Expression of N-cadherin and α-catenin in astrocytomas and glioblastomas

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Summary We examined levels of mRNA and protein for N-cadherin, the predominant cadherin in neural tissues, and mRNA levels for the cadherin-associated protein, α-catenin, in a series of gliomas and in glioblastoma cell lines. mRNA levels for N-cadherin and α-catenin were significantly higher in glioblastomas than in low-grade astrocytomas or normal brain, while the levels of intact N-cadherin protein were similar in glioblastomas, low-grade astrocytomas and brain. In addition, there was no consistent relationship between invasiveness and expression of N-cadherin and α-catenin in highly invasive vs minimally invasive tumours within the same histopathological grade. To assess further the relationship between cadherin expression and neural tumour invasion, we measured N-cadherin expression, calcium-dependent cell adhesion and motility of several glioblastoma cell lines. While all N-cadherin-expressing lines were adhesive, no correlation was seen between the level of N-cadherin expression and cell motility. Together, these findings imply that, in contrast to the role played by E-cadherin in carcinomas, N-cadherin does not restrict the invasion of glioblastomas.

Keywords: invasion; cell motility; cell adhesion; brain tumours

The prognosis for patients with malignant primary brain tumours remains poor, in large part because the invasiveness of these tumours limits the effectiveness of local therapies such as surgery (Kelly *et al.*, 1987; Burger *et al.*, 1988). Although the acquisition of invasive capacity by cancer cells during tumour progression has not been fully analysed, alterations of cell–cell adhesion, increases in cell motility and secretion of proteolytic enzymes seem likely to be involved (Liotta *et al.*, 1991). The suggestion that cell–cell adhesion might be an important determinant of invasion stems from the observation that the mutual adhesiveness of squamous cell carcinomas is weaker than that of corresponding normal cells (Coman 1944, 1947). Progress in identification and characterisation of cell–cell adhesion molecules (CAMs) (Takeichi, 1988; Cunningham *et al.*, 1990; Albelda and Buck, 1990) has made it possible to determine whether changes in expression or activity of specific CAMs contribute to the invasive or malignant potential of different tumours. Recent studies indicate that E-cadherin, the major adhesion molecule found in epithelial cells (Takeichi, 1988), inhibits the motility and invasion of carcinoma cells (Chen and Öbrink, 1991; Frixen *et al.*, 1991; Vleminckx *et al.*, 1991).

E-cadherin is one member of the cadherin family, which are transmembrane glycoproteins that mediate Ca^{2+}-dependent cell–cell adhesion (Takeichi, 1988). Classical cadherins consist of a large extracellular domain that contains five homologous segments with calcium-binding motifs, a transmembrane domain and a cytoplasmic domain. Cadherins mediate adhesion by a homophilic mechanism, i.e. cadherin-to-cadherin binding (Nagafuchi *et al.*, 1987) and the specificity of this binding is determined by the amino-terminal homologous repeat segment (Nose *et al.*, 1990). Cadherins are linked to the cytoskeleton by means of cytoplasmic proteins, termed α-, β- and γ-catenins (Ozawa *et al.*, 1989; Ozawa and Kemler, 1992), which bind to a region of 70 amino acids in the cytoplasmic domain of cadherins. Binding of catenins is essential for effective cadherin-mediated adhesion (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1990).

Although other CAMs are coexpressed with cadherins in most cells, the cadherins appear to play the predominant role in cell–cell adhesion. Blocking cadherin activity with antibodies not only prevents reassociation of dispersed cells, but also dissociates adherent cells in culture or from intact embryonic tissues (Takeichi *et al.*, 1981; Shirayoshi *et al.*, 1983). In contrast, inactivation of other adhesion molecules has little effect on adhesiveness of cells as long as cadherins are intact (Duband *et al.*, 1987). In addition to mediating adhesion, cadherins appear to affect cell differentiation and behaviour. When cadherins are introduced into fibroblasts or sarcoma cells via transfection, the cells assume a more epithelioid morphology, with the development of specialised junctions (Takeichi, 1988). Cadherin-mediated contact also induces cell differentiation (Shirayoshi *et al.*, 1983). These results imply that changes in expression of cadherins might have profound effects on cell behaviour.

