NOTE *Biochemistry*

Fibronectin modified expression of Sonic hedgehog in ATRA-mediated neuronal differentiation

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ABSTRACT. In this study, the effect of fibronectin on the neurite outgrowth from embryoid bodies (EBs) in neurodifferentiated embryonal carcinoma P19 cells was examined. The neurite outgrowth on fibronectin was maintained for a longer time in comparison with those on collagen or laminin. Quantitative RT-PCR revealed that mRNA level corresponding to sonic hedgehog (Shh) in neurodifferentiated P19 cells was upregulated on fibronectin, whereas collagen or laminin did not affect. Further knockdown of integrin αv subunit in P19 cells demonstrated that expression of Shh was mediated through interaction between fibronectin and integrin. Additionally, exogenous Shh agonist accelerated neurite outgrowth from embryonic stem cell-derived EBs without large change of neuronal phenotype expression. Taken together, fibronectin could maintain neurite outgrowth via increased Shh expression.

KEY WORDS: fibronectin, integrin, neuronal differentiation, retinoic acid, Shh

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Fibronectin plays important roles in many cellular events including adhesion, growth, migration and differentiation, and is known to interact with integrin, such as α5β1, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8 or αIIbβ3 via an arginine-glycine-aspartic acid (RGD) sequence [5, 7, 9, 13]. Importance of interaction between integrin and extracellular matrix (ECM) on axonal outgrowth along with its guidance in development and regeneration of the nervous system has been demonstrated [8]. Especially, neuronal migration and differentiation on ECM depended on integrin β1 [2], and integrin-ECM interaction controls neural network formation [13]. Axonal guidance is also regulated by several developmental morphogens including sonic hedgehog (Shh) [4]. In neurogenesis, Shh is known to act as a graded signal to induce neuronal subtypes at different concentrations [3, 15]. On the other hand, the mouse embryonal carcinoma-derived P19 cell line as well as embryonic stem (ES) cells can differentiate into neurons by induction with all *trans* retinoic acid (ATRA) [1, 11, 15, 17]. ATRA-treated P19 cells strongly expressed the integrin αvβ1 [6], and it was demonstrated that the β1 integrin-knockout ES cell line decreased the length of neurites from the embryoid bodies (EBs) [10]. These results strongly suggested that cell-matrix interaction plays a partial role on neuronal differentiation, however, it is not well known how fibronectin participates in neurodifferentiation. During evaluation of the effect of ECM on the neurite outgrowth and its survival *in vitro*, we found that ATRA-treated P19 cells showed continuous neurite outgrowth on fibronectin-coated dish. Thus, the effect of fibronectin on neuronal differentiation of P19 cells was examined.

ATRA-mediated neuronal induction of P19 cells was performed as described previously [11]. Briefly, P19 cells were pretreated with 0.5 μM ATRA (Sigma-Aldrich Chemical Co., St. Louis, MO, U.S.A.) for 4 days, and then, resulting embryoid bodies were plated onto culture dishes coated with type I collagen (AGC Techno Glass Co., Ltd., Shizuoka, Japan), laminin (EHS tumor-derived, BD Biosciences, Bedford, MA, U.S.A.), fibronectin (BD Biosciences) or a synthetic GRGDS peptide (Peptide Institute, Inc., Osaka, Japan). Gene silencing of integrin αv subunit in P19 cells was performed by transfection of siRNA (Cell Signaling Technology Inc., Danvers, MA, U.S.A.) according to the manufacturer's protocol, and then, cells were selected with puromycin and established as stable transfectants (IntαvKD-P19). Silencing of mRNA corresponding to integrin αv was verified by RT-PCR with the following primer set (upper; 5′-CCATCAGCCAGGGTGTGC-3′ designed in exon 25, lower; 5′-CTGTGCCGTAGGGGAGGG-3′ designed in exon 30). Mouse ES cells (EB3, RIKEN Bio Resource Center, Tsukuba, Japan) were propagated in feeder-free ES-define medium (StemMedium, DS Pharma Biomedical Co., Ltd., Osaka, Japan) supplemented with 1,000 U/m*l*, leukemia inhibitory factor (LIF, ESGRO, Millipore, Temecula, CA, U.S.A.) and 0.1 mM 2-mercaptoethanol (2ME) on gelatin-coated dishes. Neuronal differentiation was performed according to the previous report [14]. Briefly, EB3 cells were precultured in ADFNK medium [18] for 2 days to form EBs and then cultured for additional 4 days with 1 μM ATRA. To induce neurite outgrowth, ATRA-treated EBs were seeded on collagen-coated dished and cultured for 2 days in the presence or absence of 1 μM Shh agonist (purmorphamine, Merck Millipore, Bil-

