Invasive behaviour of glioblastoma cell lines is associated with altered organisation of the cadherin-catenin adhesion system

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Summary

As little is known about the role of cadherin-mediated cell-cell adhesion in astrocytes and its alteration in migrating and invasive glioblastomas, we investigated its molecular composition and organisation in primary cultured astrocytes and the T98G and U373MG glioblastoma cell lines. Biochemical and morphological analysis indicated that all three cell types express all of the structural components of the adhesion system, including the LIN-7 PDZ protein, a novel component involved in the organisation of the junctional domain in epithelia and neurons. However, only the astrocytes and T98G cells generated and maintained mature adhesive junctional domains to which LIN-7 was recruited. Alterations in the junctional domain of U373MG cells were associated with higher motility in a poly-L-lysine migration assay. When the T98G cells were cultured on Matrigel matrix, they acquired invasive properties but, despite unchanged cadherin adhesion system protein levels, the invasive T98G cell-cell contacts failed to accumulate LIN-7 and failed to mature. These results identify the LIN-7 PDZ protein as a marker of cell adhesion maturity and cell invasion and indicate that instability and disorganisation of cadherin-mediated junctions rather than reduced expression of cadherin-catenin system components are required to promote migration and invasiveness in glioblastoma cell lines.

Key words: Adherens junctions, Astrocytes, Migration, LIN-7 PDZ protein

Introduction

A number of intercellular junctions, including synapses and epithelial adherens junctions, are mediated by tissue-specific members of the cadherin family. Cadherins are transmembrane proteins whose extracellular domains promote calcium-dependent homotype adhesion between neighbouring cells, whereas their cytoplasmic regions are linked to the actin cytoskeleton by means of cytoplasmic proteins called catenins: α-catenin, β-catenin and γ-catenins (plakoglobins) (Aberle et al., 1996). The actin cytoskeleton appears to play a fundamental role in facilitating the formation and strengthening of these cell-cell contacts (Adams et al., 1996; Vasioukhin et al., 2000).

Cell-cell adhesion plays a central role in complex biological processes, including cell growth, proliferation, differentiation and survival. Alterations in epithelial cadherin (E-cadherin)-mediated cell-cell adhesion are associated with an increase in carcinoma cell motility, invasiveness and metastasis (Behrens et al., 1993). Conflicting results have been obtained concerning the involvement of neuronal N-cadherin in the acquisition of invasive properties. An increase in the expression of N-cadherin, or its exogenous expression, is associated with greater invasive potential in breast cancer cell lines (Hazan et al., 1997; Hazan et al., 2000), whereas no correlations have been found between N-cadherin expression and the invasive behaviour of astrocytomas or cell migration in a series of glioblastoma cell lines (Shinoura et al., 1995).

As the process of cell migration and invasion into surrounding tissue probably requires the coordinated and dynamic assembly and disassembly of cell-cell adhesions, the acquisition of invasive properties by tumour cells may depend on changes in the stability and organisation of cell-cell contacts rather than on modifications to the total expression of the molecular components of cell-cell adhesion. We therefore characterised the composition, maturity and organisation of the cadherin-mediated adhesion system in primary cultured astrocytes and in the T98G and U373MG glioblastoma cell lines, which come from highly invasive human tumours [grade III and IV according to the World Health Organisation (WHO), (Kleihues et al., 1993)] but have different migration and invasion capacities depending on the substrate in which they are cultured (Giese et al., 1994; Nakagawa et al., 1996; Belot et al., 2001). In this study, we tested the capacity of the glioblastoma cell lines to migrate on poly-L-lysine or invade a Matrigel matrix, and established a relationship between glioma cell aggressiveness and the degree of maturation and organisation of their cell-cell junctions.

The maturity of the junctional domain was evaluated on the basis of the reorganisation of the actin cortical cytoskeleton along the cell-cell contacts using morphological [filamentous (F) actin staining] and biochemical analysis [resistance of the E-cadherin–β-catenin complex to Triton X-100 (TX-100) extraction (Nathke et al., 1994; Adams et al., 1996)]. The

maturation of cadherin-mediated junctions is also accompanied by a reduction in tyrosine-phosphorylated junctional proteins (Daniel and Reynolds, 1997; Lampugnani et al., 1997), and so morphological and biochemical analyses were made to measure the phosphorylation level of the structural junctional components.

In order to characterise the process of maturation and organisation of cadherin-mediated cell-cell adhesion, we followed the junctional accumulation of the LIN-7 PDZ protein. Together with LIN-2/CASK and LIN-10/Mint/X11, this protein (also called Veli or MAL) forms a functional scaffold complex that is involved in the assembly of junctional components in neurons and epithelial cells (Borg et al., 1998; Butz et al., 1998; Kaech et al., 1998; Perego et al., 1999; Kamberov et al., 2000). The association of LIN-7 with the cadherin-catenin adhesion system is mediated by its physical interaction with the PDZ target sequence of β-catenin (Perego et al., 2000b). As the recruitment of LIN-7 in β-catenin-containing cell-cell contacts is a progressive process that is fully competed only in mature junctions, its accumulation was used to mark the degree of maturation of cadherin-mediated adhesion.

Materials and Methods

Primary astrocyte and glioblastoma cell line cultures

The cortical cells were prepared from 3-day-old Sprague Dawley rats as previously described (Perego et al., 2000a). The human T98G and U373MG cell lines (ATCC) are well characterised permanent glioblastoma cell lines derived from patients classified as having malignant anaplastic astrocytoma grade III-glioblastoma (U373MG) and glioblastoma multiforme (T98G), according to the WHO system (Kleihues et al., 1993). Cultured as suggested by the manufacturer's protocols, the cells were seeded under subconfluent (20×10^3) or confluent (20×10^4) conditions on glass coverslips or Petri dishes (35 mm) covered with 1 mg/ml poly-L-lysine (Sigma, St Louis, MO) or 20-50 μg/cm^2 Matrigel (Becton Dickinson, Bedford, MA).

Primary antibodies and immunocytochemistry

The polyclonal anti-α-catenin, monoclonal and polyclonal anti-β-catenin, monoclonal anti-Pan-cadherin (CH-19) and monoclonal anti-E-cadherin (Uvomorulin clone DECMA-1) came from Sigma. LIN-7 was detected using a specific rabbit polyclonal anti-peptide antibody (Perego et al., 2000b) or a rabbit polyclonal antiserum raised against amino acids 44-207 of mouse LIN-7A (Histidin-LIN7A fusion protein) that was recently generated in our laboratory. As similar results were obtained with both antibodies, all of the figures in this paper were obtained using the LIN-7 anti-peptide antibody. Fluorescein isothiocyanate (FITC)-labelled phalloidin (Sigma) was used to detect filamentous actin and mouse anti-phosphotyrosine antibody (Transduction Laboratories, Lexington, KY) to detect tyrosine-phosphorylated proteins.

