Mice lacking IGF-I receptor exhibit marked muscle hyperplasia and die in the neonatal period (38). Conversely, mice with overexpression of IGF-I in skeletal muscle demonstrate a profound increase in muscle mass because of myofiber hypertrophy (39, 40). We previously reported that IGFs and their receptors are highly expressed in mouse tongue muscle cells between E13 and E15 during which differentiation of tongue muscle cells actively progresses and IGF stimulates the differentiation,

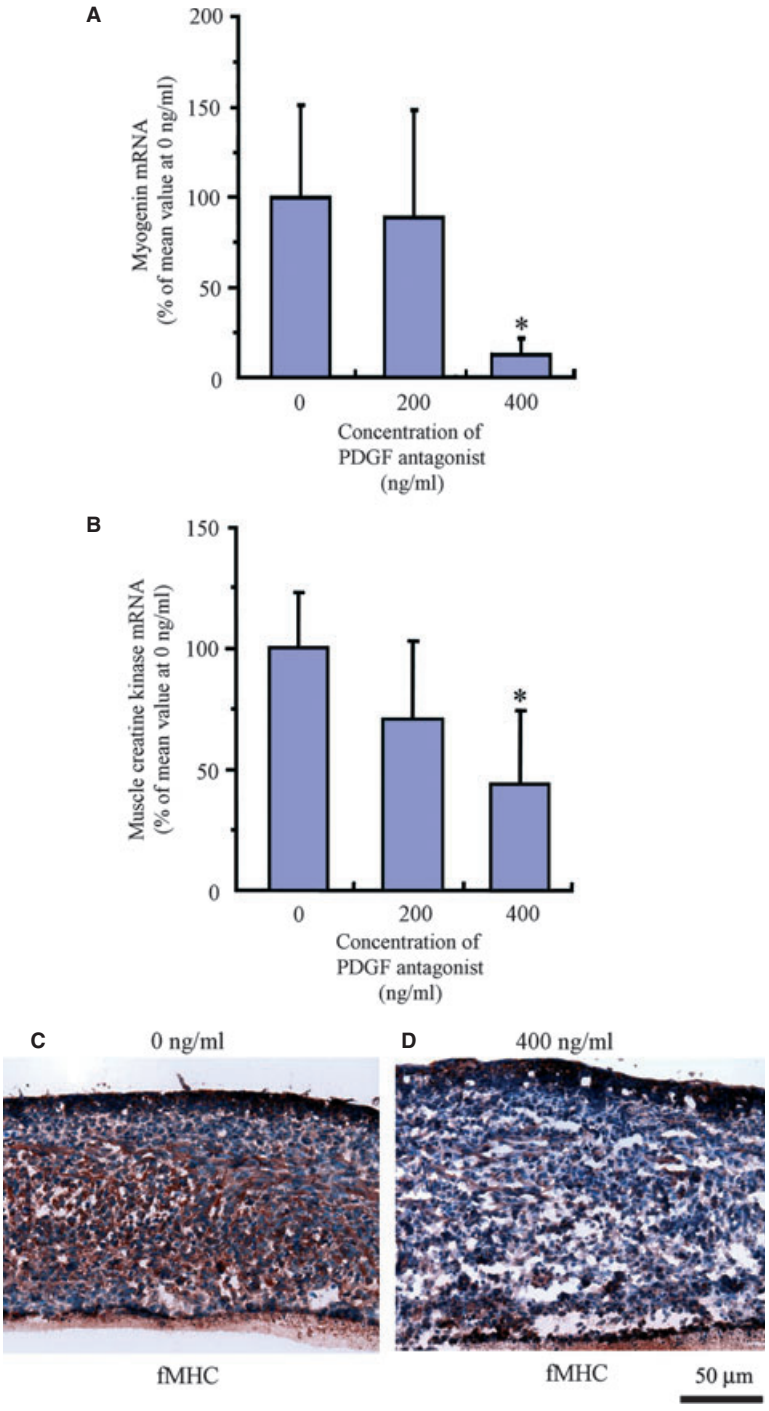

Fig. 7. Expression levels of myogenin (A) and (MCK) (B) mRNAs in E12 tongues cultured for 8 days in BGJb medium containing 0 ~ 400 ng/ml of PDGF antagonist. The longitudinal axis represents the percent value relative to the mean value at 0 ng/ml of PDGF antagonist set at 100. Each column and vertical bar represents the mean + one SD of six cultured tongues. Significant difference from 0 ng/ml of PDGF antagonist, $*p < 0.05$. Immunostaining image for fast myosin heavy chain (fMHC) in the middle portion of E12 tongues cultured for 8 days in BGJb medium containing 0 (C) and 400 (D) ng/ml of PDGF antagonist. Brown color indicates immunostaining image for fMHC.

based on studies using an organ culture system of mouse embryonic tongue (27, 41). It was previously reported that PDGFs may provide a means for fine-tuning the mass of skeletal muscle collaborated with IGFs (42). Thus, we assume that PDGF functions as a positive regulator in the differentiation of tongue muscles cells in collaboration with IGFs.