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Table 1. Primer sets for qRT-PCR

Gene	Upper	Lower	Annealing temp	Size
GAPDH	5′-CTCCCACTCTTCCACCTTCG-3′	5′-CCACCACCCTGTTGCTGTAG-3′	53.4°C	110 bp
Shh	5′-CCATCATTCAGAGGAGTC-3′	5′-TGGATTCATAGTAGACCC-3′	51.4°C	137 bp
nestin	5′-AGAGGAAGAGCAGCAAGG-3′	5′-TCAGCAAACCCATCAGAC-3′	54.5°C	180 bp
TUBB3	5′-GAGGAGGAGGGGGAGATG-3′	5′-GGCTAAAATGGGGAGGAC-3′	56.3°C	271 bp

lerica, MA, U.S.A.). Quantitative RT-PCR (qRT-PCR) was performed as described previously [11], and primer sets corresponding to Shh, nestin and class III β-tubulin (TUBB3) are listed in Table 1. The levels of each mRNA are normalized against the GAPDH mRNA level, and three independent experiments from each cell culture sample were performed. Statistical differences were determined by Mann-Whitney's *U* test, and a value of $P<0.05$ was considered significant. For immunocytochemical staining, cells were seeded in chamber slides and fixed with ethanol at −20°C, overnight. After blocking, these slides were incubated with anti-pan β-tubulin monoclonal antibody (TUB2.1, Sigma-Aldrich), followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Chemicon International Inc., Temecula, CA, U.S.A). Counterstaining was performed with DAPI (Roche Diagnostics, Mannheim, Germany), and then, images were acquired with a LSM710 confocal laser scanning microscope (Carl Zeiss Co., Ltd., Munich, Germany).

In neurodifferentiated P19 cells, qRT-PCR demonstrated that nestin and TUBB3 increased more than 100-fold compared to undifferentiated P19 cells on all ECM components examined (Fig. 1). On the other hand, expression of Shh was exclusively upregulated to 100 to 200-fold in neurodifferentiated P19 cells seeded on fibronectin as well as a GRGDS

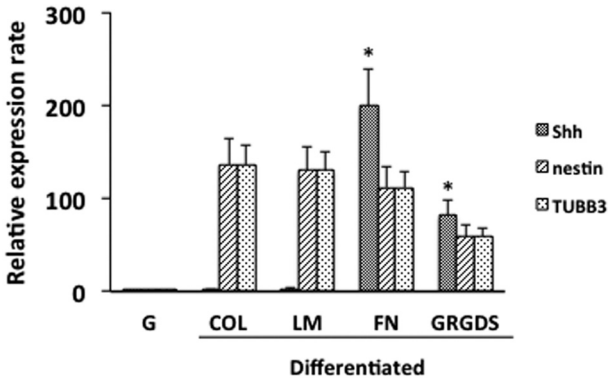

Fig. 1. Effects of coating substrates on expression of neuronal phenotypes in neurodifferentiated P19 cells were evaluated by qRT-PCR. ATRA-treated P19 cells were seeded on type I collagen (COL), laminin (LM), fibronectin (FN) and a GRGDS peptide, and then cultured for 2 days to promote neurite outgrowth (Differentiated). The expression rate of Shh, nestin and TUBB3 was expressed relative to those of undifferentiated P19 in growth phase (G). The values shown are mean ± SD of three independent experiments. Asterisks indicate a significant difference ($P<0.05$) to the value of neurodifferentiated cells on collagen.