The cells were fixed in 4% paraformaldehyde and permeabilised with 0.5% Triton X-100 (TX-100). Immunostaining with primary antibodies was followed by incubation with rhodamine-conjugated anti-rabbit, FITC-conjugated anti-mouse antibodies from Jackson Immunoresearch (West Grove, PA). The confocal images were obtained using a Bio-Rad MRC-1024 confocal microscope.

Scanning electron microscopy

Subconfluent T98G and U373MG cells grown on round coverslips coated with poly-L-lysine were fixed as a monolayer with 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.4 for two hours at 4°C, post-fixed in 1% OsO_4 in the same buffer, dehydrated using an ethanol series, and then bathed in hexamethyldisilazane (HMDS, Sigma) and allowed to dry overnight under the fume hood. The samples were mounted on stubs and gold coated (Balzers CED100) before being examined using a Philips SEM505 scanning electron microscope.

Transmission electron microscopy

Confluent T98G and U373MG cells were fixed in Petri dishes with 2.5% gluteraldehyde in 0.1M cacodylate buffer for 2 hours, post-fixed in 1% OsO_4 in the same buffer, stained en bloc using a saturated solution of uranyl acetate in 20% ethanol, dehydrated using an ethanol series and embedded in EPON 812 (Fluka, Buchs, Switzerland).

Ultrathin monolayer sections were obtained using an ultramicrotome Ultracut E (Reichert-Jung) equipped with a diamond knife (Diatome), counterstained with uranyl acetate and lead citrate and examined using a Philips CM10 transmission electron microscope.

Triton X-100 extraction and western blot analysis

Cells grown to confluence on Petri dishes were extracted using Triton X-100 as previously described (Perego et al., 2000b). The same volumes of the TX-soluble and TX-insoluble fractions were solubilised in solubilisation buffer (Perego et al., 1999), loaded onto 10% SDS-PAGE and immunostained with the indicated antibodies followed by anti-IgG or Protein A conjugated to peroxidase (Sigma). The immunoreactive bands were revealed using Supersignal West femto maximum sensitivity substrate, Pierce Chemical Co.).

Immunoprecipitation experiments

The brain and cell lysates were obtained as previously described (Perego et al., 2000b). The immunocomplexes were separated by SDS-PAGE and analysed by means of immunoblotting with the appropriate antibodies.

In order to measure the level of tyrosine phosphorylation, 5×10^5 cells were plated in 100 mm Petri dishes and cultured for 72 hours before cell lysis. In order to extract β-catenin efficiently and maintain the phosphorylated tyrosine residues, 0.02% SDS and 100 μM pervanadate were added to the lysis buffer.

Cell surface biotinylation

Cells grown to confluence on 100 mm Petri dishes were starved for 30 minutes in Dulbecco's modified Eagle's medium and biotinylated with NHS-ss-biotin (Pierce Chemical Co.) according to a previously published protocol (Sargiacomo et al., 1989). They were then lysed, and the biotinylated N-cadherin was recovered using 150 μl Ultra-link Streptavidin beads (Pierce Chemical Co.). The proteins were released from the beads by boiling the samples in SDS solubilisation buffer and then analyzed on 10% SDS-PAGE.

Affinity chromatography assay

Glioblastoma cell line or cortical astrocyte lysates were incubated with immobilised GST-mLIN-7A fusion protein (Perego et al., 1999). The bound material was resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with the β-catenin antibody.

Cell migration and invasion assay

Cell migration through poly-L-lysine-coated filters or cell invasion through Matrigel-coated filters was measured in a Boyden chamber (Albini et al., 1987), with 12 μm nuclepore polyvinylpyrrolidine (PVP)-free polycarbonate filters (Neuro Probe, Gaithersburg, MD) being coated on the side facing the cells with 200 μl of Matrigel (0.5

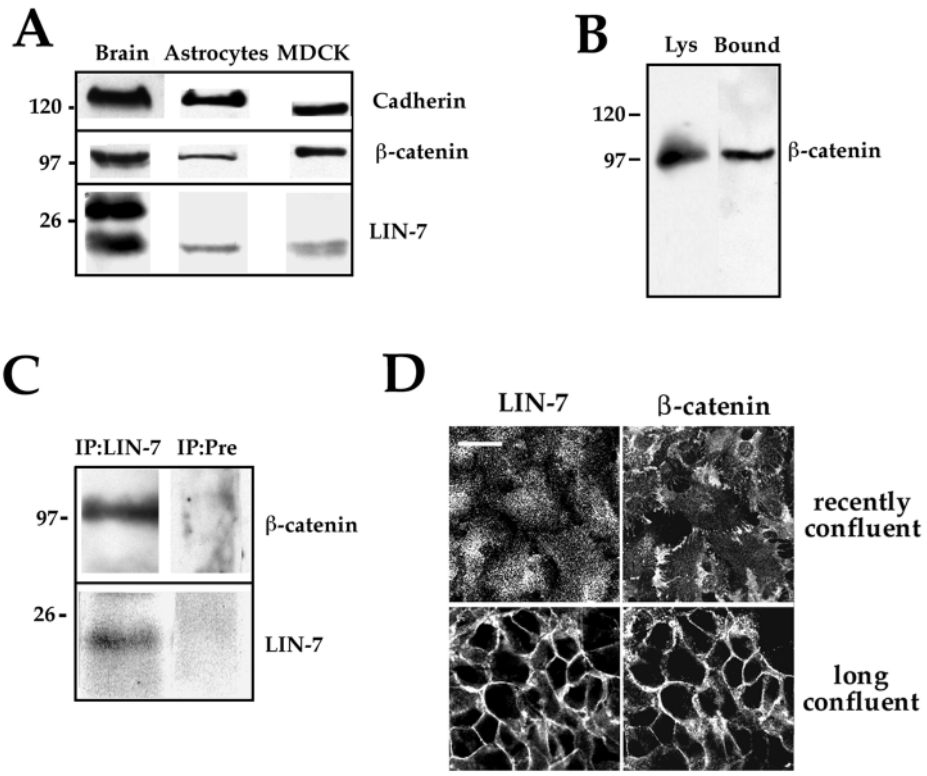

Fig. 1. Characterisation of the cadherin-mediated junctional complex in primary cultured cortical astrocytes. (A) Western blot analysis of the total expression of the components of the cadherin-based junctional system in brain, astrocytes and MDCK cells. 20 μg aliquots of homogenates were loaded onto 11% SDS-polyacrylamide gel and immunoblotted with the indicated antibodies. The MDCK cell homogenate was probed with the E-cadherin antibody, and the brain and astrocyte homogenate with the pan-cadherin antibody. (B) Affinity chromatography. The rat cortical astrocyte lysate was incubated with immobilised GST-LIN-7A fusion protein. The bound material (Bound) was resolved on 10% SDS-PAGE and immunostained for β-catenin. 10% of the total astrocyte lysate (Lys) used in the experiment was probed with the same antibody. (C) Co-immunoprecipitation of β-catenin with the LIN-7 antibody. The rat cortical astrocytes were extracted in lysis buffer and immunoprecipitated with the LIN-7 antibody (IP: LIN-7) or the pre-immune serum (IP: Pre). The immunoprecipitates were loaded onto 11% SDS-PAGE and immunoblotted with the β-catenin and LIN-7 antibodies. Molecular weight standards expressed in kDa are indicated on the left (A-C). (D) Confocal analysis of double immunofluorescence staining for LIN-7 and β-catenin in cultured primary astrocytes. The astrocytes were plated onto poly-L-lysine-coated glass coverslips, cultured until they reached confluence (recently confluent) or had been confluent for a longer period (long confluent) before fixation in 4% paraformaldehyde. Bar, 10 μm.

