SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 4: 812-816, 2012

Comparison of L1 expression and secretion in glioblastoma and neuroblastoma cells

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Received March 16, 2012; Accepted June 27, 2012

DOI: 10.3892/ol.2012.787

Abstract. The expression of cell adhesion molecule L1 has been identified in a vast spectrum of tumors; however, its expression pattern with regard to tumor type is rarely discussed. In the present study, we studied L1 levels in human glioblastomas and neuroblastomas, and compared the expression and secretion of L1 in human glioblastoma U87-MG and neuroblastoma SK-N-SH cells. Immunofluorescence staining revealed different grades of L1 staining in human glioblastoma and neuroblastoma samples. In U87-MG cells, full-length L1 was weakly detected in cell lysates (CLs), while greater levels of abundant soluble L1 were confined in conditioned culture medium (CCM). In contrast, higher levels of full-length L1 were confined in SK-N-SH CLs, while almost no soluble forms of L1 were detected in CCM. Our data indicates various expression patterns of L1 in U87-MG and SK-N-SH cells, which may underlie the different malignancies of the two neural tumor types and further stress the importance of soluble L1-mediated signaling pathways in cell malignancy.

Introduction

The L1 cell adhesion molecule (L1CAM) is a transmembrane adhesion molecule of the immunoglobulin (Ig) superfamily (1), which plays a pivotal role in neural cell migration and survival, as well as neurite outgrowth, myelination and synaptic plasticity (2-5). Several studies have indicated that L1 also plays a significant role in tumor progression and metastatic behavior (6). The extracellular portion of L1 is composed of six Ig-like domains followed by fibronectin type III (FNIII) domains. The extracellular domain is joined by a short intracellular cytoplasmic domain (ICD) via a single transmembrane sequence (Fig. 1) (7). Various functions of L1 are largely dependent on the complex homophilic and heterophilic interactions of L1 extracellular domains and other CAMs on the cell surface (8).

Glioblastomas represent a critical medical challenge due to their highly invasive and metastatic characteristics (9-12). Neuroblastomas are a type of neuroendocrine tumor derived from any neural crest element of the sympathetic nervous system (SNS), including the adrenal glands. They may also originate from nerve tissues in the neck, chest, abdomen or pelvis (13). Neuroblastomas are genetic and morphological heterogeneous tumors with a variable clinical course, manifesting as the most common type of solid tumor in childhood, with unconventional clinical behavior (14). Although L1 has been detected in glioblastomas and neuroblastomas, accumulated evidence suggests that its roles in these tumors are markedly different. L1 levels in glioblastomas are higher in those with greater malignancy and L1 is associated with aggressive clinical behavior. Additionally, L1 may also be subjected to protease cleavage to release soluble L1 of different molecular weights ranging from 140-180 kD. Cleaved L1-mediated homophilic interactions may facilitate glioma cell adhesion, migration and metastasis to sites far from the tumor-originating sites. In contrast to that in human glioblastomas, an association of L1 positivity with greater event-free and overall survival has been observed in patients with neuroblastoma. L1 negativity is independently prognostic of event-free and overall survival, and predicts a good outcome in children with neuroblastoma (15,16). Thus, L1 may function differently in the two tumor types.

Based on these hypotheses, we examined L1 expression in human glioblastoma and neuroblastoma samples. We also studied L1 expression and secretion in glioblastoma and neuroblastoma cell lines. We identified that although a high level of L1 was detected in all human glioblastoma and neuroblastoma samples, their release level was markedly different. Neuroblastoma SK-N-SH cells contain more full-length membrane-tethered L1, while glioblastoma U87-MG cells contain more soluble L1 within the conditioned culture medium (CCM).

