Regulatory effect of nerve growth factor in α9β1 integrin–dependent progression of glioblastoma

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In the present study we described the role of α9β1 integrin in glioblastoma progression following its interaction with nerve growth factor (NGF). The level of expression of α9β1 on astrocytomas is correlated with increased grade of this brain tumor and is highest on glioblastoma, whereas normal astrocytes do not express this integrin. Two glioblastoma cell lines, LN229 and LN18, that are α9β1 integrin positive and negative, respectively, were used for α9β1 integrin–dependent NGF-induced tumor progression. NGF was a significant promoter of promigratory and pro-proliferative activities of glioblastoma cells through direct interaction with α9β1 integrin and activation of MAPK Erk1/2 pathway. The level of NGF increases approximately threefold in the most malignant glioma tissue when compared with normal brain. This increase is related to secretion of NGF by tumor cells. Specific inhibitors of α9β1 integrin or gene silencing inhibited NGF-induced proliferation of LN229 cell line to the level shown by LN18 cells. VLO5 promoted α9β1-dependent programmed cell death by induction of intrinsic apoptosis pathway in cancer cells. LN229 cells were rescued from proapoptotic effect of VLO5 by the presence of NGF. This disintegrin significantly inhibited tumor growth induced by implantation of LN229 cells to the chorioallantoic membrane (CAM) of quail embryonic model, and this inhibitory effect was significantly abolished by the presence of NGF. α9β1 integrin appears to be an interesting target for blocking the progression of malignant gliomas, especially in light of the stimulatory effect of NGF on the development of these tumors and its ability to transfer proapoptotic signals in cancer cells. *Neuro-Oncology 10, 968–980, 2008 (Posted to Neuro-Oncology [serial online], Doc. D08-00033, August 1, 2008. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2008-0047)*

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Gliomas are the most common and difficult to treat tumors of the adult and child CNS.[1] According to WHO, gliomas are divided into four grades (I–IV) according to malignancy.[2] Grade II gliomas are diffused astrocytomas, grade III are anaplastic astrocytomas, and grade IV are glioblastomas. The most malignant form of glioma is glioblastoma multiforme (GBM), which is histologically characterized by cellular atypia, high mitotic activity, and focal necrosis as well as high levels of vascularization.[3,4] Although gliomas do not metastasize outside of the CNS, the survival rate for patients is very low. The standard clinical treatments such as neurosurgery, chemotherapy, and radiotherapy often fail because of a high recurrence rate.[5] The high invasiveness of cancer cells causes an absence of the sharp border between normal tissue and the tumor. This event increases the difficulty of complete surgical elimination of these brain tumors.

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The malignancy of glioma is dependent on diffusive properties of tumor cells that may invade widely as single cells anywhere in the brain.[6] Integrins are the family of adhesive receptors that promote invasiveness of glioma. These heterodimeric transcellular membrane receptors are composed of two subunits, α and β, and are the major receptors for the extracellular matrix (ECM) proteins. Currently, 18 α and eight β subunits have been identified, which may be combined in a restricted manner to form at least 24 heterodimers.[7,8] Immunohistopathological studies revealed an upregulation of α2β1, α3β1, α5β1, α6β1, and αvβ3 integrins on GBM when compared with normal brain.[9,10] The functional blocking or antisense elimination of integrins containing an αv subunit had a positive effect on the inhibition of glioma development and invasion in vitro and in vivo.[6] Similar observations were performed for the β1 subunit of integrins. Blocking monoclonal antibody against the β1 subunit inhibited adhesion, motility, and invasion of glioma cells,[11] whereas use of antisense strategy in vivo in the rat model of C6 glioma reduced tumor growth.[12] However, which α subunit, when functionally associated with β, is important in glioma progression is still an open question. Although some studies suggest that α2β1 and α3β1 integrins may participate in this process,[13,14] we show in the present study the significance of α9β1 integrin, which is a multifunctional cell surface receptor that cross-reacts with a variety of ligands. It has been characterized as a receptor for vascular cell adhesion molecule-1 (VCAM-1), is expressed on neutrophils, and is involved in transmigration of these granulocytes through the endothelium.[15–19] The novel observation is that growth factors are also the ligands for α9β1 integrin. In this context, VEGF-C and VEGF-D were first described.[20] Our very recent data revealed that NGF also belongs to the category of growth factors that interact with α9β1.[21] Based on these evaluations we focused on the interaction of α9β1 integrin with NGF as an important element of glioma progression, although three other ligands for this integrin—tenascin-C, osteopontin, and thrombospondin-1—also are involved in this pathological process. These three ECM proteins are produced by tumor cells and were found overexpressed in glioma tissue.[22–24]

NGF is an evolutionarily conserved polypeptide neurotrophin that plays a crucial role in the life of the sympathetic and sensory nervous systems.[25] This is a homodimeric molecule that showed cross-reactivity with two receptors: the high-affinity TrkA and common neurothrophin $p75^{NTR}$.[26] TrkA belongs to a family of tyrosine kinase receptors, and its complex structure with NGF has already been published.[27] $p75^{NTR}$, however, belongs to the tumor necrosis receptor family, binds all neurotrophins, and is involved in neurotrophin-induced neuronal apoptotic signals.[28–30] The ability of NGF to interact with these two receptors reveals its participation in the regulation of the cell death/survival process. The effect of NGF on survival of glioma cells was investigated in vitro and in vivo, although published data were not consistent. Under certain conditions NGF showed a stimulatory and inhibitory effect on the proliferation of glioblastoma cell lines.[31,32] This divergent effect of NGF on glioma cell growth is attributed to the expression of two receptors by these cells. After binding to TrkA, NGF transfers prosurvival and pro-proliferative signals, while $p75^{NTR}$ is involved in transferring proapoptotic signals. From this point of view the predominant level of expression of each receptor on the cell surface appears to be essential for glioma growth. In this article, we present evidence that a third receptor for NGF, α9β1 integrin, is involved in the progression of glioma in vitro and in vivo. We characterized this integrin as a low-affinity NGF receptor with a similar molecular weight (kDa) as $p75^{NTR}$. However, its biological activity is related to TrkA, because it stimulates cell growth and survival.[21]

Materials and Methods

Cell Lines, Antibodies, Snake Venom Disintegrins, and Human Tissue Samples

Glioblastoma cell lines were purchased from ATCC (Manassas, VA, USA). Primary human astrocytes were purchased from Cambrex (Rutherford, NJ, USA). Polyclonal serum against the α9 subunit of integrin cytoplasmic domain was developed commercially in rabbit (Chemicon, Temecula, CA, USA). Monoclonal antihuman antibodies—anti-α1 (FB12), anti-α2 (P1E6), anti-α3 (clone C3II.1), anti-α6 (clone GoH3), anti-α9β1 (Y9A2), anti-αvβ3 (LM609), anti-β2 (P4H9), and anti-β4 (ASC-3)—were purchased from Chemicon; anti-α4 (HP2/1), anti-α5 (SAM-1), and anti-β (Lia1/2) were purchased from Beckman Coulter, Inc. (Fullerton, CA, USA). Anti-pErk (Thr202/Tyr204) and anti-Erk polyclonal antibodies were purchased from Cell Signaling Inc. (Beverly, MA, USA). Anti-TrkA polyclonal serum was kindly provided by Dr. Louis Reichardt (University of California, San Francisco, CA, USA). Native NGF isolated from mouse submandibular glands was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Two snake venom dimeric disintegrins, VLO4 and VLO5, were purified from the venom of *Vipera lebetina obtusa* (Latoxan, Valence, France) using two steps of reverse-phase high-performance liquid chromatography as described previously.[33] The normal brain and glioma human tissue samples were collected during surgical resection from cancer patients, frozen, and stored at –70°C. The frozen samples were obtained from the Nervous System Tissue Bank (NSTB) housed at Toronto Western Hospital (Toronto, ON, Canada).