mg/ml) or 200 μl of poly-L-lysine (1 mg/ml). Conditioned media, obtained by incubating the T98G and U373MG cells for 48 hours in serum-free medium, were used as attractants in the bottom chamber (stimulated) (Yamamoto et al., 1997; Dai et al., 2001) and a serum-free medium containing 0.1% fatty acid-free bovine serum albumin (BSA) as a negative control (basal). Suspended in MEM containing 0.1% fatty acid-free BSA, the T98G and U373MG cells were added to the upper chamber at a density of 30×10^6cells/well. After 6 hours of incubation at 37°C, the non-migrated cells on the upper surface of the filter were removed by scraping. The cells that had migrated to the lower side of the filter were stained with Diff-quick stain (VWR Scientific Products, NJ), and five to eight unit fields per filter were counted using a microscope (Zeiss) at 160× magnification. The assays were run in triplicate.

Results

Biochemical and morphological characterisation of the adherens junctions in primary cultured astrocytes

The cadherin-catenin adhesion system has been extensively characterised in epithelia and neurons, but little is known about its molecular composition and organisation in astrocytes. We used immunoblotting to examine whether cultured cortical astrocytes express components of the cadherin-catenin complex. These components include the LIN-7 PDZ protein, a newly discovered component of the cadherin-based system that associates with the cadherin-catenin complex as a result of direct interaction with the C-terminal PDZ target motif of β-catenin in epithelia and neurons (Perego et al., 2000b). Fig. 1A shows the total expression of these components in homogenates derived from the brain, cultured cortical astrocytes and the Madin-Darby canine kidney (MDCK) epithelial cell line. As expected, a single band with an apparent molecular weight of ~130kDa corresponding to the electrophoretic mobility of N-cadherin was recognised by the Pan-cadherin antibody CH-19 (Sigma) in brain and astrocytes (Vázquez-Chona and Geisert, 1999), whereas E-cadherin staining (Uvomorulin clone DECMA-1, Sigma) was only observed in the MDCK cells. A band with an apparent molecular weight of ~100 kDa was revealed in all of the samples by the specific β-catenin antibody. The LIN-7 antibody recognised a band of ~23 kDa in all of the samples, whereas the ~30 kDa band corresponding to neuron-specific LIN-7 isoform(s) (Jo et al., 1999) was exclusively revealed in the brain homogenate.

To verify whether LIN-7 is a component of the cadherin-catenin complex in cultured astrocytes, as it is in neuron and epithelia, we tested the ability of a GST-mLIN-7A fusion protein to retain β-catenin from a lysate of cortical astrocytes. A fraction (3%) was specifically retained by the GST-mLIN-7A fusion protein (Fig. 1B) but not by immobilised GST (data not shown).

The in vivo association of LIN-7 with β-catenin was revealed by co-immunoprecipitation experiments (Fig. 1C). The polyclonal LIN-7 antibody (but not a pre-immune serum) co-immunoprecipitated β-catenin from the cultured astrocyte lysate.

The organisation of the cadherin adhesion system in astrocytes was investigated using confocal microscopy.