Materials and methods

Reagents and microarrays. Goat anti-human L1 antibody specifically targeting the extracellular domain of human L1 was purchased from R&D Systems (Minneapolis, MN,

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Key words: glioblastoma, neuroblastoma, L1 cell adhesion molecule (L1CAM), U87-MG glioblastoma cell line, SK-N-SH neuroblastoma cell line, tissue array

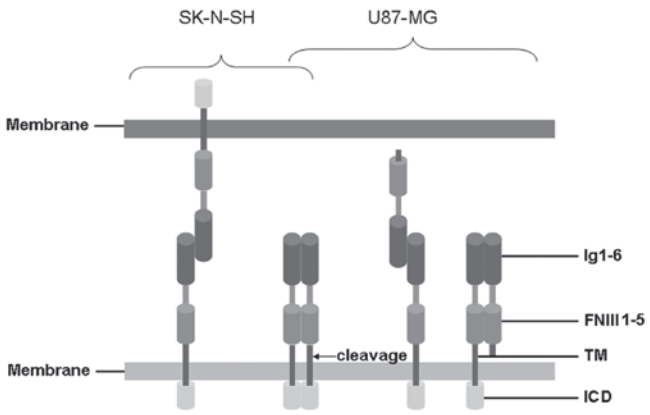

Figure 1. Schematic diagram demonstrating the homophilic and heterophilic interaction of L1 in U87-MG glioblastoma and SK-N-SH neuroblastoma cells. Ig, immunoglobulin; FN, fibronectin; TM, transmembrane protein; ICD, intracellular domain.

USA). Donkey anti-goat secondary antibody conjugated to Daylight™ 488 was obtained from The Jackson Laboratory (Bar Harbor, ME, USA). A human glioblastoma-containing tissue microarray and a neuroblastoma-containing tissue microarray were purchased from Chao Ying Biotechnology Co., Ltd. (GL 2083 and MC 803; Xi'an, Shaanxi, China) (17). The study was approved by the ethics committee of Shantou University Medical College, Guangdong, China.

Cell culture. Human glioblastoma U87-MG cells and neuroblastoma SK-N-SH cells were purchased from the Chinese Type Culture Collection (CTCC; Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Scientific Hyclone, Beijing, China) supplemented with 50 U/ml of penicillin/streptomycin mixture (Solarbio Science and Technology Co., Ltd., Beijing, China) and 10% fetal bovine serum (Sijiqing Biotechnology Co., Hangzhou, China). Cells were routinely grown in 75 cm^2 cell culture plates (Corning Inc., Corning, NY, USA) at 37˚C in a humidified 5% CO_2 atmosphere.

Immunofluorescence analysis. Paraffin-embedded 4 μm human glioblastoma and neuroblastoma tissues containing microarray sections were dewaxed, and antigen retrieval using 10 mM citrate buffer (pH 6.0) was conducted. Deparaffinized sections were rehydrated through a graded series of ethanol to phosphate-buffered saline (PBS; pH 7.4). Sections were briefly washed in PBS for 5 min each and blocked with 10% normal donkey serum in PBS at room temperature for 60 min. They were then subjected to incubation with goat anti-human L1 (1:200) primary antibody mixture. After three washes in PBS for 5 min, samples were incubated at room temperature with donkey anti-goat secondary antibody conjugated to Daylight 488 (1:500) for 90 min. Tissues were co-stained with 4',6-diamidino-2-phenylindole (DAPI) and mounted using anti-fade mounting solution (Beyotime Institution of Biotechnology, Jiangsu, China). Confocal images were obtained using the Olympus confocal system (FV-1000, Olympus, Japan). DAPI and Daylight 488 were excited at 405 and 488 nm, respectively.

Table I. Immunostaining intensity analysis of the cell adhesion molecule L1 in human glioblastoma and neuroblastoma tissues.

		L1 cell adhesion molecule				
Tumor type	n	-	+	++	+++	++++
Glioblastoma	46	0	0	3	5	38
Neuroblastoma	5	0	0	0	2	3

Evaluation of L1 immunostaining intensities in the whole field of each microarray set. Staining intensities were scored between 0 and 4 based on their immunofluorescence signal: 0 (-), no detectable signal; 1 (+), weak; 2 (++), intermediate; 3 (+++), strong; and 4 (++++), very strong.