Cell Adhesion Studies

Adhesion studies of cultured cells labeled with 5-chloromethyl fluorescein diacetate (CMFDA) (Invitrogen, Carlsbad, CA, USA) were performed using 96-well microtiter plates (BD Falcon, Frankin Lakes, NJ, USA) as described previously.[34]

Detection of NGF in Human Tissue and Proteins Secreted by Glioma Cell Lines in ELISA

Tissue lysate samples (10 μg/ml of protein) were immobilized on 96-well plates overnight at 4°C in phosphate-buffered saline (PBS). Wells were blocked with 5% nonfat milk (Bio-Rad, Richmond, CA, USA) in PBS containing 0.05% Tween-20 (PBST), and anti-NGF polyclonal serum (1:1,000; Chemicon) was added. After 1-h incubation at 37°C, the goat antirabbit immunoglobulin G (IgG), conjugated with alkaline phosphatase (AP) (Sigma Inc., St. Louis, MO, USA), was added, and incubation was continued for another 1 h. Color was developed using alkaline phosphatase substrate, p-nitrophenyl phosphate (Sigma). Plates were read using ELISA plate reader EL×800 (BioTek, Winooski, VT, USA) at a 405-nm single wavelength. The amount of NGF was calculated from the standard curve prepared in parallel on the same plate from known concentrations of human recombinant β-NGF (PeproTech, Rocky Hill, NJ, USA).

NGF content in proteins secreted by LN229 cells was evaluated after gel filtration of conditioned media, free of fetal bovine serum (FBS). Cells were incubated at 37°C in a 5% CO_2 atmosphere with serum-free Dulbecco's modified Eagle's medium (DMEM) for 72 h. Media were collected and gel filtrated on a Superdex 200 column (2.5 × 50 cm; GE Healthcare, Uppsala, Sweden). Protein peaks were collected and immobilized (10 μg/well/100 μl) on a 96-well ELISA plate overnight at 4°C and ELISA was performed as described above.

Detection of Cell Surface Receptors

Western blot analysis. The NGF receptors TrkA and $p75^{NTR}$, as well as the α9 integrin subunit, were detected from the cell lysates or tissue lysates using polyclonal antibodies as described previously.[19]

Immunocytostaining. Cells were cultured on a glass slide up to 70% confluence and fixed with 4% paraformaldehyde. The slide was blocked by 1% bovine serum albumin (BSA), and primary antibodies against NGF receptors were added. After 1-h incubation and washing, the goat antirabbit IgG conjugated with fluorescein isothiocyanate was added, and incubation was continued for another hour. Slides were analyzed using a Nikon TE-300 convolution fluorescent microscope (Nikon, Inc., Melville, NY, USA).

Immunohistochemistry

Immunohistochemistry was performed with formalin-fixed, paraffin-embedded tissue sectioned at a 5-μm thickness. The slide tissue microarrays of normal brain and different stages of glioma were purchased from US Biomax, Inc. (Rockville, MD, USA). After blocking with 5% normal horse serum, the slides were incubated overnight with polyclonal primary antibody (anti-α9 or anti–von Willebrand factor), and biotinylated secondary antibody was added. The color was developed by ABC kit (Vector Laboratories, Burlingame, CA, USA). Images were analyzed using an Olympus AX70 light microscope (Olympus America, Inc., Center Valley, PA, USA) with 400× magnification, by three investigators, including one who was blinded for experimental protocol. Analysis was performed on at least three different microscopic observation fields for each tissue sample.

Chemotaxis Experiments

Chemotaxis experiments were performed in a Boyden chamber using 3-μm thick membranes (HTS FluoroBlok inserts; BD Falcon) as described previously.[19]

Cell Proliferation Assay

Cell proliferation assay was performed using a 5-bromodeoxyuridine (BrdUrd) kit according to the manufacturer's instructions (Roche, Mannheim, Germany).

RNA silencing of α9 in LN229 cells was performed with serum-free media and by transfection with siRNA (0.1 μM; no. 16708, Ambion, Austin, TX, USA) using Lipofectamine (Invitrogen) in Opti-MEM media. After 72 h of transfection, media were changed, and the BrdUrd proliferation assay was performed.

Detection of Cell Apoptosis

Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay was performed with an in situ cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Caspase 8, 9, and 3/7 assays were performed with the CaspaTag In Situ Assay Kit (Chemicon) according to the manufacturer's instructions.

Tumor Growth in Quail Embryonic Chorioallantoic Membrane (CAM) System

An assay of tumor growth in the quail embryonic CAM system was developed based on the angiogenesis assay described earlier.[35] Briefly, fertilized Japanese quail (*Coturnix coturnix japonica*) eggs were purchased from Boyd's Bird Co. (Pullman, WA, USA). Eggs were cleaned with ethanol and maintained at 37°C until embryonic day 3. The shells were then opened with a razor blade and sterile scissors and the contents transferred into 6-well tissue culture plates and returned to the 37°C incubator. At embryonic day 6, LN229 glioma cells (1×10^7/50 μl) were applied on the top of the CAM and allowed to grow for 24 h. Embryos were divided into experimental groups, each containing at least 10 animals, and VLO5 (20 μg) and/or NGF (100 ng) in 50 μl of PBS were applied on the top of the tumor every day. The control group received a vehicle (PBS) treatment. The experiment was performed until day 12, and then the embryo was fixed with 5 ml of prewarmed gluteraldehyde (2%) and paraformaldehyde (4%) in PBS for 48 h at room temperature. The membranes containing the tumor were dissected from the embryo and transferred onto the glass slide. Tumors were carefully cut out of the membrane and weighed.

Table 1. Comparison of expression of the α9 integrin subunit on different grades of human glioma and normal brain tissues by immunostaining of paraffin sections, as percentages of positively stained astrocytic-like cells per total number of cases in each group

		Grade of Glioma		
Stage of Expression[a]	Normal Brain (n[b] = 20)	II (n = 46)	III (n = 43)	IV (n = 61)
A	100%[c] (20 cases)	28.3% (13 cases)	22.2% (10 cases)	8.2% (5 cases)
B	0% (0 cases)	32.6% (15 cases)	26.7% (12 cases)	22.9% (14 cases)
C	0% (0 cases)	23.9% (11 cases)	24.4% (11 cases)	32.8% (20 cases)
D	0% (0 cases)	15.2% (7 cases)	22.2% (10 cases)	36.1% (22 cases)

[a]Stages of expression of α9 integrin subunits were evaluated according to the percentage of astrocytic-like cell staining using the following scale: A, 0% stained cells; B, 0%–30% stained cells; C, 30%–70% stained cells; D, 70%–100% stained cells.

[b]*n* represents total number of cases investigated per grade of glioma or normal brain tissue.

[c]Data represent mean from analysis of at least three microscopic observation fields per sample performed by three investigators.

Results

Evaluation of Expression Level of a9 Integrin Subunit and NGF on Gliomas of Different Grades

Immunohistostaining of paraffin sections revealed that the intensity of α9β1 integrin expression on astrocyte-like cells is strongly associated with increased grades of glioma (Table 1, Fig. 1A). We investigated 20 cases of normal brain tissue that were randomly obtained from the biopsy and autopsy of nonglioma patients as well as from normal brain white matter adjacent to the tumor. In all cases (100%), normal tissue showed no expression of this integrin on the astrocytes, oligodendrocytes, and neurons. Positive staining in normal brain was observed only for endothelial cells of vasculature, which is in agreement with data recently published by us.[19] The percentage of positively labeled anti-α9 antibody increased in correlation with glioma grade and was the highest in GBM tissue. Although all stages of expression were present for all grades of glioma, the highest percentage negative expression for anti-α9 antibody tissues (stage A) was observed for grade II glioma (28.3%), followed by grade III (22.2%) and IV (8.2%) gliomas. The reverse tendency occurred for stage D, which showed a more than twofold higher percentage of strongly labeled cells for glioblastoma than for diffused astrocytoma. The moderate expression of α9β1 was found in anaplastic