In carcinoma cells, loss of E-cadherin correlates directly with increased malignancy and invasiveness *in vivo* (Schipper *et al.*, 1991; Shiozaki *et al.*, 1991; Sommers *et al.*, 1991; Mayer *et al.*, 1993; Rasbridge *et al.*, 1993). In addition, transfection of epithelial cells or fibroblasts with E-cadherin suppresses their invasion *in vitro*, while loss of E-cadherin correlates with enhanced invasiveness (Behrens *et al.*, 1989; Chen and Öbrink, 1991; Frixen *et al.*, 1991; Vleminckx *et al.*, 1991). These observations strongly suggest that E-cadherin-mediated adhesion regulates tumour cell invasion. This conclusion is strengthened by closer analysis of some tumours that are invasive yet retain levels of E-cadherin: in these cells, α-catenin expression is greatly reduced, resulting in reduced E-cadherin-mediated adhesion (Shimoyama *et al.*, 1992; Morton *et al.*, 1993; Kadowaki *et al.*, 1994). Although these observations establish that E-cadherin inhibits tumour cell invasion, the mechanism by which this occurs is not yet fully understood.

In contrast to E-cadherin, little is known about the relationship between expression of neural cadherins and invasion of astrocytomas. Several cadherins are enriched in neural tissue, including N-cadherin (Hatta and Takeichi, 1986; Hatta *et al.*, 1987), B-cadherin (Napolitano *et al.*, 1991), R-cadherin (Inuzuka *et al.*, 1991) and T-cadherin (Ranscht and Dours-Zimmerman, 1991). Recently, PCR methods have been used to identify additional neural cadherins (Suzuki *et*

al., 1991). Of these. N-cadherin has been most extensively investigated. Within the central nervous system, N-cadherin is expressed by both neurons and astrocytes (Hatta et al., 1987; Tomaselli et al., 1988), but the molecule is not restricted to the nervous system and is also abundant in skeletal (Hatta et al., 1987) and cardiac muscle (Duband et al., 1987). A fragment of N-cadherin can be released from the cell surface by proteolytic cleavage and accumulate in vivo, as in the vitreous body (Paradies and Grunwald, 1993). Although this soluble fragment retains adhesive activity (Paradies and Grunwald, 1993), little is known about the physiological effects of such release. In neurons, adhesion mediated by N-cadherin triggers a cascade of second messengers, culminating in the influx of calcium, which induces extension of neuronal processes (Doherty et al., 1991). The effects of N-cadherin-mediated contact on the motility and invasion of normal astrocytes and astrocytoma cells, however, have not been described.

In this study, we compared the expression of N-cadherin and α-catenin in normal brain tissue, low-grade astrocytomas and malignant gliomas, to determine whether changes in this cadherin adhesion system might play a role in the enhanced invasion of glioblastoma cells. We also examined expression of N-cadherin and α-catenin in a series of human glioblastoma cell lines and tested whether cadherin-mediated adhesion correlated with the motility of these cells in in vitro assays. The results indicate that N-cadherin and α-catenin levels remain the same or increase in high-grade gliomas. In addition, we found no relationship between cadherin-mediated adhesion and motility in the glioblastoma cell lines. Together, these findings suggest that, in contrast to E-cadherin in carcinomas. N-cadherin does not regulate tumour cell invasion in astrocytic tumours.

Materials and methods

Tumour specimens and cell lines

Tumour specimens were obtained from a tumour bank maintained by the Brain Tumour Research Center at The University of Cincinnati College of Medicine. The tissues were snap frozen in liquid nitrogen immediately after resection. The samples included one pilocytic astrocytoma, four low-grade astrocytomas, three anaplastic astrocytomas, nine glioblastomas, two mixed gliomas, two oligodendrogliomas and a specimen of normal brain obtained from an epilepsy patient. Tumour specimens were classified according to the WHO system, in which grade I corresponds to pilocytic astrocytomas, grade II to low-grade astrocytomas, grade III to anaplastic astrocytomas and grade IV to glioblastomas. Patient identity was not disclosed but the results of histopathological analysis and limited clinical information were made available for correlation with the analyses of cadherin and catenin expression. Magnetic resonance (MR) images obtained during the course of treatment were reviewed to assess whether the tumours remained localised (termed 'minimally invasive') or diffusely infiltrated surrounding brain tissue (termed 'highly invasive'). Samples of normal brain, heart, liver and placenta were analysed as controls. In addition, seven glioblastoma cell lines were analysed, including U-87MG, U-118MG, U-138MG, U-373MG, T98G, Hs683 (Ponten and MacIntyre, 1968; Owens et al., 1976; Stein, 1979), obtained from the American Type Culture Collection (Rockville, MD, USA), and SNB-19 (Gross et al., 1988), obtained from the Tumor Depository, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