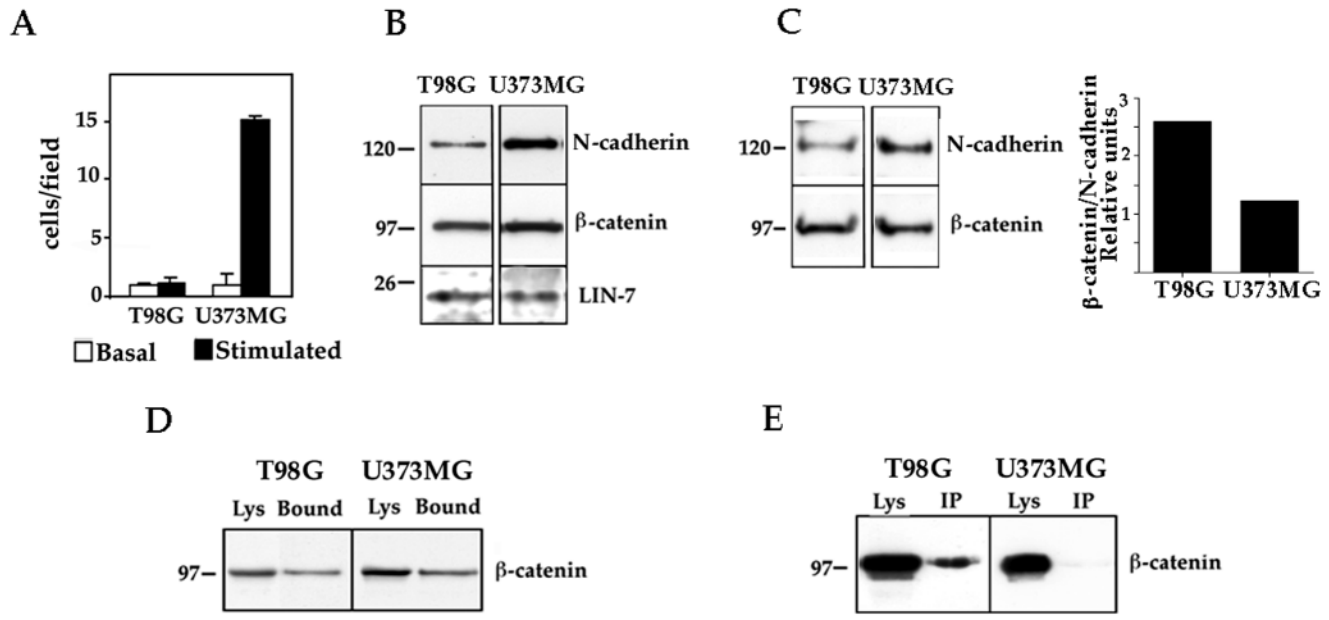

Fig. 2. Biochemical characterisation of the cadherin-mediated junctional complex in the T98G and U373MG glioblastoma cell lines. (A) In vitro cell migration assay of T98G and U373MG cells. Serum-free medium containing 0.1% BSA (basal) or conditioned media from the same cells (stimulated) were used as chemoattractants. The data are expressed as the mean number of migrating cells per field±s.d. for triplicate samples from a representative experiment. (B) Western blot analysis of total cadherin, β-catenin and LIN-7 expression in glioblastoma cell lines grown to confluence. 50 μg aliquots of total glioblastoma cell homogenates were loaded onto 11% SDS-PAGE and immunoblotted with the indicated antibodies. (C) Surface biotinylation assay. After being grown to confluence, the glioblastoma cell lines were surface biotinylated with NHS-ss-biotin. After cell lysis, the surface-biotinylated proteins were recovered using streptavidin beads, loaded onto 10% SDS-PAGE and immunoblotted with the indicated antibodies. The results were densitometrically analysed using the NIH Image 1.61 programme. The corresponding β-catenin/N-cadherin ratio (expressed as relative units) is shown on the right. (D) Affinity chromatography. Confluent cell lysates were incubated with immobilised GST-LIN-7A fusion protein: the bound material (Bound) was resolved by 10% SDS-PAGE and immunostained for β-catenin. (E) Co-immunoprecipitation of β-catenin with the LIN-7 antibody. Lysates of confluent cells were immunoprecipitated with the LIN-7 antiboby (IP), and the immunocomplexes were resolved by 11% SDS-PAGE and immunoprobed for β-catenin. 10% of the total T98G or U373MG lysate (Lys) used in the experiments was probed with the indicated antibody (D-E). Molecular weight standards expressed in kDa are indicated on the left (B-E).

Astrocyte cultures mainly contained type I glial fibrillary acidic protein-positive astrocytes (data not shown) that grew as a monolayer of flat epithelium-like cells when cultured to confluence (Perego et al., 2000a). In recently confluent astrocytes, the LIN-7 staining was predominantly cytosolic (Fig. 1D), whereas β-catenin and N-cadherin (data not shown) were enriched in irregular structures along the cell-cell contacts. The confluent astrocytes showed continuous adhesion throughout the cell-cell contact surfaces in which LIN-7 and β-catenin colocalised. LIN-7 can therefore be considered to be a marker of junctional maturity in astrocytes, as previously shown in MDCK cells and neurons (Perego et al., 2000b).

Biochemical and morphological characterisation of the adherens junctions in T98G and U373MG glioblastoma cell lines

In order to explore the role of cadherin-mediated cell-cell adhesion in astrocytes and determine whether its is affected in glial tumors, we analysed the expression of its components and its maturity and organisation in glioblastoma cell lines with different in vitro migration properties. U373MG and T98G cells come from highly invasive human tumours but, when we tested their ability to migrate and penetrate 12 μm porous Transwell filters coated with poly-L-lysine in a Boyden-modified chamber (Albini et al., 1987), we found that only U373MG permeated the poly-L-lysine barrier (Fig. 2A).

We then tested whether the motility of U373MG cells might be explained by the altered expression of the molecular components of the cadherin system. Immunoblot analysis of the cell homogenates revealed similar levels of β-catenin and LIN-7 in the T98G and U373MG cells, but the expression of total N-cadherin was lower in the former (Fig. 2B). No E-cadherin expression was detected by the specific antibody in either cell line (data not shown).

Given that only surface-expressed cadherin is involved in cell-cell contacts, we investigated the amount of functional cadherin in the glioblastomas by means of a surface biotinylation assay (Fig. 2C), which revealed a lower surface expression of cadherin in T98G cells. The N-cadherin–β-catenin association is maintained, as indicated by the presence of β-catenin in the streptavidin immunocomplex; however, the T98G cells contained a considerably higher amount of β-catenin associated with surface N-cadherin. As catenins are thought to participate in strengthening cell-cell adhesion by interacting with the actin cytoskeleton (Adams et al., 1996), an altered level of cadherin-associated β-catenin may indicate reduced adhesive strength. Alternatively, although undetectable by the available antibodies, the differences in β-catenin–N-cadherin stoichiometry might be explained by

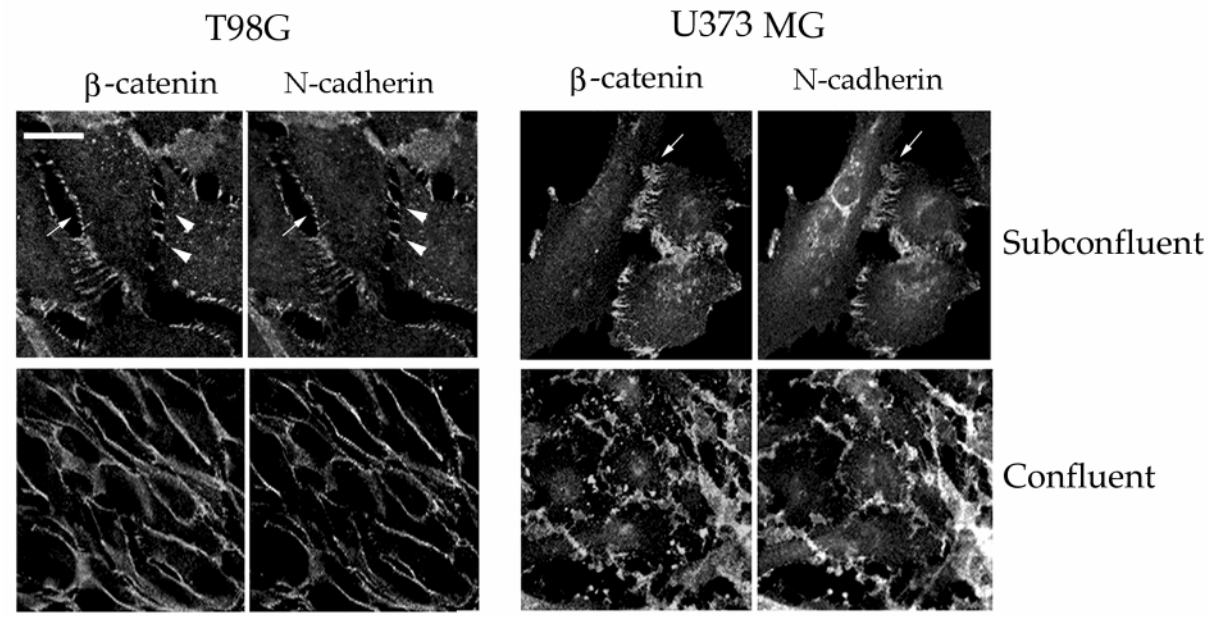

Fig. 3. Confocal analysis of β-catenin and N-cadherin distribution in subconfluent and confluent T98G and U373MG cells. The cells were plated onto poly-L-lysine-coated glass coverslips, fixed in 4% paraformaldehyde and immunostained. The arrows indicate β-catenin- and N-cadherin-enriched spots in the T98G cells and lamellae and lamellipodia-like structures connecting adjacent cells in the U373MG cells. The arrowheads indicate filopodia-connecting neighbouring cells. Bar, 10 μm.

interactions with other isoforms of cadherin (in T98G cells) or catenin (in U373MG cells).

We next tested whether LIN-7 formed a complex with the cadherin–β-catenin system in T98G and U373MG cells. Affinity chromatography experiments showed that β-catenin was specifically retained on GST-mLIN7A sepharose beads in both cell lines (Fig. 2D), thus suggesting that the β-catenin in T98G and U373MG cells is equally capable of binding to LIN-7. However, β-catenin was co-immunoprecipitated by LIN-7-specific antibodies only in the T98G cells (Fig. 2E), which excludes the possibility that the two proteins are truly associated in U373MG cells.