Western blot analysis. Equivalent quantities of cell lysates (CLs) from U87-MG and SKN-SH cells or 60 μl of serum free CCM from the cells were heated to 95˚C in 20% sample loading buffer containing 0.125 M Tris-HCl (pH 6.8), 20% glycerol, 10% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue and 5% β-mercaptoethanol. They were then resolved by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto polyvinylidene difluoride membranes (PVDFs; Millipore, Billerica, MA, USA). Non-specific protein binding sites were blocked with 5% non-fat dry milk diluted in Tris-buffered saline (TBS; pH 7.4) buffer containing 0.05% Tween-20 (TBST). Membranes were incubated with the primary antibody for human L1 (1:500; R&D Systems) and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000; Beyotime Institution of Biotechnology) overnight at 4˚C. After three washes for 5 min each, horseradish peroxidase (HRP)-conjugated goat anti-mouse and rabbit anti-goat secondary antibodies (1:1000; Boster, Wuhan, China) diluted in TBST were applied. After three washes in TBST for 5 min each at room temperature, antigens were visualized using enhanced chemiluminescence (Beyotime Institution of Biotechnology). Signal intensity was quantified using Image Tool II software (Dental Diagnosis Science, San Antonio, TX, USA) by multiplying the average densitometry by the area indexed by the number of pixels (18,19).

Statistical analysis. Data were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS version 10.0 software (SPSS, Chicago, IL, USA). Data were analyzed using the independent samples Student's t-test and $P<0.05$ was used to indicate a statistically significant difference.

Results

Immunofluorescence staining of L1. Human glioblastoma and neuroblastoma tissues demonstrated extensive positive staining for L1. In one representative glioblastoma sample, extensive L1 staining was localized to glioblastoma cells and the extracellular matrix (Fig. 2). However, in another representative neuroblastoma sample, L1 was mainly localized at or around the cell membrane (Fig. 2). These observations suggested the

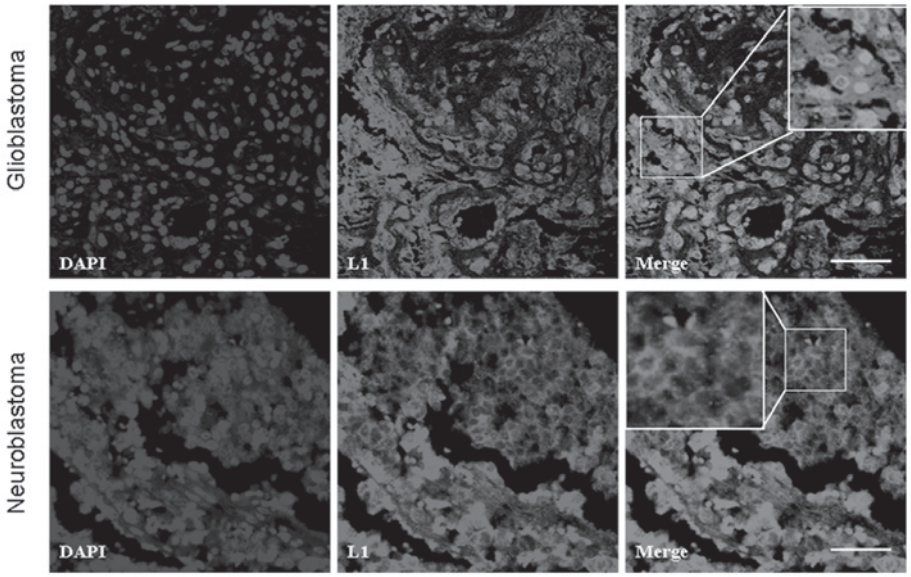

Figure 2. Representative immunofluorescence staining of L1 in human glioblastoma and neuroblastoma tissues. Human glioblastoma tissues demonstrated extensive positive staining for L1 in the glioblastoma cells and the cell matrix. Human neuroblastoma tissues demonstrated extensive positive staining for L1 at or around the cell membrane. Scale bars, 40 μm. DAPI, 4',6-diamidino-2-phenylindole.