Northern blot analysis of tumour and cell line RNA

Total RNA was extracted from human gliomas and glioblastoma cell lines by a single-step isolation method (Chomczyn-

ski and Sacchi, 1987). The levels of mRNA for N-cadherin and α-catenin were compared by Northern blot analysis. Twenty micrograms of total RNA from each sample was fractionated by denaturing agarose gel electrophoresis and then transferred to nylon membranes (Micron Separation, Westborough, MA, USA). For analysis of glioblastoma cell lines, total RNA was extracted from seven glioblastoma cell lines at different stages of confluency: semiconfluent (SC), immediately after reaching confluency (IC) or 4–5 days after reaching confluency (C).

The cDNA probe for N-cadherin was the middle EcoRI fragment (approximately 300 base pairs) of the sequence reported by Reid and Hemperly (1990). The cDNA probe for α-catenin contained the entire open reading frame of α-catenin (Oda et al., 1993). Because of the close similarity in sequence, this probe recognises both α-E-catenin and α-N-catenin. Probes for Northern blots were labelled using the Prime-It random primer labelling kit according to the manufacturer's instruction (Stratagene, La Jolla, CA, USA). As an internal control for the amount of RNA loaded in each well, a 20mer oligonucleotide probe for 18S RNA (GACAAGCATATGCTACTGGC) (McCallum and Maden, 1985) was end labelled with $[\gamma^{-32}P]dATP$ and used according to previously published methods (Church and Gilbert, 1984). Hybridisation of RNA blots was performed by standard methods (Selden, 1992). RNA blots were washed with 2 × SSC and 0.1% SDS for 30 min at room temperature and with 0.2 × SSC and 0.1% SDS for 30 min at 55°C, covered in Saran Wrap, and autoradiographed overnight at $-20°C$. Relative hybridisation intensities were determined by use of a phosphorimager. The values for N-cadherin or α-catenin were corrected for variations in loading by dividing by the values obtained for 18S RNA. For ease of comparison in Table I, all N-cadherin and α-catenin values were normalised to the values obtained from tumour GBM1, which were set to 0.2. Similarly, in Table II, all N-cadherin and α-catenin values were normalised to the values obtained from SNB-19 (IC), which were set to 1.0.

Immunoblot analysis of tumour samples and glioblastoma cell lines

Glioblastoma cell lines were washed with 0.24% Hepes buffer, scraped and centrifuged. After removal of supernatant, an equal volume of 1 × Laemmli buffer (Laemmli, 1970), 5 mM EDTA, 100 μl of phenylmethylsulphonyl fluoride, 1 mM leupeptin, 1 μM pepstatin and 100 μM iodoacetamide was added and the pellets were boiled for 5 min. Normal brain tissue removed from an epilepsy patient, astrocytomas and a sample of chicken heart (a positive control for N-cadherin) were homogenised in an equal volume of the buffer described above and boiled for 5 min. Equal volumes (10 μl per lane) of each extract were separated by using 7.5% polyacrylamide gels and transferred onto nitrocellulose membranes. After blocking with 5% dry milk in TBS (10 mM Tris–HCl pH 7.5, 150 mM sodium chloride), the membranes were incubated with primary antibodies overnight. After electrophoresis, the protein content of each extract was quantified by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) and the extracts were found to contain similar levels of protein that, in no case, varied more than 2-fold. In addition, the relative level of loading and effectiveness of transfer were verified by cutting off the lower part of the nitrocellulose filter and staining with 0.1% amido black. For detecting N-cadherin, we used the monospecific polyclonal antiserum C-NCAD (raised against a synthetic peptide corresponding to amino acids 838–856) (Lagunowich et al., 1990) (generously provided by J Grunwald), which recognises the cytoplasmic region near the carboxyl terminus. After washing, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (H + L) (Bio-Rad Laboratories). After washing, the bands were visualised with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (Gibco BRL, Gaithersburg, MA, USA).

