

## Netrin-1 Specifically Enhances Cell Spreading on Fibronectin in Human Glioblastoma Cells

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Netrins are secreted molecules and involved in axon guidance, cell migration and tumor development. Recent studies revealed that netrins perform novel functions in such processes as epithelial development and angiogenesis without operating through the classical netrin receptors, DCC (Deleted in Colorectal Cancer) and Unc5h. In the present study, we investigated the roles of netrin-1 and its receptors in cell spreading of human glioblastoma cells, and found that netrin-1 haptotactically enhanced fibronectin-induced cell spreading and focal adhesion formation in U373 glioblastoma cells. Netrin-1 binding to the U373 cell membrane was blocked by an antibody against  $\alpha v$  integrin subunit, but not by an anti-DCC or anti-Unc5h antibody. In addition, enhancement of the fibronectin response by netrin-1 was abrogated by a function blocking antibody against integrin  $\alpha v \beta 3$ . Since the  $\alpha v$  subunit of the integrin family plays an important role in the pathophysiological aspects of cell migration, including tumor angiogenesis and metastasis, our data provide important insight into the molecular mechanism of netrin function.

**Key Words:** Netrin, Fibronectin, Spreading, Integrin alpha v, Glioblastoma

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### INTRODUCTION

Netrins are a conserved family of laminin-related molecules originally discovered in midline floor plate cells of the developing spinal cord (Kennedy et al, 1994). Netrins can attract spinal commissural axons towards the midline, while repelling a subset of axons migrating away from the midline (Kennedy et al, 1994; Serafini et al, 1994; Colamarino et al, 1995). They also play a role in the regulation of cell migration during the development of the nervous system (Park et al, 2002). Additionally, recent studies have revealed that netrins serve as guidance cues for the migration of non-neuronal cells, including endothelial cells, leukocytes and colon cancer cells (Park et al, 2004; Ly et al, 2005; Wislon et al, 2006; Rodrigues et al, 2007). Thus, it is likely that netrins might be ubiquitous guidance molecules for the control of cell migration in various tissues.

The classical netrin receptors, Deleted in Colorectal Cancer (DCC) and Unc5h, participate in most functions of netrins known to be related to axon guidance and cell migration (Keino-Masu et al, 1996; Leonardo et al, 1997; Hong et al, 1999; Park et al, 2002). However, it has recently been suggested that the classical netrin receptors are not responsible for all of the diverse netrin functions. First, netrins play an important role in angiogenesis without the aid of

known netrin receptors (Park et al, 2004). Second, the heparin binding region of the carboxy-terminal of netrins (Kennedy et al, 1994; Serafini et al, 1994), which is not necessary for binding of netrin to its classical receptors, allows netrins to act as haptotactic factors when immobilized on dishes (Yebra et al, 2003). Indeed, netrin-1 binds to most of the basement membrane proteins (Kennedy et al, 1994; Serafini et al, 1994; Yebra et al, 2003), and it has recently been reported that netrin-4 participates in the formation of basement membrane by binding to laminin (Schneiders et al, 2007). Interestingly, integrin  $\alpha 6 \beta 4$  and  $\alpha 3 \beta 1$  are thought to be netrin receptors required for adhesion and migration of pancreatic epithelial cells during development (Yebra et al, 2003). Thus, it seems that netrins might function as extracellular matrix (ECM) proteins by employing integrins as their novel receptors.

In order to elucidate the roles of netrin-1 and its receptors in nervous system-derived tumor cells, we investigated the effects of netrin-1 on the cell spreading of U373MG human glioblastoma cells, and demonstrated that netrin-1 specifically enhanced fibronectin-induced cell spreading of human glioblastoma cells. Furthermore, a function blocking anti-integrin antibody was sufficient to prevent netrin-1 from binding to the cell surface and inhibit the ability of netrin-1 to enhance cell spreading by fibronectin. These findings indicate that the netrin-1/integrin interaction regulates cell spreading *in vitro*.

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**ABBREVIATIONS:** DCC, deleted in colorectal cancer; ECM, extracellular matrix; FAK, focal adhesion kinase.