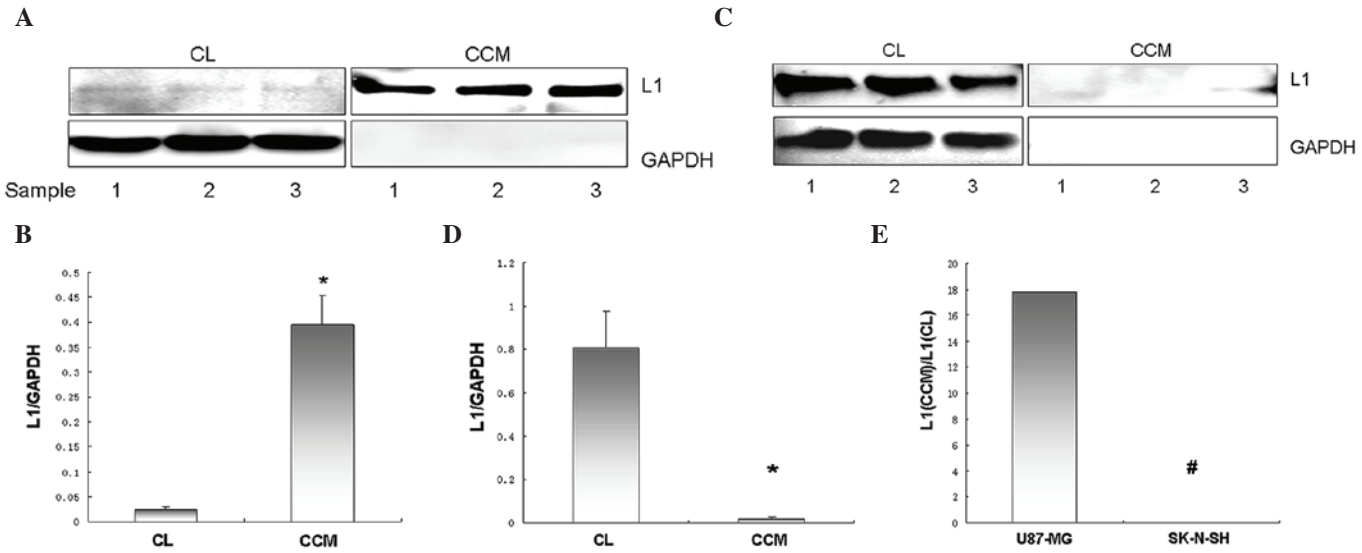

Figure 3. Western blot analysis of full-length L1 and released soluble L1 expression in glioblastoma U87-MG and neuroblastoma SK-N-SH cells. (A) In U87-MG cells, full-length L1 in the CL was weakly detected, while the level of soluble L1 in the CCM was markedly high. (B) Released soluble L1 levels were significantly higher compared to those of membrane-tethered full-length L1 in U87-MG cells. (C) In contrast, full-length L1 in the SK-N-SH CL was strongly detected, while soluble L1 in the CCM was almost undetectable. (D) Full-length L1 levels were significantly higher compared to those of released soluble L1 in SK-N-SH cells. (E) The ratio of soluble L1 to full-length L1 in U87-MG cells was significantly higher than that in SK-N-SH cells. GAPDH was used to indicate the equal loading volume and ensure no significant cell leaking into the CCM. *P<0.05; #P<0.01; n=3. CL, cell lysate; CCM, conditioned culture medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

differential localization pattern of L1 in glioblastomas and neuroblastomas.

Immunostaining intensities of L1 in glioblastoma and neuroblastoma samples. Positive immunostaining for L1 was detected in all 46 glioblastoma samples. Three, five and 38 samples demonstrated moderate, strong and very strong immunostaining, respectively. L1 was also detected in five neuroblastoma samples. Two samples demonstrated strong and three samples demonstrated very strong staining intensity (Table I).

Western blot analysis of full-length L1 and released soluble L1 expression in U87-MG and SK-N-SH cells. In U87-MG cells, full-length L1 in CLs were weakly detected, while the level of soluble L1 in the CCM was notably high (Fig. 3A).