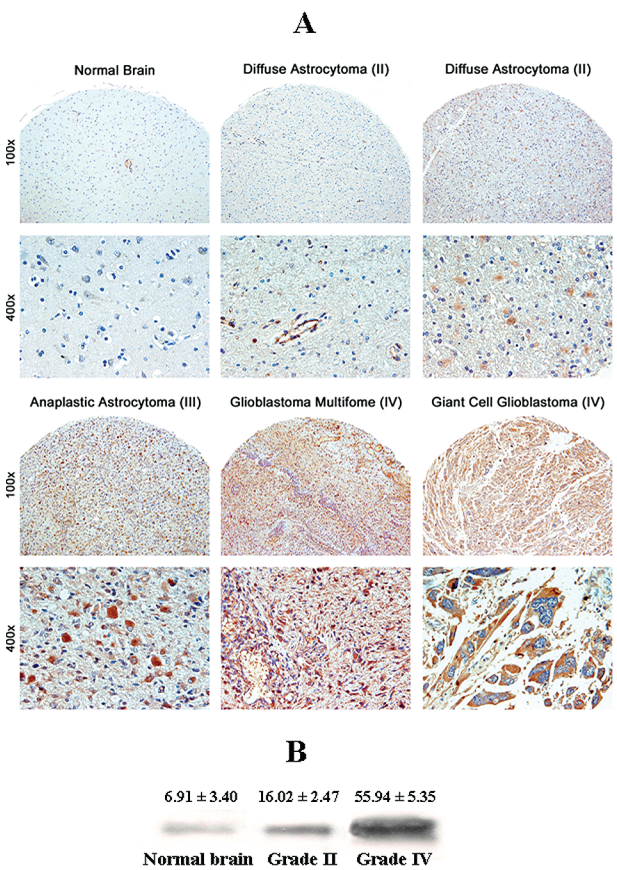


Fig. 1. (A) Representative images of immunohistochemical analysis of normal brain and different grades of gliomas stained with anti-α9 polyclonal antibody. The tissue array plates were blocked with 5% normal horse serum and incubated with a primary antibody overnight at room temperature in a humidified chamber. Biotinylated antirabbit immunoglobulin G was added and incubated for 1 h. Color was developed by incubation with avidin-biotin complex and diaminobenzidine. Images were analyzed using an Olympus AX70 light microscope with 100× and 400× magnification. (B) Comparison of expression of α9β1 integrin in normal brain and different grades of glioma tissues by Western blot analysis. Frozen samples of human tissues were obtained from patients' surgical resection. Tissues were homogenized in a lysis buffer–containing cocktail of protease inhibitors, and soluble fractions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis with a total amount of 20 μg of protein per sample. After electrophoresis, proteins were transferred onto the polyvinylidene fluoride membrane and incubated first with primary anti-α9 polyclonal antibody, and then horseradish peroxidase–conjugated goat anti-rabbit secondary antibody. The bands were visualized using a chemiluminescent Western detection kit. The numbers above the bands represent value of average pixels, reflecting the intensity of the bands, which were digitalized using Un-Scan-It gel software (Silk Scientific, Inc., Orem, UT, USA). Error values represent SD for normal brain (n = 5), grade II glioma (n = 3), and grade IV glioma (n = 7).

astrocytoma, and this grade of glioma had the intermediate percentages between grades II and IV for each stage of expression (Table 1). Interestingly, a very high level of α9β1 integrin was found on giant-cell glioblastoma (Fig. 1A).

Immunohistochemistry results were confirmed by Western blot analysis of tissue lysates obtained from the surgery of glioma patients (Fig. 1B). The intensity of bands related to the α9 subunit of α9β1 integrin was the highest for the most malignant glioma, whereas the lowest was observed for the normal brain white matter samples. Based on the immunohistochemistry, the presence of α9β1 in the normal brain tissue lysates is related to its expression on the microvascular endothelial cells.

Identification of α9β1 Integrin, TrkA, and p75^{NTR} on Glioblastoma Cell Lines

Glioma malignancy is dependent on the integrins expressed on the cancer cell surface because they are responsible for the regulation of the diffusive and proliferative properties of gliomas. To evaluate the role of α9β1 integrin interaction with NGF in glioma progression we selected the two most malignant glioblastoma cell lines: LN229, which expresses α9β1, and LN18, which does not. Presence of the α9 integrin subunit on LN229 and absence on LN18 cell lines were verified using several assays such as monoclonal antibody adhesion microarray (Fig. 2A), Western blot analysis of cell lysates (Fig. 2B), flow cytometry (Fig. 2C), and immuno-