Cell aggregation assay

This assay was carried out as described previously (Brackenbury *et al.*, 1977, 1981). In brief, glioblastoma cell lines growing in tissue culture dishes were rinsed with Hepes buffer containing 1 mM calcium chloride, then incubated in 0.04% recrystallised trypsin (Worthington) in Hepes buffer supplemented with 10 mM calcium chloride and 100 µg ml^{-1} DNAse. Cells were then centrifuged and resuspended in Hepes buffer with 1 mM calcium chloride and 100 µg ml^{-1} DNAse. Then, 2×10^6 cells were added to approximately 2 ml of Hepes buffer with 1 mM calcium chloride or 1 mM EDTA. Initial cell numbers (N_0) were determined using a Coulter counter (Coulter Electronics, Hialeah, FL, USA) and, after shaking incubation for 30 min at 37°C, the cell numbers (N_{30}) were counted again. The percentage aggregation was calculated from the formula $N_0 - N_{30}/N_0 \times 100$. Assays were repeated at least three times and averages were calculated.

Wound filling assay

Glioblastoma cell lines were grown to confluency in tissue culture dishes. At three places within each dish, a small region was scraped with a P-200 plastic pipette tip to produce a cleared path ('wound') of almost constant width. The wounds were observed by phase-contrast microscopy at roughly 12 h intervals after scraping, and the time when each wound was totally filled by cells was noted. In two experiments, the times of complete filling did not vary.

Results

Expression of N-cadherin and α-catenin mRNA in astrocytomas, gliomas and glioblastoma cell lines

To determine whether alterations in the N-cadherin adhesion system might contribute to invasive potential, we compared the expression of mRNA for N-cadherin and the associated protein α-catenin in normal brain and gliomas of varying grades. RNA was extracted from snap-frozen tissues and analysed by Northern blots. Figure 1 shows data from Northern blots hybridised with radiolabelled probes for N-cadherin and α-catenin. An N-cadherin cDNA probe recognised RNA transcripts of 4.0 and 4.3 kb, as previously reported (Walsh *et al.*, 1990). The α-catenin probe reacted with a single transcript of 3.8 kb (Oda *et al.*, 1993). To compare the levels of N-cadherin and α-catenin mRNA in different samples, the blots were also hybridised with an oligonucleotide probe for 18S RNA as a measure of the amount of RNA loaded. These blots and others were then quantified by scanning with a phosphorimager, the amounts of mRNA for N-cadherin and α-catenin in each sample were normalised relative to 18S RNA, and the values tabulated (Table I). Replications of the same tumour specimens were generally consistent, and comparison of the different tumour specimens revealed that N-cadherin was expressed at significantly higher levels in anaplastic astrocytomas and glioblastomas than in low-grade astrocytomas and normal brain (Table I). The difference between these groups was highly significant ($P < 0.05$ in Student t-test). The α-catenin transcript was also expressed at significantly higher levels in high-grade gliomas than in low-grade astrocytomas and normal brain, which contained very low levels of N-cadherin and α-catenin mRNAs.

Although higher grade tumours are generally more invasive, this is not invariably the case. We therefore compared the expression of N-cadherin among tumours within a single grade that varied in invasiveness, as assessed by clinical records, particulary MR scans. Although this analysis was necessarily restricted by the limited number of samples, no consistent relationship was seen between N-cadherin expression and invasion. For example, among the grade IV glioblastomas, GBM1, GBM6, GBM7 and GBM9 all express similar amounts of N-cadherin mRNA; GBM1 and GBM7