## METHODS

### Materials

Laminin, fibronectin and vitronectin were obtained from Sigma (St. Louis, MO). Netrin-1, recombinant ectodomain of UNC5, anti-Unc5h antibodies were purchased from R&D system (Minneapolis, MN). Anti-his antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein-or Cy3-conjugated secondary antibodies were obtained from Amersham (Piscataway, NJ). Tissue culture plastics were obtained from Corning Costar (High Wycombe, UK), and culture media were from Life Technologies (Paisley, UK). Human U373MG glioblastoma cell line was purchased from the American Type Culture Collection. All anti-integrin antibodies and the anti-phosphotyrosine antibody were obtained from BD bioscience (San Jose, CA).

### Cell spreading assay

A cell spreading assay was performed with U373MG glioblastoma cell line, a commonly used cell line for glioma cell researches. Coating of dishes with various extracellular matrix proteins was performed as previously described (Lee et al, 2006). Briefly, 24-well culture plates were incubated with fibronectin (10  $\mu$ g/ml), laminin (10  $\mu$ g/ml), vitronectin (2  $\mu$ g/ml), and various concentration of netrin-1 (10 ng/ml ~ 500 ng/ml) overnight at 4°C. Cells were dissociated after a brief exposure to 0.1% trypsin-EDTA, and recovered by subsequent incubation in 10% fetal bovine serum (FBS) containing Dulbecco's Modified Eagle's Medium (DMEM) for 45 min. Cells were then plated on the different adhesion substrates ( $4 \times 10^4$  cells/well), and the attached cells were photographed after 1 or 2 h. The areas of spreading cells (more than 200 cells per experiment) were measured from randomly selected areas (200 $\times$ 200  $\mu$ M) using a software, accompanied with the LSM 510 confocal microscope (Carl Zeiss, Germany). (Wheeler and Ridley, 2007).

For blocking experiments, cells were preincubated with commonly-used function blocking anti-integrin antibodies (5  $\mu$ g/ml), or an antibody against DCC (clone; AF-5, Pharmingen) or an antibody against Unc5h3 for 30 min before plating. Cells were incubated with antibodies for the duration of the experiment. We employed mouse IgG (20  $\mu$ g/ml, Sigma) as a non-specific antibody control for this experiment.

### Western blot analysis for FAK phosphorylation

Cells were serum-starved for 24h in 0.5% serum before the experiment. Cells were detached with minimum trypsinization, washed with ice-cold 1% bovine serum albumin (BSA)/DMEM three times, and resuspended in pre-warmed 1% BSA/DMEM supplemented with 20 mM HEPES (pH 7.2) for 30 min. Next, cells were allowed to adhere to the coated dishes for the indicated time. Adherent cells were washed with ice-cold phosphate buffered saline (PBS) and lysed with boiled 2 $\times$  sodium dodecyl sulfate (SDS) sample buffer. Protein extracts were resolved by 8 or 10% SDS-polyacrylamide gel electrophoresis, and transferred electrophoretically to nitrocellulose membranes (Amersham). The blotted membranes were blocked with 5% non-fat milk in PBS containing 0.05 % Tween-20 (PBST), and then were incubated with primary antibodies (anti-phospho-focal adhesion kinase (FAK) (pY397)) diluted in PBST containing 3% non-fat milk for 1 h at room temperature. After three

washes in PBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham, 1 : 3,000) for 1 h at room temperature, and then washed again with PBST. The reaction was visualized with an enhanced chemiluminescence reaction (Amersham).

### Immunostaining for detection of focal adhesion formation

For immunolabeling of focal adhesions, attached cells were fixed with 4% paraformaldehyde (PFA), and immunostained with an anti-phosphotyrosine antibody (Barry and Critchley, 1994) or an anti-vinculin antibody (Sigma). A Cy3-conjugated anti-mouse IgG (1 : 800) was used as a secondary antibody, and the staining was viewed using a laser confocal microscope (LSM510, Carl Zeiss, Germany).