Full-length L1 and soluble L1 levels derived from the same amount of U87-MG cells, as indexed by L1/GAPDH, were 0.023±0.006 and 0.394±0.06, respectively. Thus, released soluble L1 levels were significantly higher compared to membrane-tethered full-length L1 levels in U87-MG cells (P<0.01; Fig. 3B). In contrast, full-length L1 in SK-N-SH CLs was strongly detected, while soluble L1 in the CCM was almost undetectable (Fig. 3C). Full-length L1 and soluble L1 levels derived from the same amount of SK-N-SH cells, as indexed by L1/GAPDH, were 0.807±0.17 and 0.02±0.01, respectively. Thus, full-length L1 levels were significantly higher compared to released soluble L1 levels in SK-N-SH cells (P<0.05; Fig. 3D). The ratio of soluble L1 to full-length L1 in U87-MG cells (17.833:0.024) was significantly higher compared to that observed in SK-N-SH cells (0.024:0.008) (P<0.01; Fig. 3E). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to indicate equal loading volume and ensure no significant leaking of cell content into the CCM.

Discussion

Although it has been demonstrated that glioblastomas and neuroblastomas contain high levels of L1, their biological behaviors are distinct. In this study, we identified the different expression patterns of L1 between U87-MG and SK-N-SH cells, suggesting that this pattern may partially explain the heterogeneity of two tumor types in cell migration, metastasis and proliferation.

L1 stimulates glioblastoma cell motility (20), and promotes growth and survival of glioma stem cells in an autocrine/paracrine manner, which can be inhibited by siRNA and L1 ectodomain-binding antibodies (20,21). It has been identified that L1 is poorly expressed in gemistocytic astrocytomas, which are less prone to invading the brain parenchyma (22). Unlike previous results of neuroblastoma cells, cells in the invasive fronts of glioblastoma express more L1 and display greater invasive potential (23). The interaction of another important CAM member, NCAM, and integrin has been studied in neuroblastoma SK-N-5Y cells, where the NCAM and β1-integrin interaction contributes to cell differentiation and decreases malignancy (24). Thus, more full-length L1 may interact with molecules including integrin. However, in more malignant glioblastoma cells, native interaction of L1 with integrin was impaired, and the soluble L1-based signal responsible for cell proliferation and migration increased. Soluble L1 derived from the full-length cleavage in the third FNIII domain may disrupt its arginine-glycine-aspartic acid (RGD)-independent interaction with integrin through a sequence within this domain on the cell membrane. As a consequence, cell differentiation signaling was impaired. In contrast, dispersed soluble L1 may significantly increase the heterophilic interaction of L1 with integrin through the RGD sequence in the sixth Ig-like domain, and signaling responsible for metastasis and migration may be augmented. Transformation from the full length to the protease cleavage form of L1 may lead to the transition of cell differentiation to malignancy-related cell behaviours. L1 was identified to be preferentially and markedly expressed in glioma-related gliomatosis cerebri characterized by glial fibrillary acidic protein expression and cell invasion into preferentially the white matter (22). Thus, it was proposed that the cleaved L1 ectodomain and/or exosomal vesicles containing L1 support the extensive and diffuse migratory behavior of glioblastoma cells within the brain tissue, forming the basis of an autocrine/paracrine model for glioblastoma invasion (20). *In vivo* existence of soluble L1 allows the interaction of L1 with more diverse molecules on the surface of cells from the cleaving site (Fig. 1). By contrast, cell-cell adhesion through full-length L1-based homophilic interaction or L1/integrin heterophilic interaction should be the predominant form in SK-N-SH or other neuroblastoma cells, which are more prone to differentiate with less malignancy.

Based on our observations regarding the differential expression patterns of L1 between glioblastoma and neuroblastoma cells, we suggest that soluble L1 may contribute to malignancy-related cell migration and metastasis. It also suggests methods which may be able to interfere with glioma cell migration by targeting L1 and related molecules clinically.

Acknowledgements

The author is grateful for the support of the General Program of the National Natural Science Foundation of China (grant no. 81171138).

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