were highly invasive tumours while GBM6 and GBM9 were minimally invasive. Similarly, GBM5 and GBM8 expressed roughly similar amounts of N-cadherin mRNA, higher than the level expressed in GBM1, GBM6, GBM7 and GBM9; of these tumours, GBM5 was highly invasive while GBM8 was
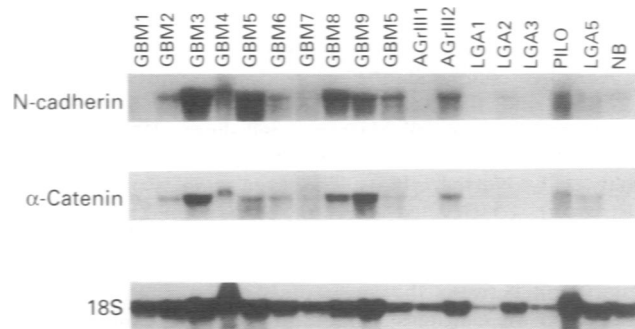
Figure 1 N-cadherin and α-catenin mRNA levels in astrocytomas. The figure shows an autoradiograph of Northern blot analysis of total RNA (20 µg per lane) extracted from glioblastoma multiforme tumours (GBM) classified as WHO grade IV; anaplastic astrocytomas (AGrIII) classified as WHO grade III; low grade astrocytomas (LGA) classified as WHO grade II; a pilocytic astrocytoma (PILO) classified as WHO grade I; and a normal brain sample (NB). In one case (GBM5) two different samples from the same tumour were analysed. Blots were hybridised with cDNA probes for N-cadherin (top) and α-catenin (middle) and with an oligonucleotide probe for 18S rRNA (bottom).
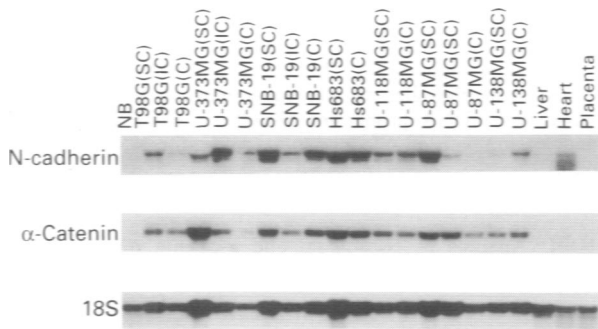
Table I Analysis of N-cadherin and α-catenin mRNA levels in gliomas

Tumour[a]	N-cadherin[b]	α-catenin[b]
High-grade gliomas		
GBM1	0.2, 0.2	0.2, 0.2
GBM2	0.61, 0.58	0.35, 0.70
GBM3	0.70, 0.32	0.54, 0.35
GBM4	0.50, 1.05	0.48, 0.73
GBM5	0.60, 0.57	0.46, 0.40
GBM6	0.31	0.30
GBM7	0.26	0.24
GBM8	0.47	0.48
GBM9	0.27	0.58
AGrIII1	0, 0.75	0, 0.68
AGrIII2	0.3, 0	0.33, 0
AGrIII3	0	0.13
Low-grade gliomas		
LGA1	0, 0	0, 0
LGA2	0.19, 0	0.14, 0
LGA3	0, 0.09	0, 0.33
LGA5	0.12, 0.21	0.12, 0.17
Other tumours		
PILO	0.11, 0.12	0.17, 0.30
MG1	0	0
MG2	0	0
OLIGO1	0	0
OLIGO2	0	0
Control tissues		
Normal brain	0.18	0.07
Heart	0.72, 0.95	0.42, 0
Liver	0, 0.17	0, 0
Placenta	0, 0	0, 0

[a]RNA was extracted from tumour specimens (see legend to Figure 1), analysed by Northern blot and quantified by use of a phosphorimager, as described in Materials and methods. The table presents data from the blot shown in Figure 1 combined with data from other similar blots. [b]Relative hybridisation intensities were determined by use of a phosphorimager. The values for N-cadherin or α-catenin were corrected for variations in loading by dividing by the values obtained for 18S RNA. For ease of comparison, all N-cadherin and α-catenin values were normalised to the values obtained from tumour GBM1, which were set to 0.2.

minimally invasive. As another example, within grade III, AGIII2 was highly invasive, whereas AGIII3 was very localised; when corrected for differences in loading, these tumours expressed similar levels of N-cadherin mRNA. Finally, although there was significant variation in the level of mRNAs for N-cadherin and α-catenin in different gliomas (Table I), the ratio between the two mRNAs was generally similar (linear regression and correlation analysis yielded values of $r = 0.838$ and $P = 0.000$).

We also compared the expression of mRNA for N-cadherin and the associated protein α-catenin in several established human glioblastoma cell lines. RNA was extracted from cells harvested at different states of confluence analysed by Northern blots. The seven glioblastoma cell lines also expressed elevated levels of N-cadherin and α-catenin mRNA relative to normal brain (Figure 2 and Table II). In contrast to the tumour specimens, however, there was no correlation between the levels of N-cadherin and α-catenin mRNA in the cell lines. In addition, mRNA levels for both molecules varied as a function of cell density, although there was no consistent pattern of density-dependent expression among the various cell lines.