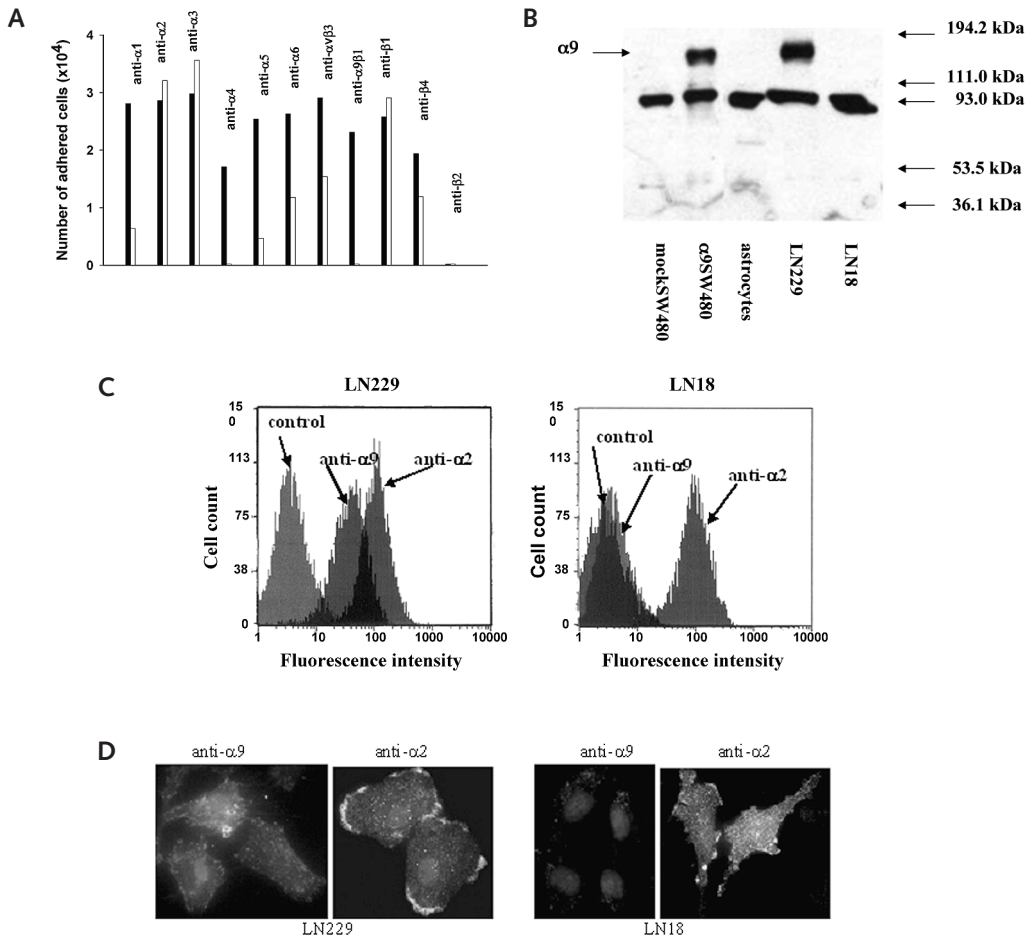


Fig. 2. Identification of integrins on human glioblastoma cell lines. (A) Monoclonal antibodies against integrins were immobilized overnight at 4°C on a 96-well plate in phosphate-buffered saline. LN18 (light bars) and LN229 (dark bars) cell lines were labeled with 5-chloromethylfluorescein diacetate and added (1×10^5/100 μl) to the wells, previously blocked by bovine serum albumin. Incubation was performed at 37°C for 30 min in Hank's balanced salt solution buffer containing calcium and magnesium. After washing the unbound cells, Triton X-100 was added to the wells, and the plate was read using a fluorescence microplate reader at an excitation wavelength of 485 nm using a 530-nm emission filter. The number of adhered cells was calculated from the standard curve prepared in parallel on the same plate from a known number of cells. The data represent the mean from three independent, duplicated experiments. (B) Western blot analysis of cell line lysates with polyclonal anti-α9 antibody. The molecular weight markers are indicated by arrows. (C) Immunocytostaining of glioblastoma cell lines by polyclonal antibody against α9 and monoclonal anti-α2 antibody (clone P1E6). Images of cells were analyzed in the presence of 4′,6-diamidino-2-phenylindole using a fluorescent convolution microscope (Nikon TE-300) with 400× magnification. (D) Expression of α9β1 and α2β1 integrins analyzed by flow cytometry in LN229 and LN18 glioblastoma cell lines. Cells were stained with Y9A2 or P1E6 monoclonal antibodies against α9 and α2 integrin subunits, respectively, and analyzed using the Guava EasyCyte flow cytometry system (Guava Technologies, Hayward, CA, USA).

cytostaining (Fig. 2D). Very high expression of the α9 integrin subunit was observed only on the LN229 cell line. Interestingly, Western blot analysis using polyclonal anti-α9 antibody confirmed our immunohistochemistry studies showing the absence of this integrin subunit on normal astrocytes (Fig. 2B). In this assay, SW480 human colon carcinoma cell line transfected with α9 subunit was used as a positive control, and mock-transfected SW480 cell lysate was used as a negative control. Further, expression of α9β1 integrin on LN229 and lack of it on LN18 was confirmed by flow cytometry and immunocytostaining analysis (Fig. 2C, D). We selected the α2 integrin subunit in both assays as a positive staining for integrin present in all astrocytic cell lines and normal astrocytes (data not shown).

The investigation of cellular responses following binding of NGF to α9β1 integrin on LN229 and LN18 cells required evaluation of the level of two other NGF receptors, TrkA and $p75^{NTR}$. Western blot analysis of cell lysates and immunocytostaining revealed negligible expression of TrkA on both types of cells, and a stable expression of $p75^{NTR}$ in comparison with PC12 neuronal cells used as a positive control (Fig. 3). Interestingly, the level of $p75^{NTR}$ appeared to be lower on LN18 cells, which are α9β1 integrin deficient, than on LN229 cells, which are α9β1 integrin positive. This correlation was previously observed in the mRNA level of SW480 cells transfected with α9 integrin subunit.[21] Cells transfected with the α9 subunit showed a potent increase of $p75^{NTR}$, whereas mock-transfected cells had a significantly lower expression of this NGF receptor. The opposite situation occurred for TrkA, suggesting that this specific high-affinity NGF receptor has a functional complementary association with α9β1 integrin.[21]

Estimation of NGF Contents in Human Tissue Lysates and Proteins Secreted by LN229 Glioma Cells

The presence of NGF in normal brain and glioblastoma tissue lysates, as well as in proteins secreted by LN229 cells, was evaluated using direct ELISA. The amount of NGF per milligram protein present in tissue lysate of normal brain white matter was estimated to be 0.59 ± 0.08 ng ($n = 5$). The level of this growth factor potently increased in the most malignant glioma tissue lysate up to 1.83 ± 0.41 ng ($n = 7$). This approximately threefold increase of NGF content in cancer tissue indicates its possible role in the development of brain malignancy. The higher concentrations in cancer tissue strongly suggested that tumoral cells are the source of this additional NGF. Thus, the presence of this growth factor was investigated in the serum-free media harvested from

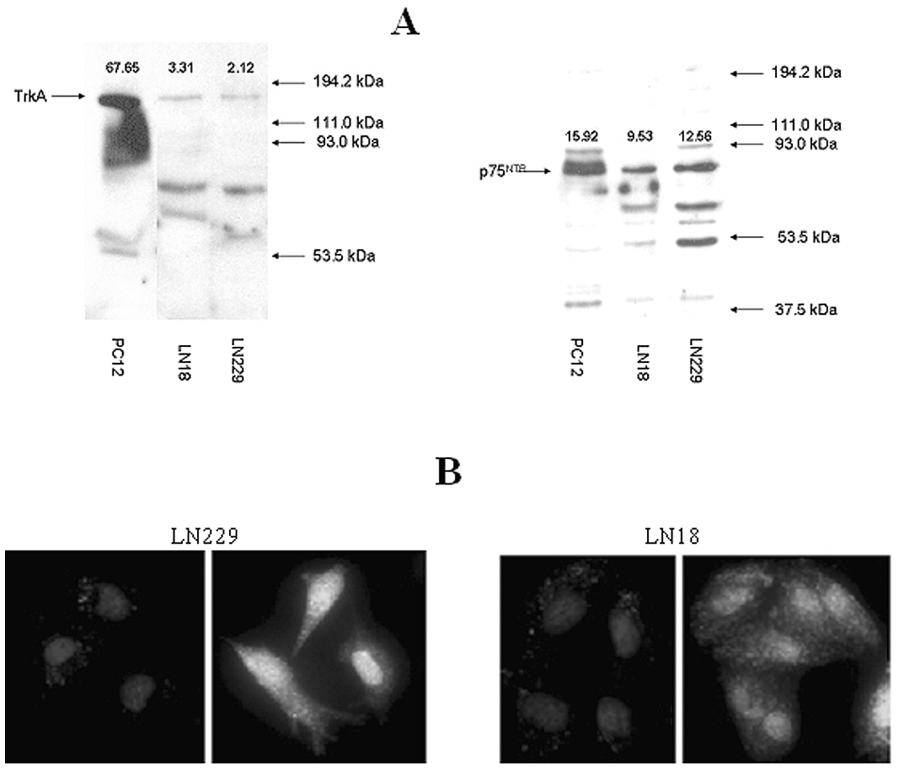


Fig. 3. Identification of TrkA and $p75^{NTR}$ on glioblastoma cell lines. (A) Western blot analysis of cell line lysates using polyclonal serum against TrkA (left panel) and polyclonal antibody against $p75^{NTR}$ (right panel). The numbers above the bands represent the values of average pixels, reflecting the intensity of the bands, which were digitalized using Un-Scan-It gel software (Silk Scientific, Inc., Orem, UT, USA). (B) Immunocytostaining of LN229 and LN18 glioblastoma cell lines with polyclonal anti-TrkA and anti-$p75^{NTR}$. Images of cells were analyzed using a fluorescent convolution microscope (Nikon TE-300) with 400× magnification. Left images show staining with anti-TrkA, whereas right images show staining with anti-$p75^{NTR}$ polyclonal primary antibodies.