Taken together, the results of these experiments suggest an altered organisation of the cadherin-mediated system in migrating U373MG cells. In order to analyse the degree of maturation and organisation of cell-cell adhesion in the glioblastoma cell lines, we followed the morphological phases of cell-cell contact formation in cells grown at different densities and stained for N-cadherin and β-catenin. Confocal analysis showed colocalisation of β-catenin and N-cadherin on the cell surfaces of both cell lines, regardless of the cell density (Fig. 3). However, the morphology and genesis of the cell-cell contacts were different. In the subconfluent T98G cells, neighbouring cells appeared to be connected by filopodia enriched in β-catenin and cadherin (arrowheads); these cultures often contained neighbouring cells sporadically enriched in β-catenin and cadherin along their juxtaposed surfaces (arrows). In the confluent cell cultures, neighbouring cells were sealed together by continuous contacts along their entire surfaces, in which β-catenin and N-cadherin accumulated.

In the subconfluent U373MG cells, β-catenin and N-cadherin accumulated at high levels in lamellae and lamellipodia-like structures (arrows), connecting adjacent cells, and these markers remained irregularly distributed along the cell-cell contacts, even in the cells that had been confluent for a long time.

To investigate the detailed structure of the cell-cell contacts, we used scanning electron microscopy to analyse non-confluent T98G and U373MG cells (Fig. 4a,b). The T98G cells (a) grew flat on the substratum, were polygon-shaped and had many parallel, long and thin filopodial processes concentrated in the regions facing neighbouring cells (arrows), which they contact using the tips of the filopodia. The filopodia contained β-catenin and N-cadherin and seemed to be rich in actin filaments, as shown by FITC-phalloidin staining and transmission electron microscopy (data not shown). The U373MG cells (b) were irregularly shaped and had borders characterised by two main features: broad lamellae whose edges had lamellipodia-like structures (arrows) and sparse, thin and branched processes (arrowheads) making contacts with the substratum but without any significant cytoskeletal organisation (data not shown). The U373MG intercellular contacts appeared to be mediated by the lamellipodia.

The detailed ultrastructure of the contact sites in confluent cells was analysed using a transmission electron microscopy (Fig. 4c-f). Ultrathin sections cut perpendicularly to the substratum showed that the T98G cells formed a monolayer with every cell lying adjacent to the other in an epithelia-like organisation (c); as a result, the cell edges in the sections parallel to the substratum are very sharp, and it is possible to observe many adherens junctions along the plasma membranes (e, arrows). On the other hand, the confluent U373MG cells tended to crawl over each other (d) and, because of this, the plasma membranes of two cells in thin sections cut parallel to the substratum are barely visible and no adherens junctions can be seen (f).

Taken together, these data indicate stronger cell-cell adhesion in non-migrating T98G than in migrating U373MG

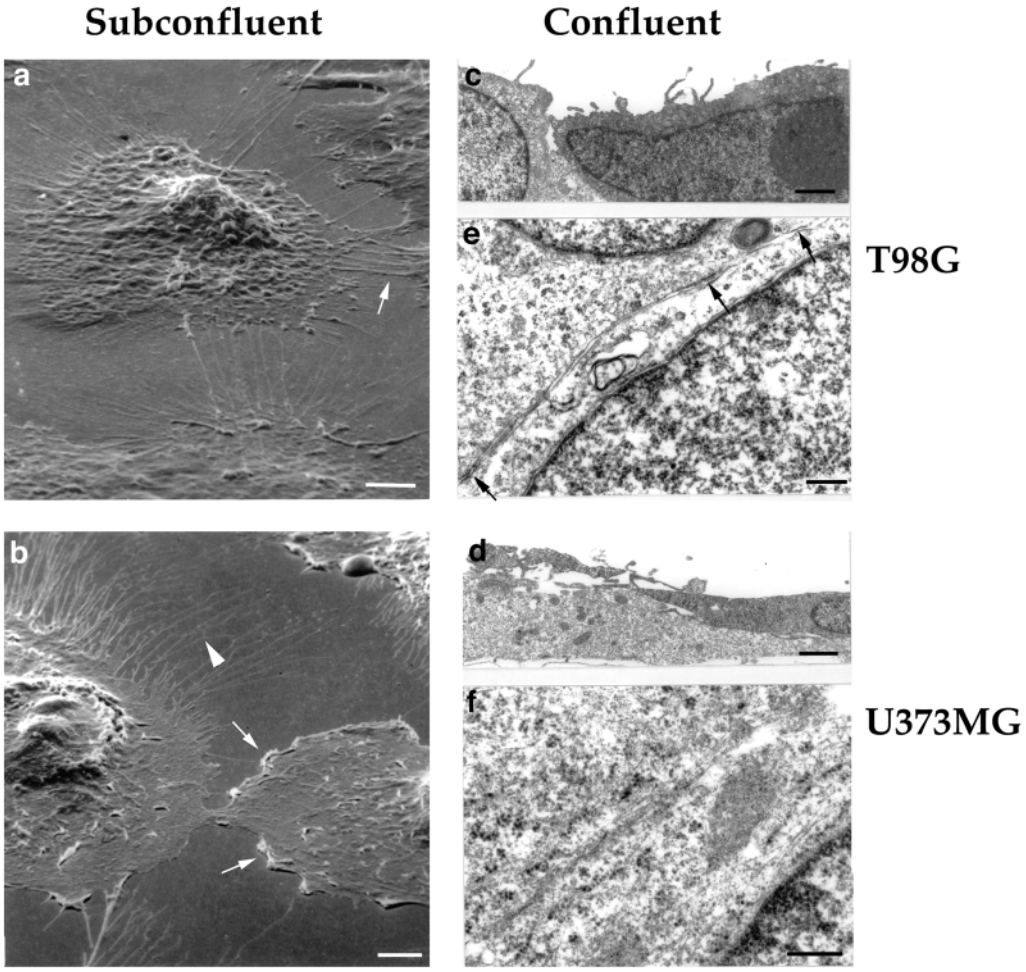

Fig. 4. Ultrastructural analysis of cell-cell contacts. (a,b**)** Scanning electron microscope analysis of subconfluent T98G (a) and U373MG cells (b). The arrows indicate thin parallel cellular processes contacting neighbouring T98G cells (a) or lamellipodia-like structures (b). The arrowheads indicate sparse, branched and thin cellular processes making contact with the substratum (b) (Bars, 5 μm). (c-f) Transmission electron microscope analysis of confluent T98G (c,e) and U373MG (d,f) cells, showing perpendicular (c,d) and parallel sections (e,f). The arrows in (e) indicate well defined adherens junctions. (c,d, bars, 1 μm) (e,f, bars, 0.3 μm).

cells. To characterise the state of maturation and organisation of the cell-cell contacts in both types, we used immunofluorescence staining to follow the junctional recruitment of LIN-7 and α-catenin and the organisation of F-actin (Fig. 5).