Expression of N-cadherin protein in gliomas and glioblastoma cell lines

To assess the levels of N-cadherin protein, tumour samples and glioblastoma cell lines were extracted with non-ionic detergents. Equal volumes of each extract were separated by gel electrophoresis, transferred to nitrocellulose and blotted with antibodies against N-cadherin. Subsequent protein determinations indicated that roughly equal amounts of protein were loaded. As shown in Figure 3, antibodies to N-cadherin detected a band of approximately 120 kDa in extracts of all astrocytomas and in normal brain that co-migrated with a band in the positive control extract from chicken heart. The different astrocytoma tumour samples generally contained similar levels of N-cadherin. Although slightly lower levels of N-cadherin were seen in the GBM11 extract, the pattern of amido black staining suggested that there was some degradation of the proteins in this extract. N-cadherin was also detected in extracts of all glioblastoma cell lines (Figure 4), although the levels varied somewhat. The U-87MG and T98G lines expressed relatively low levels of N-cadherin, while the U-373MG and SNB-19 lines expressed relatively high levels.

Figure 2 mRNA expression of N-cadherin and α-catenin in glioblastoma cell lines at different cell densities. The levels of mRNA for N-cadherin (top) and α-catenin (middle) isolated from seven glioblastoma cell lines at different cell densities were assessed by Northern blotting as described in the legend to Figure 1. RNA was extracted from cells that were semiconfluent (SC), immediately after the cells became confluent (IC) or 4–5 days after the cells became confluent (C). Normal brain (NB) was used as a positive control.
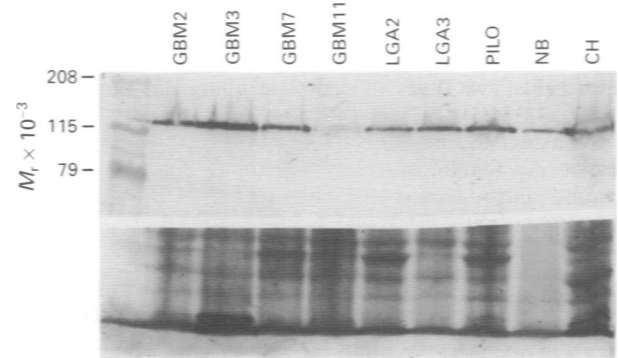

Figure 3 Protein expression of N-cadherin in astrocytomas and glioblastoma cell lines. Relative levels of N-cadherin protein in extracts from astrocytomas were determined by immunoblot analysis using a polyclonal antiserum raised against a synthetic peptide from the cytoplasmic domain of N-cadherin (Lagunowich et al., 1990) as described in Materials and methods. The lower part of each filter was stained with amido black to verify the loading and transfer of extracts. Abbreviations used are: GBM, glioblastoma multiforme; LGA, low-grade astrocytoma; PILO, pilocytic astrocytoma; NB, normal brain; CH, chicken heart.

Table II Analysis of N-cadherin and α-catenin mRNA levels in glioblastoma cell lines

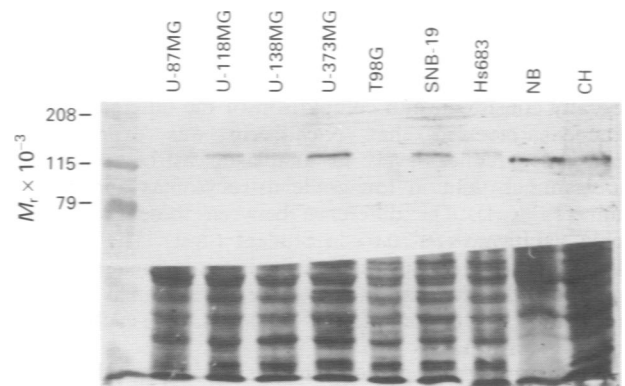| Cell line[a]	N-cadherin[b]	α-catenin[b]
T98G(SC)	0.71	0.71
T98G(IC)	0.40	0.97
T98G(C)	0.36	1.90
U-373MG(SC)	1.59	0.91
U-373MG(IC)	0.83	0.53
U-373MG(C)	1.21	1.03
SNB-19(SC)	0.70	0.84
SNB-19(IC)	1.00	1.00
SNB-19(C)	0.71	0.95
Hs683(SC)	0.79	0.85
Hs683(C)	0.71	0.84
U-118MG(SC)	0.68	0.63
U-118MG(C)	0.83	0.92
U-87MG(SC)	0.38	0.84
U-87MG(C)	0.24	0.72
U-138MG(SC)	0.29	0.78
U-138MG(C)	0.63	0.77

[a]RNA was harvested from indicated cell lines at different stages of confluency – semiconfluent (SC), immediately after reaching confluency (IC) or 4–5 days after reaching confluency (C) – and analysed by Northern blot. [b]Relative hybridisation intensities were determined by use of a phosphorimager. The values for N-cadherin or α-catenin were corrected for variations in loading by dividing by the values obtained for 18S RNA. For ease of comparison, all N-cadherin and α-catenin values were normalised to the values obtained from SNB-19 (IC), which were set to 1.0.