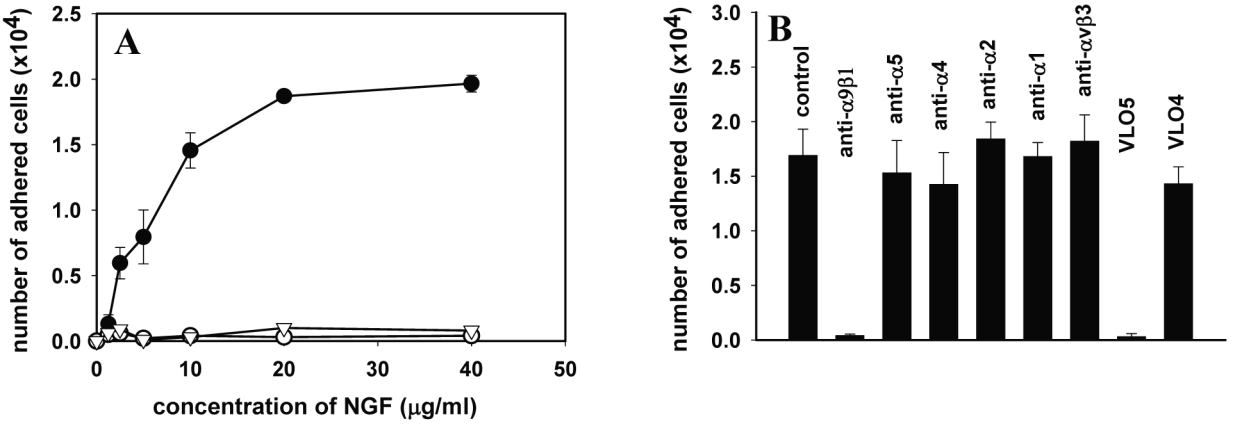


Fig. 4. Interaction of normal human astrocytes and glioblastoma cell lines with nerve growth factor (NGF) in adhesion assay and effect of integrin antibodies and disintegrins on adhesion of LN229 cell line to immobilized NGF. (A) Adhesion of normal primary human astrocytes (triangles) and LN18 (open circles) and LN229 (filled circles) glioblastoma cell lines to immobilized mouse NGF (mNGF). Different concentrations of mNGF were immobilized on a 96-well, and adhesion of 5-chloromethylfluorescein diacetate (CMFDA)–labeled cell lines was performed as described in the Fig. 2 caption. Error bars represent SD from three independent duplicated experiments. (B) Effect of monoclonal antibodies against various integrins and snake venom disintegrins on the adhesion of LN229 cell line to immobilized mNGF. mNGF (10 μg/ml) was immobilized on a 96-well plate, and CMFDA-labeled cells were added to the wells in the absence or presence of blocking integrin antibodies (10 μg/ml) or disintegrins (1 μM). Error bars represent SD from three independent duplicated experiments.

the LN229 cells after 72-h incubation. ELISA screening of different amounts of total protein in media showed no detectable level of immobilized NGF. However, after gel filtration, media proteins were divided into three major fractions according to their molecular weight. Applications of proteins obtained after gel filtration on ELISA showed the presence of NGF in the third fraction, which had the longest elution time from the column, and proteins with low molecular weight. NGF as a dimer has a molecular weight of only 26 kDa, which is consistent with the observed elution time. The calculation of NGF content per milligram of protein present in the media was 15.5 ± 2.3 ng.

Interaction of NGF with LN229 Cells is α9β1 Integrin Dependent

NGF was previously characterized by us as a low-affinity ligand for α9β1 integrin in an adhesion assay with the SW480 cell line transfected with α9 integrin and with purified receptor in ELISA.[21] In this article, we present the effect of this interaction on the progression of glioma. The specific cross-reactivity of NGF with α9β1 integrin was investigated in an adhesion assay of normal human astrocytes and two glioblastoma cell lines (Fig. 4A). Only LN229 cells bind to immobilized mouse NGF (mNGF) in a dose-dependent manner, whereas other cells that do not express this integrin showed no adhesion to mNGF. Adhesion of LN229 to this growth factor depends on α9β1 integrin, because the integrin was inhibited by the blocking anti-α9β1 monoclonal antibody Y9A2 and by the methionine-leucine-aspartic acid (MLD)-disintegrin VLO5 (Fig. 4B). The monoclonal antibodies against various other integrins were not able to inhibit that adhesion. Moreover, VLO4, an arginine-glycine-aspartic acid (RGD) disintegrin that inhibits RGD-dependent integrins such as α5β1 and αvβ3, was also not active as an inhibitor of adhesion to mNGF. Although LN229 and LN18 highly express another NGF receptor, $p75^{NTR}$ (Fig. 3), its interaction with mNGF in the adhesion assay appeared to be negligible because it is only a signaling receptor.

NGF Induces Migration of LN229 Cells

The promigratory activity of glioma cells is an important element in a tumor's invasion of healthy tissue in the brain. Integrin α9β1 promotes chemotaxis of LN229 cells by using its ligands as chemoattractants (Fig. 5). A significant increase of chemotaxis was observed for both VLO5 and Y9A2 as well as for mNGF when compared with random migration. Interestingly, chemotaxis of LN18 cells was observed above a random level only for FBS. NGF had no chemoattractive properties for this cell line, suggesting that neither TrkA nor $p75^{NTR}$ are receptors involved in NGF-induced cell migration. In this context, α9β1 looks to be a major promigratory receptor that is involved in NGF-induced glioma invasion.

NGF Induces α9β1-Integrin–Dependent Proliferation of LN229 Cells

The modulatory effect of mNGF on the proliferation of two of the most malignant glioma cell lines, LN229 and LN18, was tested in a BrdUrd assay (Fig. 6). This growth factor increased the proliferation ratio of LN229 cells, whereas a reverse effect was observed for LN18 cells (Fig. 6A). The pro-proliferative effect of NGF was dependent on α9β1 integrin, because silencing of the α9 subunit by siRNA totally inhibited NGF-induced pro-

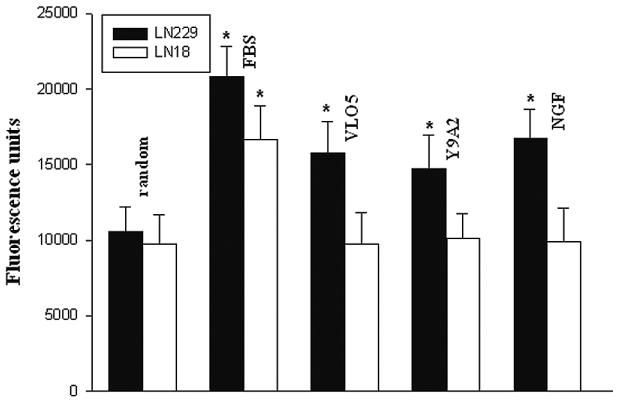
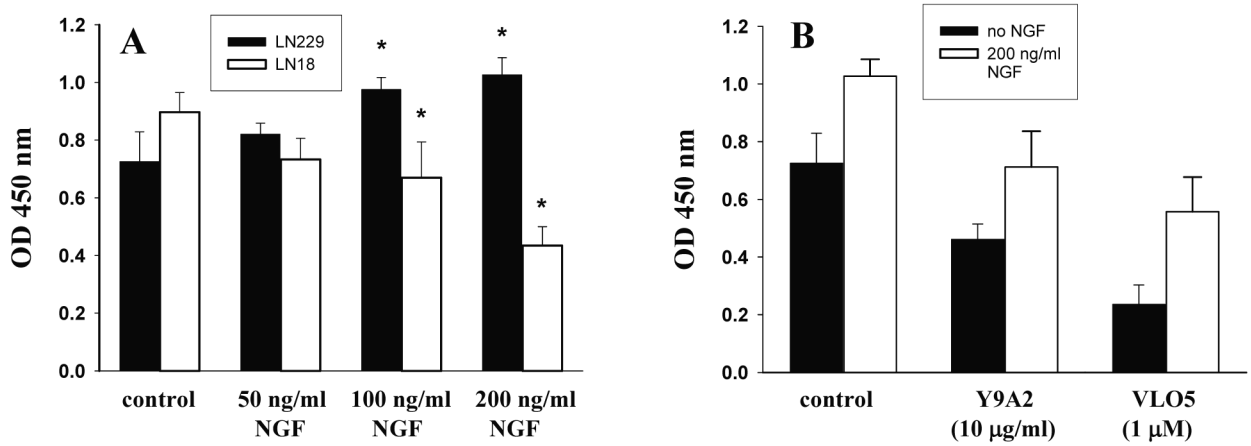


Fig. 5. Chemotaxis of astrocytoma cell lines induced by different chemoattractants. Experiment was performed using a Boyden chamber with fluoroblock membranes (3 μm). Cells were labeled with calcein in the culture by incubation for 1 h. After detaching and washing, cells were applied to the upper chamber in Dulbecco's modified Eagle's medium, whereas chemoattractants were applied to the lower chamber: 2% fetal bovine serum, VLO5 (1 μM), Y9A2 (10 μg/ml), NGF (1 μg/ml). Incubation was performed on a 24-well plate at 37°C for 1 h. Plate was read by a fluorescence plate reader with bottom reading option at an excitation wavelength of 485 nm using a 530 nm-emission filter. The asterisks indicate $p < 0.05$ in comparison with random migration, as statistically significant differences.