As in cultured astrocytes, LIN-7 in T98G cells colocalised with β-catenin in areas with continuous cell-cell contacts (arrowheads) but was absent from regions characterised by irregular β-catenin staining (arrows). No surface colocalisation of LIN-7 with β-catenin was ever observed in the U373MG cells, regardless of the cell density (data not shown). Both T98G and U373MG cells were rich in α-catenin at the β-catenin-containing cell-cell contacts (arrowheads and arrows). Phalloidin staining revealed the presence of organised filaments of F-actin running parallel to the cell-cell contact areas and colocalising with β-catenin at the junctional domain of the T98G cells (arrowheads). On the contrary, the U373MG cells had untidily distributed short filaments of F-actin, and no colocalisation of F-actin with β-catenin was observed at the cell-cell contacts (arrows).

The inability of the U373MG cell line to form mature and organised cell-cell adhesion was tested biochemically by measuring the distribution of β-catenin and LIN-7 in the Triton X-100 soluble and insoluble fractions after the detergent extraction of cells cultured to confluence for four days (Fig. 6A). Fifty percent of β-catenin and LIN-7 were found in the Triton-X-100-insoluble fraction of T98G cells, but only 20% were in the insoluble fraction of U373MG cells. This result is similar to that described during junctional maturation in MDCK cells, when the amount of LIN-7 and β-catenin recovered from the Triton-X100-insoluble fraction increases from 20% in subconfluent cells to 50% in fully polarised cells (Perego et al., 2000b). The lack of LIN-7 junctional recruitment and cytoskeleton reorganisation in U373MG cells strongly suggests a defect in the maturation process of their cadherin-mediated cell-cell contacts.

Given that junctional maturation is accompanied by dephosphorylation of components of the adhesion system (Daniel and Reynolds, 1997; Lampugnani et al., 1997), and that the tyrosine phosphorylation of junctional molecules is frequently associated with pathological alterations in cadherin-mediated cell-cell adhesion (Behrens et al., 1993), we expected to find the immature cell-cell contacts of the U373MG cells highly phosphorylated in tyrosine residues.

P-tyrosine (P-tyr) staining revealed greater total tyrosine phosphorylation in cell lysates and phosphorylated α- and β-catenin in the immunoprecipitates of U373MG cells (Fig. 6B-D). No detectable tyrosine phosphorylation of N-cadherin was found in either cell line (data not shown). Further confirmation of the inability of U373MG cells to form mature junctions came from the confocal analysis of cells double-stained for P-tyr and β-catenin. Phosphorylated proteins

accumulated in the 'free' surfaces of both cell lines, but only the U373MG cell-cell contacts were clearly positive for P-tyr (Fig. 5, arrows).

Invasive behaviour of glioblastoma cell lines correlates with alterations in their adherens junctions

Taken together, our data suggest that only cells with immature adherens junctions are capable of migrating and penetrating the poly-L-lysine barrier. To validate the relationship between immature junctions and cell aggressiveness in the same cellular context, we took advantage of the reported ability of glioma cells to acquire invasive properties when plated on particular extracellular matrix components (Nakagawa et al., 1996; Belot et al., 2001). As expected, the T98G cells showed highly invasive properties when plated on Matrigel (Albini et al., 1987) (Fig. 7A), but the acquisition of this invasive behaviour was not promoted by an increase of N-cadherin expression. Western blot analysis revealed unchanged levels of cadherin, β-catenin and LIN-7 regardless of the substrate in which the cells were plated (Fig. 7B).

Morphological analysis of the intercellular contact maturity and organisation of T98G cells cultured on Matrigel-coated glass coverslips (20-50 μg/cm^2) (Fig. 7C,D) revealed a dramatic change from a non-invasive epithelia-like morphology when grown on poly-L-lysine to an invasive fibroblastoid morphology when grown on Matrigel. The cells formed branching invasive colonies and had a tendency to grow over each other (Fig. 7C). Immunostaining for β-catenin and N-cadherin revealed their colocalisation on the cell surface, where they mainly accumulated in irregular intercellular contacts (Fig. 7D) which, in most cases, seemed to be caused by overlap rather than the juxtaposition of neighbouring cell surfaces. LIN-7 was found intracellularly, but virtually no LIN-7 staining was observed even in the cell-cell contacts that showed regular β-catenin staining (arrows).

Similarly, phalloidin staining revealed the presence of untidily distributed filaments of F-actin in the cells and no colocalisation of F-actin with β-catenin at the cell-cell contacts (arrows). These contacts had a high P-tyr content (arrows), thus further confirming their immaturity.

The culturing of T98G cells on Matrigel therefore induced the disorganisation of the actin cytoskeleton and cell-cell adhesion, modifications that seemed to drive their invasive behaviour.

Discussion

Little information is available concerning cell-cell adhesions in astrocytes. We show here for the first time that pure cortical astrocytes cultured in vitro express and organise molecular components of cell-cell adhesion to form mature cadherin-mediated adhesions. Furthermore, we also show that the LIN-7 PDZ protein (i) physically interacts with the PDZ target sequence of β-catenin, (ii) forms an in vivo complex with the cadherin–β-catenin system, (iii) is progressively associated with cadherin-mediated adhesion and (iv) is completely