### Cell surface binding and immunofluorescent staining

We tested whether the binding of netrin-1-His to the cell surface could be observed by a live cell binding assay followed by immunofluorescent staining (Kim et al, 2005). Thus, cells were grown in 16 well Lab-tek dishes (Nunc, Rochester, NY) overnight in DMEM containing 10% FBS, washed twice with serum-free DMEM, and then incubated with serum-free DMEM containing netrin-1 (500 ng/ml) for 1 h at 37°C. Unbound netrin-1 was removed by washing cells twice with serum-free DMEM, and cells were then fixed with 4% PFA for 15 min at room temperature. After several washes with PBS, the cells were blocked with 2% BSA in PBS for 30 min. Cells were incubated with a rabbit anti-His antibody (1 : 1,000) for 16 h at 4°C and washed with PBS. Next, cells were incubated with Cy3-labeled donkey anti-rabbit IgG and viewed using a laser confocal microscope. For the blocking experiments, cells were preincubated with function blocking anti-integrin antibodies ( $\alpha$  1-6,  $\alpha$  v,  $\beta$  1-3, 5  $\mu$ g/ml) 20 min before the addition of netrin-1.

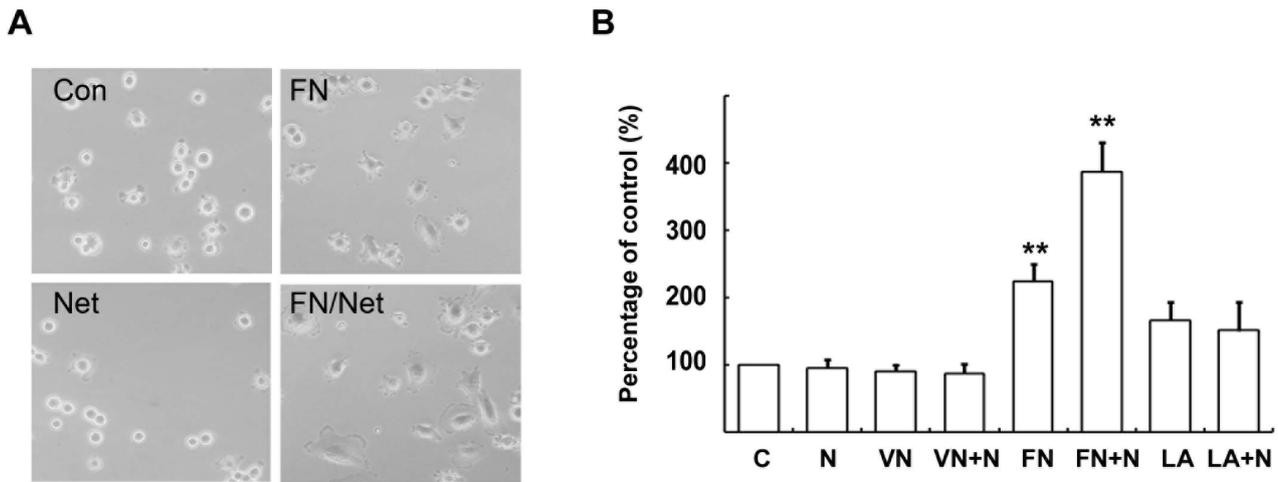
### Statistical Analysis

The means of the collected data were determined for each time point and experimental group. The statistical significance of differences in the counts between two groups was tested by Student's t-test. Statistical significance was set at  $p < 0.05$ .