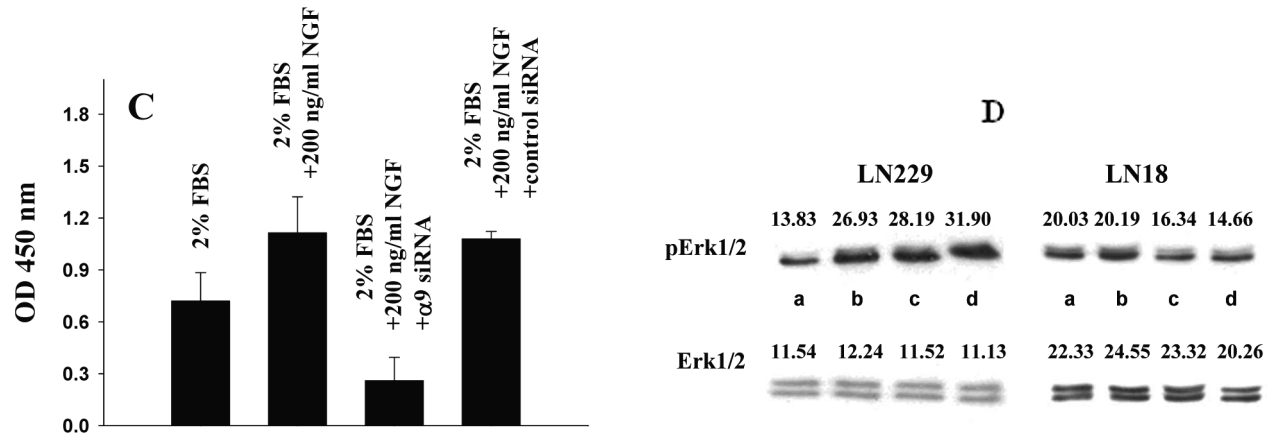


Fig. 6. Effect of nerve growth factor (NGF) on proliferation and signal transduction in glioblastoma cell lines. (A) Effect of different concentrations of mouse NGF (mNGF) on proliferation of LN229 and LN18 cell lines. The asterisks indicate $p < 0.05$ in comparison with the untreated mNGF control cells, as statistically significant differences. (B) Effect of α9β1 integrin inhibitors on proliferation of LN229 cell line stimulated or not with mNGF. (C) Effect of α9 gene silencing on proliferation of LN229 cell line. Cells were grown on a 96-well plate up to 70% confluence and then treated for 48 h with appropriate concentrations of mNGF in the absence or presence of Y9A2 or VLO5 in the medium containing 2% fetal bovine serum. α9 gene silencing was performed in LN229 cells using two duplexes of pre-design siRNA (Ambion, Inc., Austin, TX, USA). 5-bromodeoxyuridine color development assay was performed according to manufacturer's instructions (Roche). Error bars represent SD from triplicated experiments. (D) NGF-induced Erk1/2 phosphorylation in LN229 and LN18 cell lines. Cells were cultured to the confluence of about 90% on a 6-well plate and starved for 48 h. Cells were stimulated with NGF at concentrations of 100 ng/ml (line b), 200 ng/ml (line c), and 500 ng/ml (line d), or not (line a) and lysed for 30 min. Lysates were applied on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membrane, and incubated with primary anti-pErk or anti-Erk polyclonal antibodies. The bands were visualized using a chemiluminescent Western detection kit. The numbers above the bands represent values of average pixels, reflecting the intensity of the bands, digitalized using Un-Scan-It gel software (Silk Scientific, Inc., Orem, UT, USA).

liferation of LN229 cells even below a level of control induced by only 2% FBS (Fig. 6C). Moreover, the monoclonal antibody Y9A2 and the disintegrin VLO5 significantly inhibited FBS-induced proliferation, whereas NGF increased the proliferation of LN229 cells treated with these inhibitors (Fig. 6B). Therefore, NGF showed LN229 cell protecting ability by diminishing the inhibitory effect of α9β1 integrin antagonists. This conclusion is also supported by cell signaling experiments (Fig. 6D). NGF significantly induces prosurvival cell signaling pathway by the phosphorylation of MAPK Erk1/2 only in LN229 cells. No increasing effect on the activation of this pathway was observed in LN18 cells. A slight decrease in the phosphorylation level of Erk1/2 below the control was observed for higher concentrations of NGF, suggesting that this cell prosurvival signal may be affected by proapoptotic signals induced by $p75^{NTR}$, in the absence of prosurvival NGF receptors such as α9β1 integrin, and TrkA in the functionally active amount.

α9β1 Integrin Is Involved in Transferring Proapoptotic Signals

The mechanism of inhibitory effect of the snake venom disintegrin VLO5 on the proliferation of LN229 cells is dependent on its proapoptotic activity. Cells treated with VLO5 extensively changed morphology and detached from the wells (Fig. 7A). Addition of NGF significantly reduced VLO5's effect, suggesting its protective role on cell survival. The ability of VLO5 to induce apoptosis in LN229 cells was confirmed by TUNEL assay. Treatment of LN229 cells with 1 μM of VLO5 increased the percentage of positively stained apoptotic cells in the TUNEL assay proportionally to the incubation time (Fig. 7B). Addition of 10-ng/ml of mNGF reduced the number of apoptotic cells to the control levels tested in the presence of NGF alone and PBS. VLO5 induces apoptosis in a caspase-dependent manner. Activation of caspase 3/7 in LN229 cells occurred following their pretreatment with 1 μM of VLO5 in the presence of 2% FBS (Fig. 7C). However, a difference was observed between caspase 8 and 9 activation. An increase of caspase 9 activity and lack of any effect on caspase 8 indicate that the binding of VLO5 to α9β1 integrin induces apoptosis by the intrinsic pathway. Presence of NGF in the caspase assays significantly reduced the proapoptotic effect.

NGF Increases In Vivo Tumor Growth Induced by LN229 Cells

The investigation of tumor growth induced by LN229 cells was performed in vivo in a shell-less quail egg assay (Fig. 8). In this assay, tumoral cells were implanted on the top of CAM, and the developed cancer was directly treated with mNGF and VLO5. Tumor growth, evaluated by spreading area (Fig. 8A, left panels) and tumor weight (Fig. 8B), significantly increased following treatment with mNGF. Treatment with VLO5 decreased both parameters of tumor growth; however, addition of mNGF significantly elevated the ratio of tumor development. This is consistent with the rescue effect of NGF against VLO5 (Figs. 6 and 7). Interestingly, as is shown on fixed isolated CAMs (Fig. 5A, right panes), LN229 cells induced high vascularization of entire CAM, whereas VLO5 significantly decreased this proangiogenic effect.

Discussion

The identification of selectively expressed markers on tumor cells is one of the major goals of all laboratories that conduct research on designing cancer therapies that are effective and have no side effects. Integrins are the cell surface receptors that are considered attractive targets for cancer therapy because of their functional relevance for cell physiology. They may behave as structural receptors responsible for the formation of appropriate tissue structure and may also work as the signaling receptors activating internal cell pathways leading to the regulation of the cell proliferation, migration, and survival/death processes. Therefore, identification of the integrin that would be selectively expressed on the cells undergoing malignant transformation but not on the origin cells is an ideal systemic approach for cancer targeting. The antagonist of this integrin, which would generate a proapoptotic signal in cancer cells, will be an excellent pharmaceutical compound for cancer therapy. In this article, we present α9β1 integrin as a target for astrocyte-derived glioma cells and the snake venom disintegrin VLO5 as a biologically active, proapoptotic molecule that is an antagonist of this integrin. Moreover, we explain the role of α9β1 integrin in glioma malignancy, which, as a receptor for NGF, participates in increasing the diffusive properties, proliferation, and survival of cancer cells.