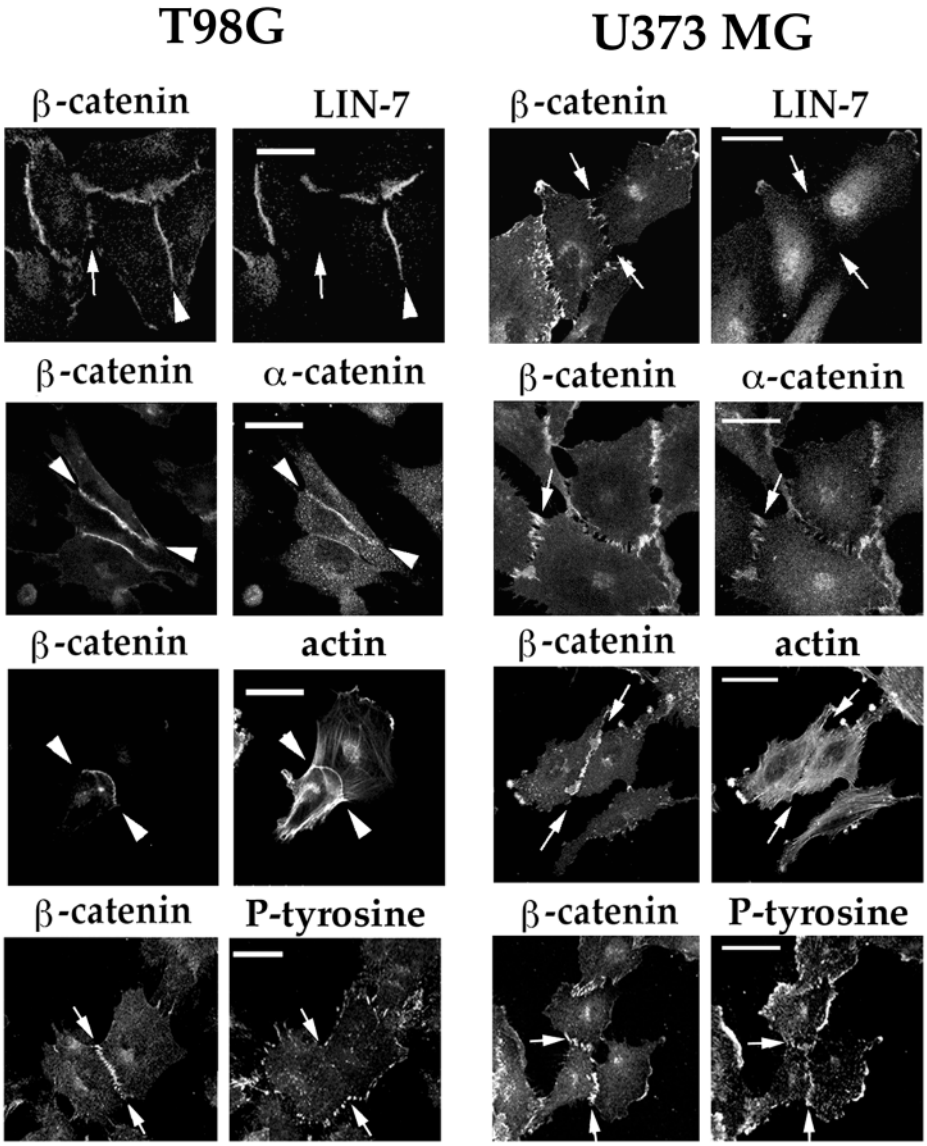

Fig. 5. Morphological analysis of the degree of maturation of cell-cell contacts in T98G and U373MG cell lines. Confocal analysis of immunofluorescence staining. The glioblastoma cell lines were plated onto poly-L-lysine-coated glass coverslips, fixed in 4% paraformaldehyde and double-stained for β-catenin and the indicated antibodies or the FITC-conjugated phalloidin to stain actin. The arrowheads indicate mature cell-cell contacts, and the arrows indicate immature cell-cell contacts. Bar, 10 μm.

associated with the junctional domain only in long-confluent astrocytes with morphologically mature intercellular adhesions. LIN-7 can therefore be considered to be a junctional maturity marker in primary cultured astrocytes, as previously shown in neurons and polarised epithelial cells (Perego et al., 2000b).

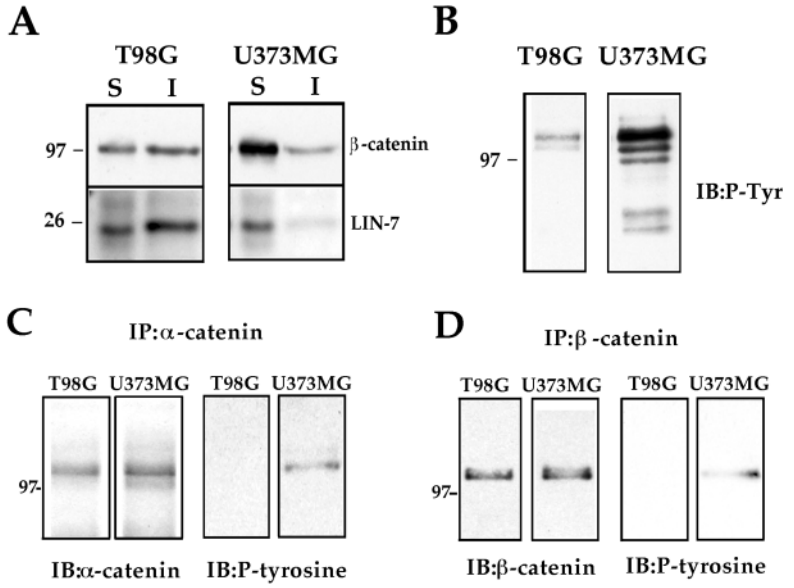

Fig. 6. Biochemical analysis of the degree of maturation of cell-cell contacts in T98G and U373MG cell lines. (A) Western blot analysis of the amount of LIN-7 and β-catenin recovered in the TX-100-soluble (S) and TX-100-insoluble (I) fractions. T98G or U373MG glioblastoma cells grown to confluence were extracted in TX-100. Equivalent volumes of the soluble or insoluble fractions were separated by 10% SDS-PAGE and immunostained using the indicated antibodies. (B) Total P-tyr content in T98G and U373MG cells. 5×10^5 cells were plated, cultured for 72 hours and extracted in lysis buffer containing 0.02% SDS and 100 μM pervanadate. Five percent of the cell lysate used in the immunoprecipitation experiments was probed for P-tyr. (C,D) Tyrosine phosphorylation of α- and β-catenin. The cell lysates were immunoprecipitated with anti-α-(C) or anti-β-catenin (D) antibodies and probed for P-tyr. To confirm similar protein loading, 10% of the immunoprecipitates were probed for α- (C) or β-catenin (D). Molecular weight standards expressed in kDa are indicated on the left.