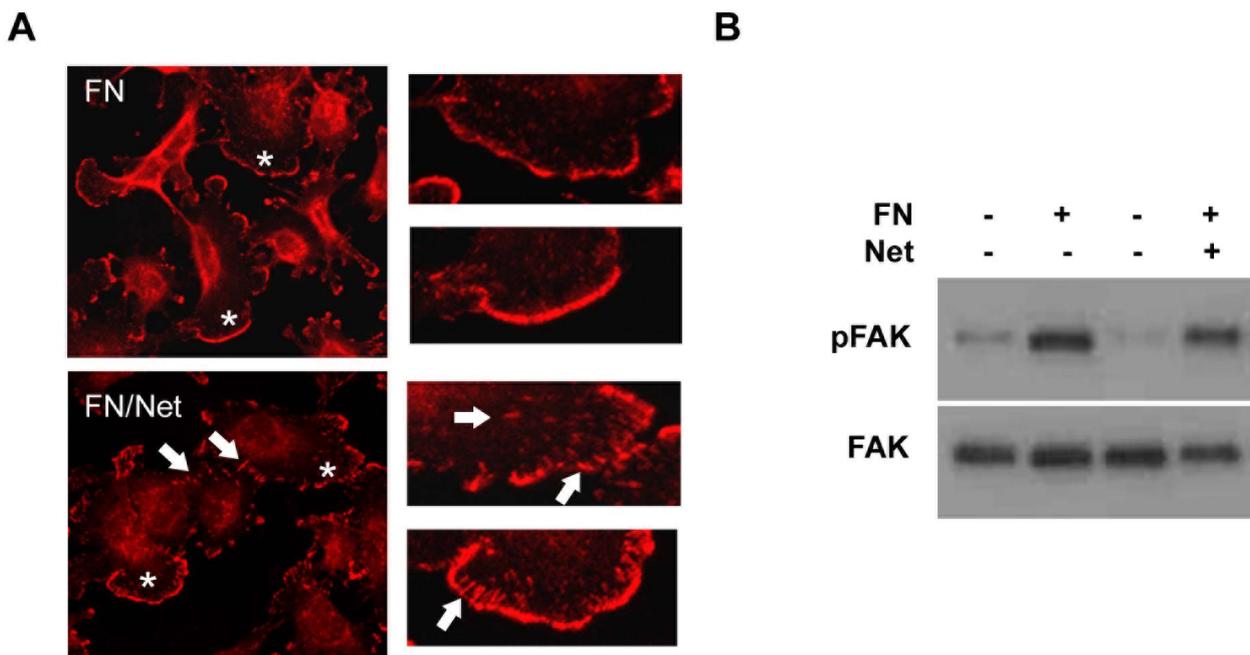
## RESULTS

### Netrin-1 selectively enhances fibronectin-induced cell spreading

We analyzed the ability of netrin-1 to increase the spreading of human U373MG glioblastoma cells, and found that glioblastoma cells spread very efficiently onto fibronectin, compared to laminin and vitronectin (Fig. 1). In fibronectin-coated dishes, spreading cells showed many cellular processes and membrane ruffles within 1 h of attachment. We determined a spreading index by measuring areas occupied by cells on dishes, and this index showed that fibronectin enhanced cell spreading more than 2 fold compared to untreated controls. We also examined whether netrin-1 can haptotactically enhance glioblastoma cell spreading, and found that netrin coating (up to 500 ng/ml) alone did not



**Fig. 1.** Netrin-1 enhances fibronectin-induced cell spreading. (A) U373 cells were attached to ECM protein-coated dishes for 1 h, and photographed. Netrin-1 (Net, 500 ng/ml) alone had no positive effect on cell spreading, but netrin-1 (50 ng/ml) enhanced fibronectin (FN)-induced cell spreading. (B) The extent of cell spreading was analyzed by measuring areas occupied by randomly selected cells 1 h after plating. C, no coating; N, netrin-1 (500 ng/ml); VN, vitronectin; FN, fibronectin; LA, laminin. Values shown are means±s.d. from three independent experiments. \*\**p*<0.01.



**Fig. 2.** Netrin-1 enhances focal adhesion formation without affecting FAK activation. (A) U373 cells were attached to ECM protein-coated dishes for 2 h, fixed and immunostained with an antibody to phosphotyrosine to localize focal adhesion. Arrows indicate focal adhesions, and asterisks indicate the areas magnified in the panels to the right. (B) U373 cells were attached to ECM protein-coated dishes for 1 h, and cell lysates were immunoblotted with an anti-phospho-FAK (pY397) antibody. FN, fibronectin; Net, netrin-1.

induce cell spreading.

We next examined whether netrin-1 would affect cell spreading in the presence of other ECM proteins. Thus, we attached cells to dishes which had been co-coated with ne-

tratin-1 and other ECM proteins, and found that netrin-1 dramatically enhanced cell spreading on fibronectin, but not vitronectin or laminin (Fig. 1A, B). The effect of netrin-1 on the fibronectin-induced cell spreading was dose-depend-