Figure 4 Protein expression of N-cadherin in astrocytomas and glioblastoma cell lines. Relative levels of N-cadherin protein in extracts from glioblastoma cell lines were determined by immunoblot analysis using a polyclonal antiserum raised against a synthetic peptide from the cytoplasmic domain of N-cadherin (Lagunowich et al., 1990) as described in Materials and methods. The lower part of each filter was stained with amido black to verify the loading and transfer of extracts. Abbreviations used are: NB, normal brain; CH, chicken heart.

Table III Cell aggregation and wound filling assays of glioblastoma cell lines

Cell line	Cell adhesion[a]	Wound filling[b]
U-87MG	40% (7%)	20 h
U-118MG	65% (4%)	NF
U-138MG	74% (7%)	60 h
U-373MG	56% (21%)	56 h
T98G	19% (4%)	68 h
SNB-19	39% (9%)	20 h
Hs683	39% (16%)	10 days

[a]Each cell line was analysed in three aggregation assays, as described in Materials and methods. The table shows the average values with standard deviations in parentheses. [b]Wound filling assays were carried out twice, in triplicate, and the table shows the values that were repeatedly obtained. NF, the wound was not filled completely.

Cell aggregation and motility of glioblastoma cell lines

To test whether cadherin-mediated adhesion regulates cell motility in the glioblastoma cell lines, we measured the aggregation and motility of these cells in vitro and correlated the results of these two assays with determinations of N-cadherin expression. For cell aggregation assays, cells were removed from culture dishes under conditions that retain cadherins and were tested for their ability to reassociate. As shown in Table III, the different glioblastoma lines showed significant variation in calcium-dependent adhesion, ranging from 19% (T98G) to more than 50% (U-138MG, U-118MG and U-373MG).

To assess the motility of these cells, we employed an in vitro assay that measures the ability of the cells to refill a 'wound' introduced into a confluent monolayer of cells by scraping a path. U-87MG showed the shortest time for wound healing (20 h), while the wound healing times of U-118MG and Hs683 were very long (Table III). The times required for refilling the wound varied substantially among the glioblastoma cell lines and showed no consistent relationship to the level of adhesiveness, as revealed in the cell aggregation assays. In addition, motility in the wound refilling assay did not show a consistent correlation with expression of N-cadherin.

Discussion

Previous work suggests that the loss of E-cadherin that occurs as tumours progress to less differentiated forms is an important determinant of enhanced invasiveness (Takeichi, 1993). N-cadherin is the predominant cadherin in brain tissues and appears to play a major role in maintaining the multicellular structure of brain (Hatta et al., 1985). We therefore tested whether expression of N-cadherin might regulate the invasive behaviour of astrocytomas. We found that N-cadherin and α-catenin expression increases or remains the same as astrocytomas progress to high-grade glioblastomas. In addition, no correlation was observed between cadherin expression and cell motility in a series of glioblastoma cell lines. As discussed below, these results indicate that the relationship between N-cadherin expression and invasion is quite different from that of E-cadherin.

To analyse the relationship between cell adhesion and invasiveness of astrocytomas, we first surveyed the expression of several cell adhesion molecules, including N-cadherin, N-CAM, L1, E-cadherin and P-cadherin, in tumour samples. Results from this initial survey (data not shown) indicated that only N-cadherin and N-CAM, which contributes to cell adhesion to a lesser extent than N-cadherin (Duband et al., 1987), showed appreciable expression. The remainder of our studies focused, therefore, on N-cadherin and the associated protein, α-catenin.