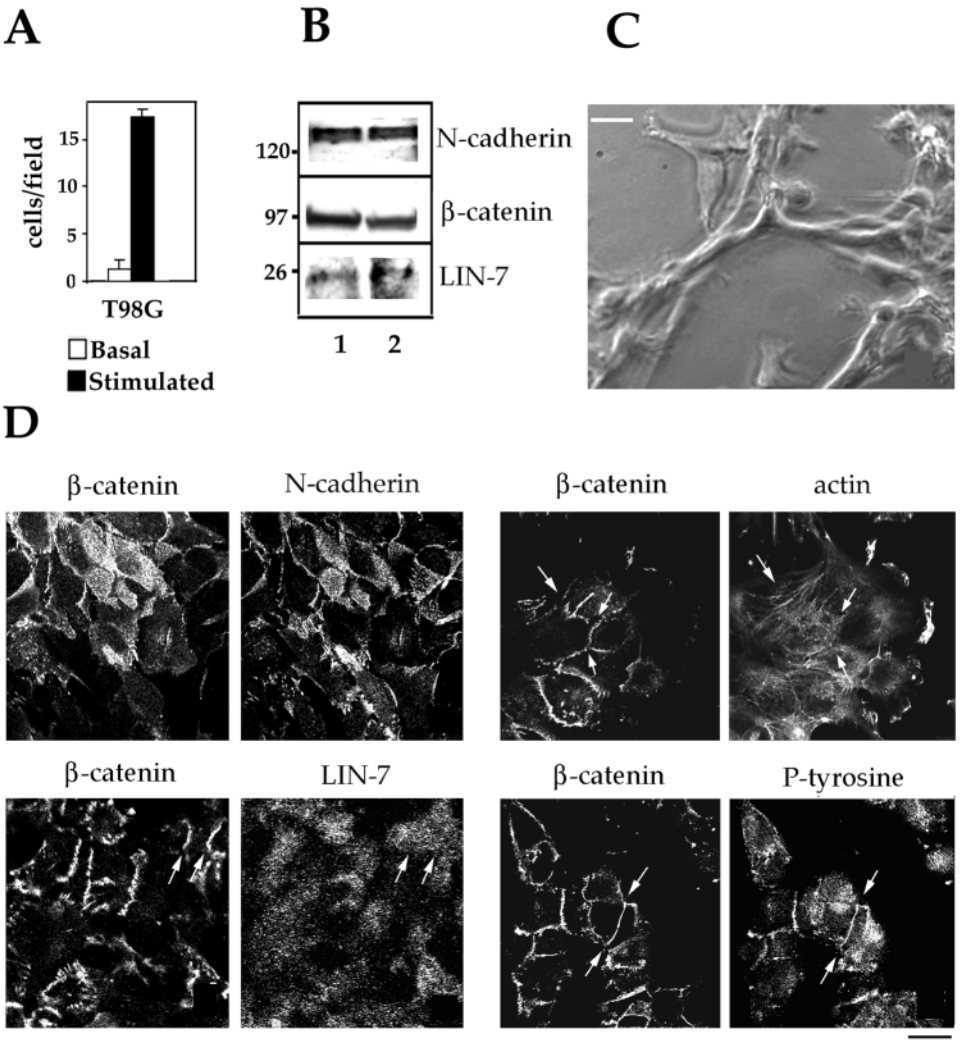

Fig. 7. Analysis of cell-cell adhesion in invasive T98G cells. (A) Matrigel invasion assay using as chemoattractants either serum-free medium containing 0.1% BSA (basal) or conditioned media from the same cells cultured in Matrigel-coated Petri dish (stimulated). The data are the mean number of cells per field±s.d. of triplicate samples from a representative experiment. (B) Western blot analysis of cadherin, β-catenin and LIN-7 total expression in T98G cells cultured on poly-L-lysine (lane 1) or Matrigel (lane 2). The same amounts of total cell homogenates were loaded onto 11% SDS-PAGE and immunoblotted with the indicated antibodies. Molecular weight standards expressed in kDa are indicated on the left. (C) Brightfield and (D) confocal laser microscopy of T98G cells cultured in Matrigel. The cells (2×10^5 cells/35 mm diameter dish) were seeded onto Matrigel-coated glass coverslips and cultured for three days. After fixation in 4% paraformaldehyde, the cells were double-stained for β-catenin and the indicated antibodies. The arrows indicate β-catenin-enriched cell-cell contacts with disorganised filaments of F-actin, virtually no LIN-7 and the accumulation of phosphorylated proteins. Bar, 10 μm.

The expression of adherens junction components has been previously characterised in human glioblastomas and in a number of astrocytoma and glioblastoma cell lines (Shinoura et al., 1995; Roth et al., 2000); however, no data were reported concerning the organisation of their junctions, and no correlation was found between N-cadherin expression and the migration/invasion properties of the glioblastoma cell lines. We show that alterations in the organisation of cadherin-mediated junctions rather than in the expression of N-cadherin are associated with the migration and invasive properties of glioblastoma cell lines. Despite their lower expression of N-cadherin, the T98G cells formed mature and organised cadherin-mediated junctional domains, as assessed by morphological and biochemical criteria, whereas the alterations in the status of the cell-cell adhesion of U373MG cells were associated with their higher cell motility in the poly-L-lysine migration assay. Similarly, the T98G cells showed altered cell-cell adhesion and invasive properties without any alteration in the total expression of junctional components when cultured on a Matrigel matrix (Fig. 7B). These data also exclude the hypothesis that an increase in N-cadherin expression is associated with the acquisition of invasive properties by glioblastoma cells, as has been shown to be the case with breast cancer cells (Hazan et al., 1997; Hazan et al., 2000) and thus further suggest that cell-cell adhesion alterations rather than changes in the expression of cadherin system components are required to promote cell motility and invasiveness.

Our data demonstrate that the LIN-7 PDZ protein is a component of the mature cadherin-based junctional domain of primary cultured astrocytes and non-migrating T98G glioblastoma cells (cultured in poly-L-lysine), whereas the immature junctions of migrating U373MG and invasive T98G cells (cultured in Matrigel) fail to accumulate LIN-7. As the absence of LIN-7 marks immature contacts of migrating and invasive cells, its presence can be considered to be a marker of junctional maturity and invasiveness. It is still not known whether LIN-7 recruitment to intercellular adhesion is the cause or a consequence of the stabilisation of this domain. Together with LIN-2 and LIN-10, LIN-7 may be required to accumulate signalling molecules with crucial junctional stabilisation functions, as in the case of the PTEN tumour suppressor protein, whose PDZ-mediated recruitment into protein complexes enhances its lipid phosphatase activity (Vazquez et al., 2001) and is critical for stabilising intercellular junctions and reverting invasiveness (Kotelevets et al., 2001).

T98G cells derive from a grade IV human glioblastoma multiform tumour and do not grow when implanted subcutaneously or intracerebrally in nude mice unless they are suspended on reconstituted basement membrane Matrigel (Rubenstein et al., 1999). Our results indicate a similar in vitro behaviour, which suggests that these cells have a more selective integrin-dependent invasive phenotype than U373MG cells rather than defects in the intracellular machinery involved in cell migration and invasion. Furthermore, they had more invasive properties than U373MG cells when cultured in Matrigel, which can be explained by their greater secretion of the specific proteases required to remodel the surrounding ECM component of the matrix (C.P, C.V., S.M. et al., unpublished). It is not known whether the cell-substrate-dependent behaviour of T98G cells is a typical feature of the original tumour or an acquired characteristic of the cell line.

Different ECM-dependent migration capacities have been previously documented in various astrocytoma and glioblastoma cell lines (Giese et al., 1994), and we found that invasion properties and cell-cell adhesions can also be modulated by modifying the extracellular matrix. Our results are in line with the in vivo behaviour of astrocytoma and glioblastoma tumours, whose characteristic features include the ability to permeate brain regions and the infrequency of extracranial metastases (Berens et al., 1990).

Cell-cell and cell-ECM adhesion proteins influence cell locomotion through the reorganisation of the actin cytoskeleton (Bissel and Nelson, 1999; Hemler and Rutishauser, 2000). It is therefore possible that competitive pathways mediate the organisation of the cytoskeleton and that, when cell-substrate interactions prevail, the cytoskeleton is organised in order to allow locomotion, and this affects the strength of cell-cell adhesions. In line with this possibility, a progressive reduction in the interaction of adherens junction proteins with the cytoskeleton has been shown during integrin-induced epithelial tubule formation in MDCK cells (Ojakian et al., 2000), and we found that migrating U373MG (Fig. 5) and invasive T98G cells (Fig. 7D) have immature cell-cell adhesions and a disorganised cortical actin cytoskeleton. Further experiments will be required in order to establish whether extracellular matrix receptors are involved and to elucidate how the cell-substrate and the cell-cell adhesion systems are integrated.

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