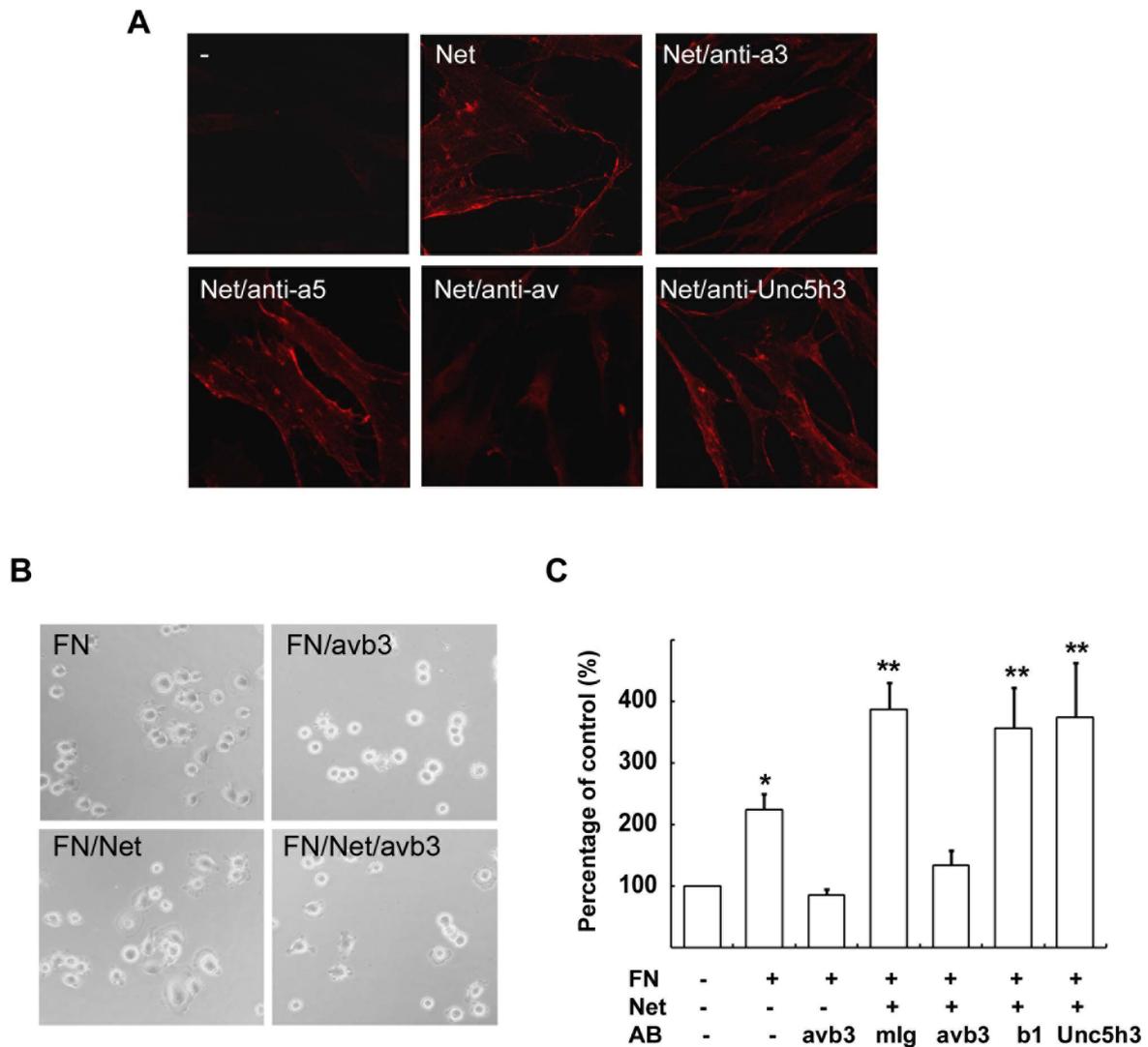
ent, and 50 ng/ml of netrin-1 was sufficient to elicit the effect.

**Netrin-1 increases focal adhesion formation by fibronectin**

Since cell spreading on ECM proteins involves formation of focal adhesions, we investigated whether netrin-1 alters fibronectin-induced focal adhesion formation by using immunofluorescent staining with an anti-phosphotyrosine antibody (Fig. 2A) or an anti-vinculin antibody (data not shown). The result revealed that netrin-1 alone could not induce focal adhesion formation, but did enhance formation of focal

adhesions in the presence of fibronectin. As shown in Fig. 2A, the focal adhesions were larger and more prominent in cells attached to fibronectin/netrin-1-coated dishes, compared to the cells on fibronectin-coated dishes.