In tumours, higher levels of mRNA for N-cadherin were found in anaplastic astrocytomas (AA) and glioblastomas

(GBM) than in low-grade astrocytomas or normal brain. This observation was consistent, in that 8/11 AAs or GBMs showed increased levels, although there was some variation in the magnitude of this increase. If increased invasive capacity resulted in part from loss of N-cadherin, then higher grade gliomas might have been expected to show reduced levels of N-cadherin expression, as is the case for E-cadherin in adenocarcinomas (see Takeichi, 1993). These findings thus suggested that loss of N-cadherin-mediated adhesion was not a key determinant in the enhanced invasion of higher grade tumours. One limitation to this analysis was that, although higher grade tumours are generally more invasive, this is not invariably the case. We therefore compared the expression of N-cadherin among tumours within the same grade that were judged, by examination of MR scans, to be highly or minimally invasive. This analysis showed that there is no consistent relationship between invasion and N-cadherin expression, confirming the conclusion that alterations in N-cadherin levels do not appear to contribute to acquisition of invasive capacity.

It was striking that, in all of the tumours examined, increases in N-cadherin mRNA were accompanied by approximately proportional increases in the levels of α-catenin mRNA. The α-catenin probe used recognises both α-E-catenin and α-N-catenin, but only α-N-catenin is expressed in normal brain (Hirano et al., 1992). We presume therefore that the elevated levels of α-catenin mRNA detected in tumours correspond to α-N-catenin. If the correlation between increased N-cadherin mRNA and α-catenin mRNA is not simply fortuitous, this finding may suggest that expression of the two genes is coordinately controlled. In contrast, α-catenin expression is greatly reduced in some epithelial tumours that retain high levels of E-cadherin (Shimoyama et al., 1992; Morton et al., 1993; Kadowaki et al., 1994), apparently contributing to the invasive ability of the tumour cells.

In contrast to the increases in mRNA for N-cadherin, immunoblot analysis suggested that normal brain, low-grade astrocytomas and high-grade astrocytomas all contained roughly similar levels of intact N-cadherin protein. Although this apparent discrepancy could arise in several ways, one intriguing possibility is that the N-cadherin protein produced in the high-grade tumours is not completely destroyed, but, by analogy with the situation known to occur during normal retinal development (Paradies and Grunwald, 1993), is cleaved to produce a soluble fragment that might retain some biological activity. This possibility is consistent with our findings, but could not be directly tested, because the antibody used to detect N-cadherin reacts with the carboxyl-terminal portion of the molecule, and thus would not detect the soluble fragment.

Chen and Öbrink (1991) have observed that cell contact mediated by E-cadherin inhibits cell motility and have suggested that this may be the means by which cadherins inhibit invasion. We therefore evaluated the motility, calcium-dependent adhesion and N-cadherin expression of several human glioblastoma cell lines, to analyse further whether there is any relationship between cadherin-mediated adhesion and invasion of glioblastoma cells. As in the glioblastoma tumours, all seven lines showed higher levels of N-cadherin mRNA expression than found in normal brain or low-grade astrocytomas. There were significant variations in calcium-dependent adhesion among the different cell lines and, given that other neural cadherins might be variably expressed by these lines, it was perhaps not surprising that the variations in adhesion did not appear to reflect differences in the levels of N-cadherin expression. The lines also showed significant variations in motility, as assessed by their ability to refill 'wounds' made in a confluent monolayer of cultured cells (the same assay used by Chen and Öbrink). In contrast to the relationship between E-cadherin mediated adhesion and motility (Chen and Öbrink, 1991), no consistent relationship was seen in the glioblastoma cell lines between cadherin expression and motility, although such a correlation could have been obscured by variations in intrinsic levels of motility.

The experiments described here were motivated by studies of the relationship between E-cadherin expression and invasion in carcinoma cells (Takeichi, 1993). The results of our studies strongly suggest that changes in expression of N-cadherin that occur during tumour progression differ from those seen with E-cadherin and, further, that N-cadherin does not appear to inhibit cell motility as E-cadherin does (Chen and Öbrink, 1991). These conclusions are further supported by our recent studies on the effects of E-cadherin and N-cadherin on the motility and invasion of an astrocyte-like cell line, WC5 (Paradies et al., 1995). In those experiments, E-cadherin expression reduced motility and invasion of WC5 cells, whereas expression of N-cadherin had little effect on the rate of invasion.

In conclusion, these studies show that the expression of N-cadherin mRNA and protein remain at equal or higher

levels in high-grade astrocytomas relative to low-grade astrocytoma or normal brain. Together with the finding that glioblastoma cell lines show no consistent relationship between N-cadherin expression and cell motility, the results imply that expression of N-cadherin does not regulate invasion of high-grade astrocytomas.

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