The majority of integrins that are expressed on malignant glioma cells are also expressed on normal origins, such as astrocytes for astrocytomas, although many of them undergo upregulation. In this context, α9β1 integrin appears to be an exception. It was not found on normal astrocytes, whereas its high expression was revealed on glioblastoma. Normal brain tissue was negative for staining with anti-α9 antibody in immunohistochemistry, whereas the level of its intensity in malignant cells was correlated with increased grades of glioma and was highest in GBM. Strong immunodetection of other integrins such as α2β1, α3β1, α5β1, α6β1, and αvβ3 has been described by Gingras et al.[9] and Mahesparan et al.[10] On the other hand, screening for the expression of integrin subunits in an mRNA array analysis of a subset of 11 glioblastomas showed significant upregulation of only the β8 subunit.[36] In the same article, the authors reported no expression of α2β1 integrin on 30 GBM cases, whereas at the protein level, this integrin was highly expressed in all gliomas. A similar situation occurred with α9β1 integrin, which was not detected on the mRNA level,[36] but showed a high presence on tissue glioblastoma tissues in the present study. This discrepancy indicates that genetic studies may sometimes generate different results than protein data.

High expression of α9β1 integrin on malignant gliomas suggests its importance for pathological

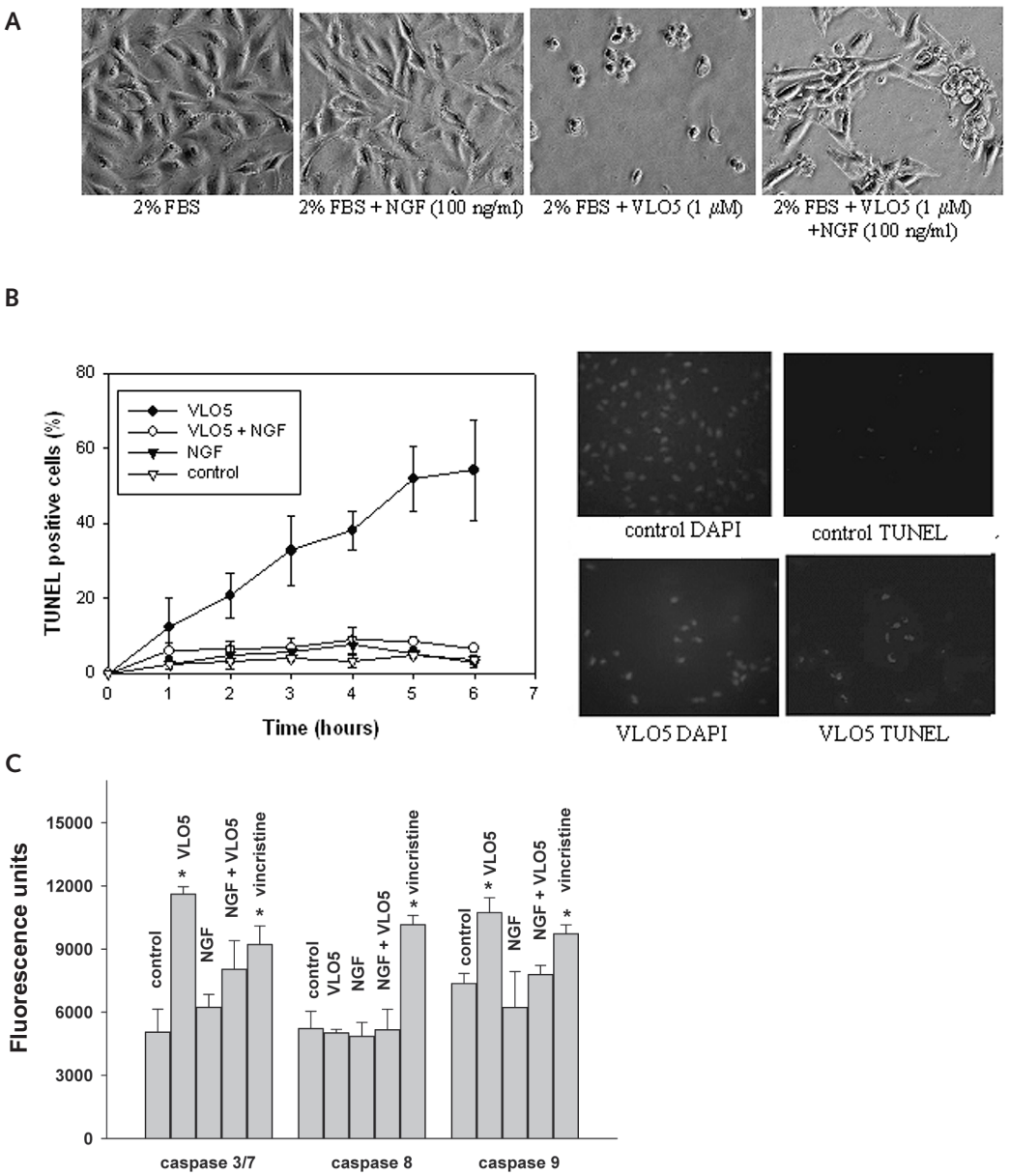


Fig. 7. Activity of VLO5 and nerve growth factor (NGF) in regulation of LN229 cell apoptosis and survival. (A) Effect of treatment of LN229 cells in the culture by NGF and VLO5. Cells were grown on a 6-well plate up to 90% confluence in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Treatment was performed in DMEM containing 2% FBS. After 24 h cells were analyzed under a contrast phase microscope (Nikon TE-300) using 400× magnification. (B) Time-dependent analysis of apoptosis in LN229 cells following treatment with VLO5 and NGF. Cells were grown on a 96-well plate up to 70% confluence, and after washing with serum-free DMEM were incubated with 1 μM VLO5 in the presence or absence of 100-ng/ml NGF in the media containing 2% FBS. Cells were fixed by 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate on ice for 2 min. Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) reaction mixture was added and incubated in the dark for 1 h at 37°C. Finally, the mounting buffer containing 4′,6-diamidino-2-phenylindole was added and slides were analyzed under fluorescence microscopy (Nikon TE-300). Error bars represent cell counts from three different observation fields under microscope, performed by three investigators blinded for experimental protocol. (C) VLO5-dependent activation of caspases in LN229 cells. The LN229 cells were grown in 6-well plates up to 70% confluence. The cells were treated in culture with VLO5 (1 μM), NGF (100 ng/ml), or both reagents together. Vincristine (50 μg/ml) was used as a control. Cells were detached and caspases 3/7, 8, and 9 were detected using CaspaTag In Situ Assay Kit (Chemicon). Error bars represent SD from three experiments. Asterisks show statistically significant differences ($p < 0.01$) according to control.