We next investigated whether netrin-1 enhances integrin signaling pathways that are activated by fibronectin, such as FAK (Fig. 2B). We previously reported that the classical netrin receptors also signal through FAK as a downstream effector (Liu et al, 2004). Cells attached to fibronectin showed phosphorylation of FAK (pY397) within 1 h. However, adhesion of cells to netrin-1-coated dishes did not result in pY397 levels (up to 500 ng/ml, data not shown). The coating of dishes with netrin-1/fibronectin did not further increase



**Fig. 3.** Netrin enhancement of cell spreading on fibronectin is blocked by a function blocking antibody against  $\alpha v \beta 3$ . (A) A live cell binding assay with netrin-1-His (Net) demonstrates specific membrane binding of netrin-1-His in controls. Pretreatment with a function blocking antibody against  $\alpha 3$  or  $\alpha v$  significantly reduced netrin-1 binding, whereas anti- $\alpha 5$  or anti-Unc5h3 did not affect the cell surface binding of netrin-1-His. (B) Cell spreading on fibronectin (FN) or fibronectin/netrin-1 (FN/Net) substrate was inhibited by incubation with an antibody to  $\alpha v \beta 3$ . (C) Quantitative result showing the specific effect of an antibody to  $\alpha v \beta 3$  on cell spreading. AB, antibody; mIg, mouse Ig G. Values shown are means $\pm$ s.d. from three independent experiments. \*\*p<0.01.

the phosphorylation of FAK, compared to fibronectin coating alone. This finding indicates that netrin enhancement of fibronectin-induced cell spreading and focal adhesion formation might not be caused simply by increased FAK signaling.

***An antibody against alpha v integrin subunit blocks netrin binding to the cell surface***

It has previously been reported that netrin-1 haptotactically functions through its C-terminal domain by binding to integrins, and that netrin binding to the cell surface is blocked by specific function blocking antibodies against integrin  $\alpha 6\beta 4$  and  $\alpha 3\beta 1$  in epithelial cells (Yebra et al, 2003). Therefore, we employed this method to identify integrin receptors in U373 MG cells that might bind to netrin-1. Cells were pretreated with either mouse IgG or function-blocking antibodies to each of the integrin subunits (Table 1,  $\alpha 1-6$ ,  $\alpha v$ ,  $\beta 1-4$ ) for 30 min, and were then incubated with His-tagged netrin-1. This binding assay showed that blocking of  $\alpha 3$  or  $\alpha v$  integrin with specific function blocking antibodies prevented netrin-1 from binding to the cell surface (Fig. 3A). In contrast, anti-DCC antibody (AF-5), anti-Unc5h3 antibody, and other anti-integrin antibodies used (Materials and Methods) had no effects (Fig. 3B and data not shown). This finding indicates that netrin-1 might interact with  $\alpha v$  and  $\alpha 3$  integrin in U373MG cells.

***Netrin-1 may enhance fibronectin response by binding to integrin receptor- $\alpha v\beta 3$***

In order to examine how netrin-1 could enhance fibronectin-induced cell spreading, we first tried to identify integrin receptors for fibronectin in U373MG cells using function blocking antibodies against integrin receptors, and found that an anti- $\alpha v\beta 3$  antibody, but not  $\alpha 5\beta 1$  (a commonly identified fibronectin receptor (Danen et al, 2001)), completely blocked cell spreading on a fibronectin substrate (Fig. 3B, C), indicating that  $\alpha v\beta 3$  integrin is the main receptor for fibronectin in U373MG cells. This finding suggests that netrin-1 and fibronectin share the same integrin receptor in U373MG cells, and that anti- $\alpha v\beta 3$  antibody blocks the netrin enhancement of fibronectin-induced cell spreading. Consistent with this hypothesis, anti- $\alpha v\beta 3$  integrin inhibited not only fibronectin-induced cell spreading,

but also netrin enhancement of cell spreading. Anti-DCC antibody (data not shown), anti-Unc5h3 antibody, anti- $\beta 1$  integrin antibody had no effect (Fig. 3C).