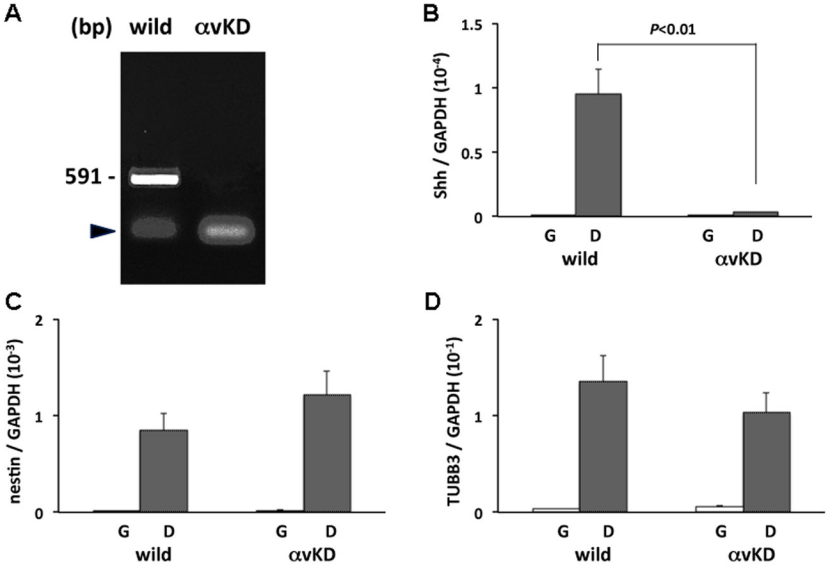

Fig. 2. RT-PCR of mRNA corresponding to αv integrin subunit in wild and IntαvKD (αvKD) P19 cells. A 591 bp single band and a primer dimer (arrowhead) are indicated (A). Comparison of mRNA level corresponding to Shh (B), nestin (C) and TUBB3 (D) between wild and IntαvKD-P19 cells in growth (G) and neurodifferentiation (D) phases was performed by qRT-PCR. The values shown are mean ± SD of three independent experiments, and a significant difference is indicated as *P* value.

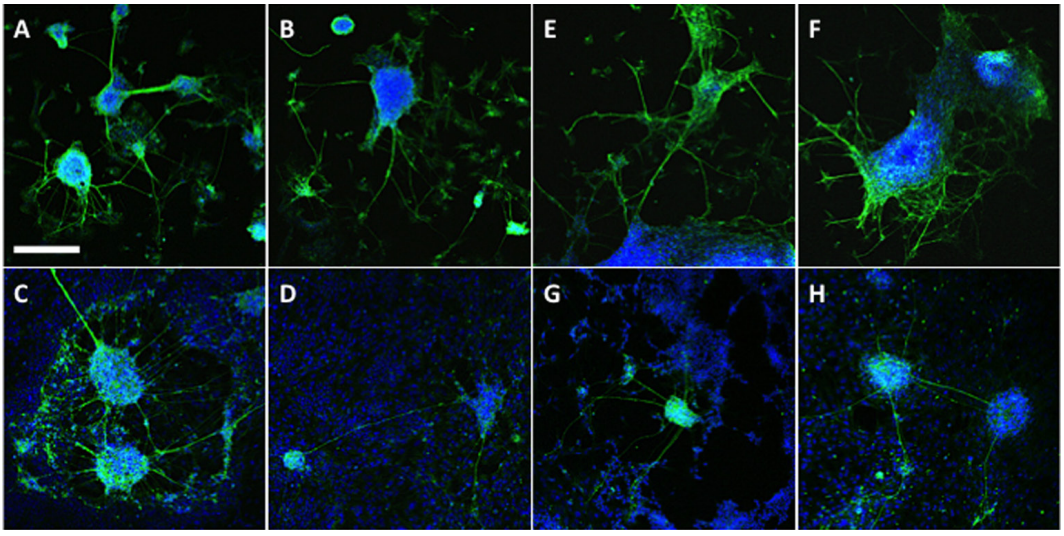

Fig. 3. Morphological features of neurite outgrowth from P19-derived EBs on fibronectin (A, C, E and G) or type I collagen (B, D, F and H) were examined. Wild (A, B, C and D) and IntαvKD-P19 cells (E, F, G and H) were cultured for 2 days (A, B, E and F) or 8 days (C, D, G and H). Immunofluorescent staining was performed with anti-pan β-tubulin (green) and DAPI (blue). Images were obtained at an original objective magnification of × 20 (Bar=100 μm).