Clinical relevance

This article provides information on the function of (PDGF) in the differentiation and proliferation of tongue muscle cells. By doing so, PDGF could be applied in clinical settings, for example, to promote the wound healing and regeneration of tongue tissue.

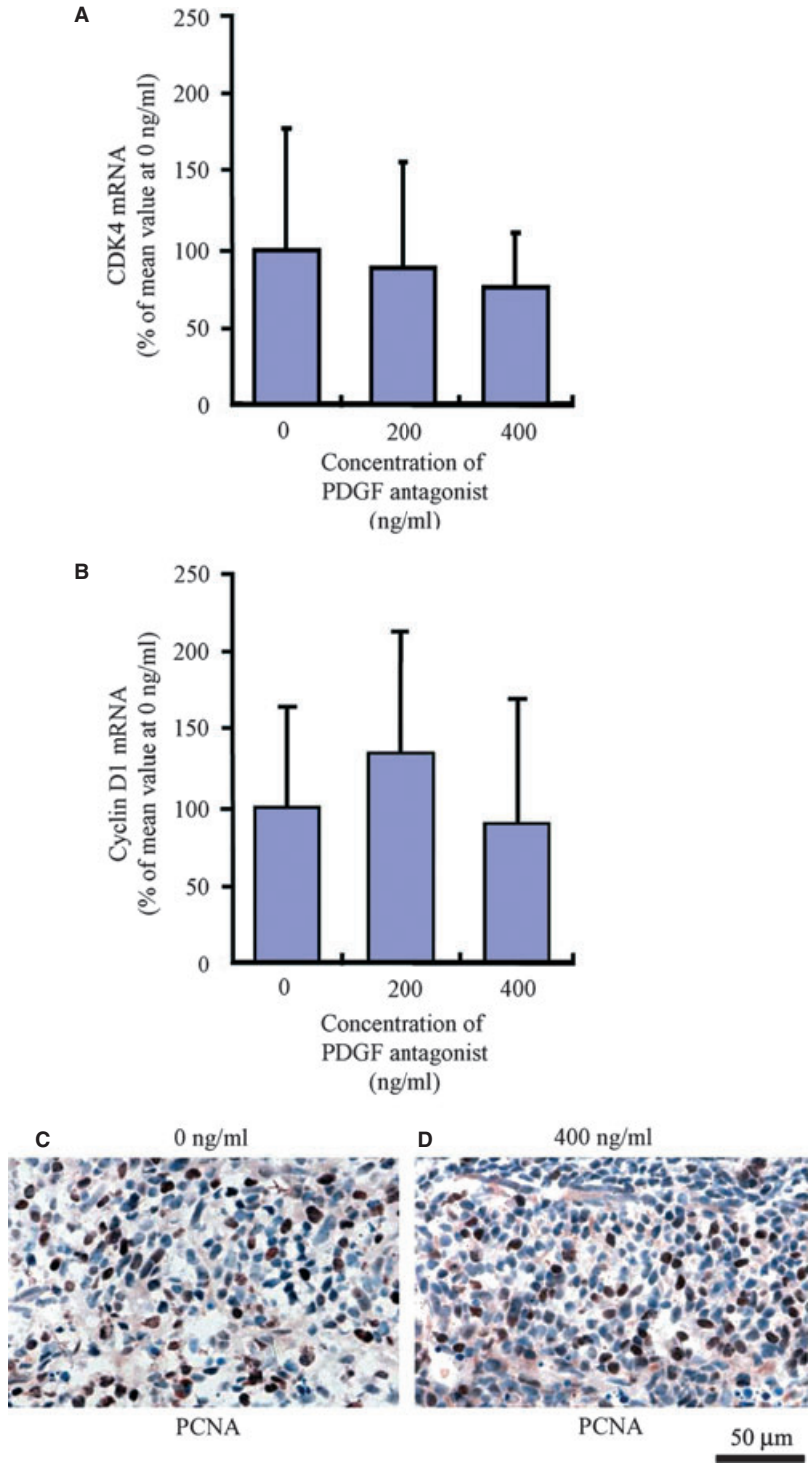

Fig. 8. Expression levels of cyclin-dependent kinase 4 (CDK4) (A) and cyclin D1 (B) mRNAs in E12 tongues cultured for 8 days in BGJb medium containing 0 ~ 400 ng/ml of PDGF antagonist. The longitudinal axis represents the percent value relative to the mean value at 0 ng/ml of PDGF antagonist set at 100. Each column and vertical bar represents the mean + one SD of six cultured tongues. Immunostaining image for proliferating cell nuclear antigen (PCNA) in the middle portion of E12 tongues cultured for 8 days in BGJb medium containing 0 (C) and 400 (D) ng/ml of PDGF antagonist. Brown color indicates immunostaining image for PCNA.

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