## DISCUSSION

Localization of netrins to the basement membrane and their interaction with ECM proteins suggest that netrins are haptotactic factors regulating cell adhesion, spreading and migration. In this study, we found that netrin-1 may bind to an integrin receptor  $\alpha v$ , and that the interaction results in the sensitization of fibronectin-induced tumor cell spreading. It has previously been reported that integrin  $\alpha 6\beta 4$  and  $\alpha 3\beta 1$  mediate netrin-1 function in epithelial cell migration (Yebra et al, 2003). Consistent with this study, we also showed that binding of netrin-1 to the cell membrane was blocked by an anti- $\alpha 3$  antibody, implying that netrin-1 binds to  $\alpha 3$  integrin subunit. Thus, it is likely that the target integrin receptors for netrin-1 may include  $\alpha 6\beta 4$ ,  $\alpha 3\beta 1$  and  $\alpha v$  integrins, and that the identity of netrin receptors in cells might be dependent on the cellular expression profile of integrin subunits. On the other hand, the amino-terminal V and VI domains of netrins bind to classical netrin receptors and seem to be sufficient for axonal outgrowth of commissural neurons by netrin-1 (Kennedy et al, 1994; Serafini et al, 1994; Hong et al, 1999). In contrast, the carboxy-terminal of netrins seems to be responsible for interaction with integrins. Our preliminary result showed that a recombinant Unc5h ectodomain (which binds to the amino-terminal of netrin-1) failed to inhibit netrin-induced enhancement of cell spreading (data not shown). Thus, the amino-terminal netrin-1 may not play a role in cell spreading. Although the molecular mechanism of interaction between netrin and integrin  $\alpha v$  is unknown at present, a plausible hypothesis is that a haptotactic function of netrins might be mediated through integrin receptors, but not through DCC or Unc5h.

Netrin-1 binding to integrin  $\alpha v$  may reduce available integrin  $\alpha v$  for fibronectin binding, thereby reducing fibronectin response. In the present study, however, netrin-1 enhanced fibronectin-induced cell spreading, suggesting that steric hindrance between netrin-1 and fibronectin binding does not occur, and that the binding site of integrin  $\alpha v$  to netrin-1 may be different from that of integrin  $\alpha v$  to fibronectin. Unexpectedly, netrin-1 alone could not induce cell spreading. It may be possible that the affinity of netrin-1 binding to integrin  $\alpha v$  may not be enough for inducing integrin signaling, but sufficient for changing the integrin affinity to fibronectin. In addition, it should also be mentioned that the netrin family has 4 members (netrin-1~4) (Cirulli and Yebra, 2007), and that netrin 2~4 may bind to integrin  $\alpha v$  with high affinity to produce their own integrin signaling. Further studies on the molecular mechanism of the interaction between netrins and integrins are required to ascertain these hypotheses.

It was recently reported that the role of the classical netrin receptors, DCC/Unc5, in netrin-induced angiogenesis was questioned by Wilson et al (2006). Even though our data were obtained from glioma cells, but not from endothelial cells, our findings may provide possible ideas for clarifying this question. First, since ECM proteins, including fibronectin, play an important role in angiogenesis (Serini et al, 2006), netrin sensitization of fibronectin receptors may attribute to the netrin-induced increase of angiogenesis.

**Table 1.** List of antibodies

Integrin subunit	Clone Name	Function blocking
$\alpha 1$	FB12	O
$\alpha 2$	P1E6	O
$\alpha 3$	P1B5	O
$\alpha 4$	P1H4	O
$\alpha 5$	P1D6	O
$\alpha 6$	NKI-G6H3	O
$\alpha V$	P3G8	O
$\beta 1$	P4C10	O
$\beta 2$	P4H9	O
$\beta 3$	25E11	O
$\beta 4$	ASC-9	O
$\alpha V\beta 3$	LM609	O
$\alpha V\beta 5$	P1F6	O

Second, integrin  $\alpha v$  mediates many aspects of endothelial function in angiogenesis, serving additional roles beyond its role as a receptor for fibronectin (Eliceiri et al, 2000; Serini et al, 2006). Thus, further studies on the functional interaction between netrin-1 and integrin  $\alpha v$  in endothelial cell migration and angiogenesis are needed to explain the diverse effects of netrin-1 in angiogenesis.

In conclusion, we found that netrin-1 affected glioma cell spreading on fibronectin, possibly by binding to integrin  $\alpha v$ . It is, therefore, likely that netrin employs two different receptor systems, classical netrin receptors and integrins, to perform its diverse cellular functions. The interaction between netrins and integrins seems to play an important role in the tumor cell adhesion, spreading and migration.

### ACKNOWLEDGEMENT

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