peptide in compared to cells on type I collagen and laminin. ATRA-treated ES cells also showed increased Shh expression on fibronectin, but not on collagen or laminin (data not shown). These results suggested that fibronectin specifically affected Shh expression via integrin-mediated response. To investigate participation of the fibronectin receptor, gene silencing of integrin αv was performed (Fig. 2A). Neuronal induction of the IntαvKD-P19 cells was performed in the same way as wild P19 cells, and then, these cells were seeded on a fibronectin-coated dish. As a result, upregulation of Shh mRNA was not observed in the neurodifferentiated IntαvKD-P19 cells seeded on fibronectin (Fig. 2B), whereas other neuronal phenotypes, nestin (Fig. 2C) and TUBB3 (Fig. 2D) increased similar to wild P19 cells. Next, the effects on neurite outgrowth by the knockdown experiment were morphologically examined (Fig. 3). On 2 days after seeding of EBs on several ECM, active neurite outgrowth was observed on fibronectin (Fig. 3A and 3E), collagen (Fig. 3B and 3F) and laminin (data not shown), and there is no apparent difference between wild (Fig. 3A and 3B) and IntαvKD-P19 cells (Fig. 3E and 3F). These results showed that the early stage of neurite outgrowth was not affected by ECM components or knockdown of integrin αv. On 8 days after seeding, an active neurite outgrowth from EBs of wild P19 cells was maintained on fibronectin (Fig. 3C), whereas those on collagen declined (Fig. 3D). On the other hand, EBs derived from the IntαvKD-P19 cells could not show active neurite outgrowth even on fibronectin (Fig. 3G) as well as on collagen (Fig. 3H) or laminin (data not shown). These results suggested that upregulation of Shh expression on fibronectin participated the maintenance of neurite outgrowth. Thus, to evaluate effect of Shh on neurite outgrowth, ES-derived EBs were cultured on collagen-coated dishes in the presence or absence of purmorphamine. As a result, addition of purmorphamine promoted neurite outgrowth from EBs (Fig. 4A), whereas the neurites remained within the EBs without Shh agonist (Fig. 4B). Under these conditions, exogenous Shh agonist did not affect expression of Shh and TUBB3 in ES cells (data not shown).

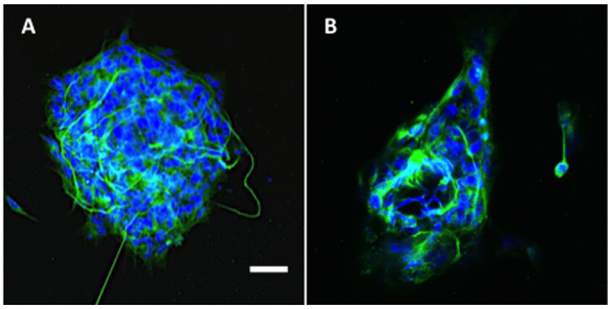

Fig. 4. Effects of Shh agonist on neurite outgrowth in neurodifferentiated ES cells were immunocytochemically evaluated. EBs derived from ATRA-treated ES cells were seeded on type I collagen-coated chamber slides and culture for 2 days to promote neurite outgrowth in the presence (A) and absence (B) of purmorphamine. Immunofluorescent staining was performed with anti-pan β-tubulin (green) and DAPI (blue). Images were obtained at an original objective magnification of × 20 (Bar=50 μm).

Fibronectin is widely distributed in the ECM of the nervous system and functions in neuronal cell adhesion, migration and the guidance of axons during development and regeneration in addition to neuronal survival [12, 13, 16].

Among 24 integrin heterodimers, integrin αvβ1 and αvβ3 were major ECM receptors in P19 cells; the αvβ1 integrin was strongly induced in ATRA-mediated neurodifferentiation

of P19 cells, but expression of the αvβ3 integrin increased to a smaller extent [6]. In another cell line, rat PC12 cells, neurite outgrowth was induced by upregulating αv integrin [10]. These results indicated tight relationship between expression of αv-related integrin and neuronal differentiation, and it was suggested that several cellular events observed on fibronectin were mainly mediated via αvβ1 as shown in this study. Next, the knockdown of αv integrin subunit showed specifically inhibited fibronectin-mediated Shh expression with no effect on expression of other neuronal phenotypes, suggesting that fibronectin-integrin interaction did not affect ATRA-mediated neuronal differentiation of P19 cells, but the maintenance of neurite outgrowth. These speculations were supported by the result that exogenous Shh agonist promoted the neurite outgrowth from ES cell-derived EBs. It was reported that ATRA treatment induced ES cells into spinal nerve cells and the following addition of Shh protein or its agonist to these cells induced the motor neuron [18]. Taken above into account, it was thought that continuous neurite outgrowth on fibronectin was brought by upregulation of Shh in ATRA-mediated neuronal differentiation.

In this study, we showed a new function of fibronectin that could regulate neuronal differentiation. These findings may contribute to the area of cell technology in the field of regenerative medicine.

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