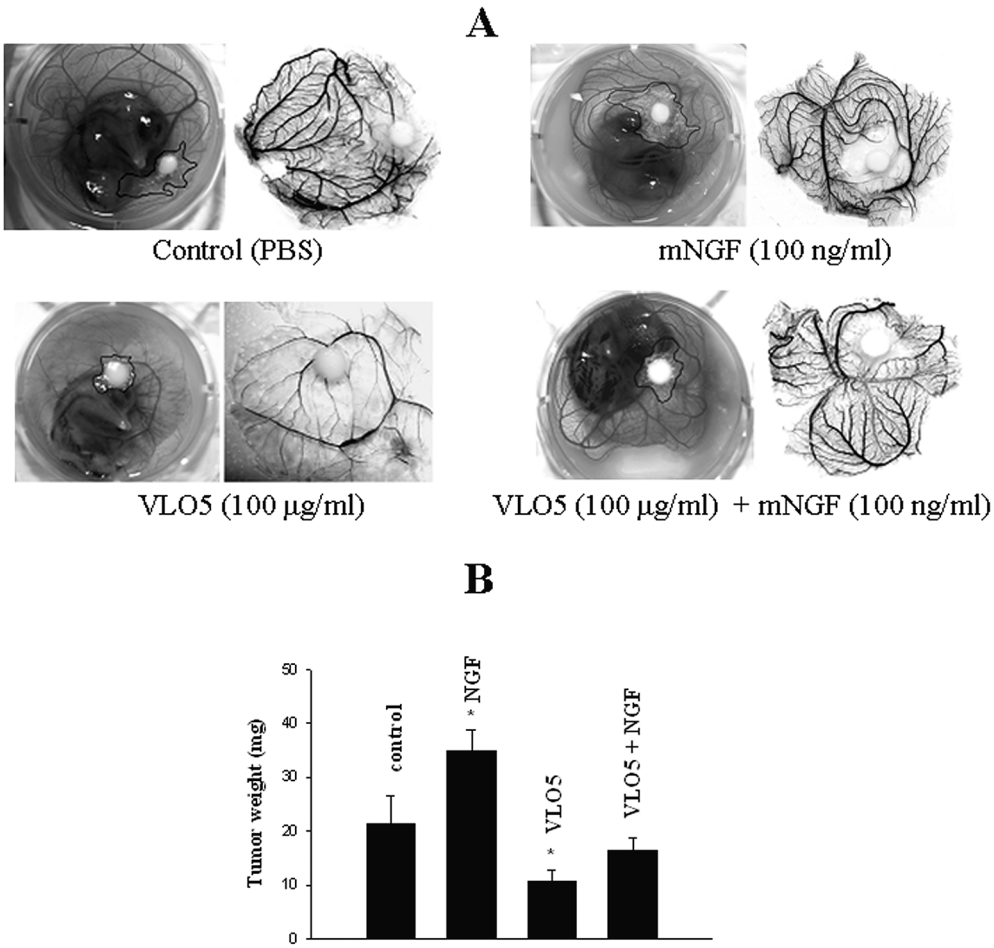


Fig. 8. Effect of mouse nerve growth factor (mNGF) and VLO5 on development of glioma tumor induced by implantation of LN229 cell line on quail chorioallantoic membrane (CAM). (A) Images of embryos with fully developed tumor on day 12 (left panels), and pictures of dissected, fixed CAMs with fully developed tumor on day 12 (right panels). (B) Comparison of weight of tumors treated and untreated with mNGF and VLO5. Embryos were grown on 6-well plates until day 7, and LN229 cells (2×10^7 per embryo per 50 μl) were placed on the top of the CAM. Tumors were allowed to grow for 24 h and then were treated with mNGF and/or VLO5 every day until day 12 by direct topping. Control embryos received vehicle treatment (50 μl phosphate-buffered saline [PBS]). On day 12 embryos were fixed and CAMs containing tumor were dissected. Tumors were dissected from the CAMs and weighed. The areas of the tumors' growth on the CAMs are framed on the embryos' pictures. The asterisks indicate $p < 0.01$ in comparison with control PBS-treated embryos, as statistically significant differences.

tumor progression and possibly tumor spreading to nonaffected areas of brain. This integrin was characterized as a multifunctional receptor interacting with several endogenous ligands. Interestingly, some of them are upregulated in tumor tissue, and their secretion by malignant cells has been observed as a significant contributor to tumor growth. These ligands include three extracellular matrix proteins such as tenascin-C, osteopontin, and thrombospondin-1. In this study, we also found that NGF is upregulated in the glioma tissue by its secretion by malignant cells. It is possible that the interaction of tumor cells with these proteins occurs at least with α9β1 integrin, increasing their prosurvival and promigratory abilities. Recently, we observed significant α9β1-dependent induction of chemotactic activity of endothelial cells by TSP-1.[19] However, direct interaction of α9β1 integrin with NGF is the most interesting. We recently also found a direct interaction of NGF with α9β1 in adhesion assays of SW480 and K562 cell lines transfected with this integrin, as well as in ELISA with the purified receptor.[21] Therefore, α9β1 integrin is a third receptor for NGF that has a similar effect on cell physiology as the high-affinity receptor TrkA. However, α9β1 is a low-affinity receptor resembling $p75^{NTR}$ that in the absence of TrkA works like a death receptor. Moreover, α9β1 as a proadhesive receptor binds NGF in the immobilized form and may work as a signaling receptor by activating internal cell pathways. TrkA and $p75^{NTR}$ showed no proadhesive activity that was tested in an adhesion assay of immobilized NGF. Cells expressing a high level of TrkA, such as PC12, did not adhere to immobilized NGF (data not shown). On the other hand, adhesion of LN229 cells was α9β1 dependent. Thus, glioma cells expressing α9β1 integrin have a unique ability to inter-

act with NGF that may be assembled in the solid tissue of the brain. This property of α9β1 may increase the invasive and proliferative abilities of the malignant cells. Interestingly, α9β1 integrin appears to be a complementary receptor for TrkA. Increased expression of α9β1 usually decreases expression of TrkA. Transfection of the SW480 colon carcinoma cell line with the α9 integrin subunit almost eliminates TrkA from the cell surface, whereas in mock-transfected cells, the level of this receptor in comparison with wild-type cells was almost identical to the protein and mRNA levels.[21] Intriguingly, LN18 cell line implanted into nude mice formed small tumors that grew slowly, while LN229 cell line formed large, fast-growing tumors.[37] These results may lead to a conclusion that the lack of prosurvival and pro-proliferative NGF receptors on glioma cells decreases the cells' ability to grow. Moreover, our experiments showed that a higher concentration of NGF (200 ng/ml) inhibited the proliferation of LN18 cells, as well as LN229 cells silenced by the siRNA α9 integrin subunit, suggesting that in the absence of α9β1, NGF binds to another low-affinity receptor, $p75^{NTR}$, which can induce a proapoptotic signal. The concept that TrkA and α9β1 integrin may work in a complementary manner is supported by data obtained by Wadhwa et al.[38] They found that TrkA is overexpressed on low-grade (I and II) astrocytomas, whereas in the advanced malignant forms (grade IV), expression is very low. The expression pattern of α9β1 on glioma is opposed to TrkA (Table 1). On the other hand, NGF is secreted by the glioma cells and its modulatory effect on tumor growth in experimental models has been reported.[39] Based on this, we conclude that the expression of prosurvival and pro-proliferative NGF receptors, as well as NGF, is dynamically regulated in glioma tissue to obtain optimal conditions for intensive tumor growth and spreading. Further studies, especially using immunohistochemistry, are required to verify this conclusion by finding a correlation with the expression of TrkA and α9β1 and NGF in glioma tissues. Possible tumors that are limited in expression of these receptors should also have a diminished level of NGF.

Data presented in this article confirm that integrins may transfer prosurvival and pro-proliferative signals in the case of their binding to endogenous ligands such as extracellular matrix proteins or growth factors. However, under certain conditions they may act as death receptors. It is well known that integrins induce proapoptotic signals following the loss of cell contact (anoikis) with the ECM,[40] or attachment to soluble or degraded ECM.[41] This integrin-mediated death (IMD) was transferred through the extrinsic pathway, which involves activation of caspase 8. VLO5-mediated death was dependent on activation of caspase 9, suggesting the participation in this process by the intrinsic (mitochondrial) pathway. This is a new observation that may be explained by the type of integrin, which transfers an apoptotic signal. IMD was characteristic for αvβ3 and its RGD-containing ligands, whereas through the α9β1 integrin a death signal may be transferred differently. Very interesting is the protecting role of NGF on the VLO5-induced apoptosis and inhibition of tumor growth in vivo. This phenomenon appears to be associated with the competition of these two compounds to bind to α9β1 integrin and modulate the cell death/survival process. However, we cannot exclude induction of a prosurvival signal by the interaction of NGF with TrkA. Although we show that this high-affinity NGF receptor is expressed on LN229 and LN18 negligibly and its participation in stimulating proliferation in LN229 cells was excluded by siRNA experiments, the possibility exists that the binding of NGF even to the very low number of copies of TrkA is effective to induce prosurvival signals.

Acknowledgments

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