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Expression of E-cadherin-catenin complex in human benign schwannomas

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Summary. The Ca²⁺-dependent cell adhesion molecule E-cadherin has been known to express in normal and reactive Schwann cells in rodents, and to play an important role in Schwann cell-Schwann cell adhesion and maintenance of peripheral nervous tissue architecture. However, little is known about expression of E-cadherin in schwannomas. The aim of the present study was to investigate the cellular expression and localization of E-cadherin, and its associated protein, alpha E-, alpha N- and beta-catenins in human schwannomas, which are supposed to derive from Schwann cells. We tested the hypothesis that these proteins might show an altered expression/distribution in schwannoma cells which correlates with their neoplastic behavior, including sparse cell-cell contact, as seen those in meningiomas and various carcinomas. In human schwannomas, however, E-cadherin, alpha E-catenin, and beta-catenin were detected by western blotting and immunohistochemistry, whereas alpha N-catenin was not. Immunoprecipitation using anti-E-cadherin antibody resulted in alpha E-catenin forming a complex with Ecadherin. SSCP analysis revealed no mutations in the transmembrane domain or in intracellular cateninbinding site of E-cadherin. These data suggest that the Ecadherin-alpha E-catenin complex is well preserved in human schwannoma cells, which is compatible with its benign behavior, and these molecules might be used as additional cell markers of Schwann cell-derived tumors.

Key words: Brain tumor, Cell adhesion molecule, Human schwannoma, Immunohistochemistry, Immunoprecipitation, Peripheral nerve sheath tumor, Tumorigenesis

Introduction

The Ca²⁺-dependent cell adhesion molecule Ecadherin and its associated cytoplasmic proteins, alpha-, beta-, and gamma-catenins, are known to play a crucial role in epithelial cell-cell adhesion and maintenance of tissue architecture (El-Bahrawy and Pignatelli, 1998). Pathological examinations have shown that reduced Ecadherin immunoreactivity is associated with dedifferentiation and metastasis in primary human tumors in vivo, including those of head and neck (Schipper et al., 1991), breast (Gamallo et al., 1993), and prostate (Umbas et al., 1992). Perturbation in the expression or function of any of these molecules results in loss of intercellular adhesion, with possible consequent cell transformation and tumor progression. Normal and reactive Schwann cells synthesize several neurite outgrowth-promoting molecules and localize them in either the extracellular matrix (e.g. laminin) or on the plasma membrane (e.g., L1/NgCAM). Recently, our studies, together with others, have demonstrated that E-cadherin (Uchiyama et al., 1994; Fannon et al., 1995; Hasegawa et al., 1996) and catenins (Muramatsu et al., submitted) are also expressed in normal and reactive Schwann cells in rodents, suggesting that Ecadherin/catenin complex may play a role in supporting the architecture of the Schwann cell cytoplasmic channel network. However, little is known concerning cadherin/catenin complex in normal human nerves and schwannoma cells, which are supposed to be derived from Schwann cells (Cravioto, 1969). E-cadherin is known to be expressed in myelin sheath in normal human nerves (Roche et al., 1997), and there has been confusion concerning E-cadherin expression of schwannoma cells; schwannoma cells have been reported negative for E-cadherin in some report (Schwechheimer et al., 1998) or positive in others (Roche et al., 1997). Therefore, the question has been raised as to whether schwannoma cells, which are individually wrapped by basal lamina with no relation to neural processes, still constantly preserve the expression

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of these proteins, or not. Interestingly, in this study, the expression of E-cadherin, alpha E-catenin and beta-catenin were well preserved in the schwannoma cells.

Materials and methods

Tissues

Intracranial and spinal schwannomas were collected surgically from 18 patients (age 22-69). Specimens of ten meningotheliomatous meningiomas, one low-grade glioma, one normal brain surgically obtained, and 6 normal peripheral nerves obtained at autopsy were also used.

Immunoblot analysis

Tissues fragments from all samples were dispersed mechanically and dissolved in a 2% sodium dodecyl sulfate sample buffer, sonicated and boiled in the same buffer containing 5% 2-mercaptoethanol. After the total amounts of proteins applied to each lane were adjusted to equal concentrations, samples were separated by SDSpolyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in TBS-Ca and incubated with primary antibodies. The following monoclonal antibodies were used as primary antibodies; HECD-1 for E-cadherin (Takara Co, Japan), NCAT-2 for alpha Ncatenin (Hirano et al., 1992) (courtesy of Dr. M. Takeichi), alpha-18 for alpha E-catenin (Nagafuchi et al., 1994) (courtesy of Dr. A. Nagafuchi), and mouse antibeta-catenin monoclonal antibody (Transduction Laboratories, Lexington, KY, USA). Alkaline phosphatase-conjugated goat anti-rat or mouse IgG antibody (Zymed, CA) and alkaline phosphatase substrate kit (Vector Labs) were used for signal detection.

Immunoprecipitation analysis

All steps in the following immunoprecipitation protocol were carried out either on ice or at 4 °C. To 200 µl of protein A-Sepharose (Pharmatica) 10 µg of HECD-1 was added and the mixture was incubated for 2 hrs. The tissues (n=2) were homogenized in the extraction buffer (0.5% Nonidet P-40, 2mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 2mM CaCl₂ in 50mM TBS), and centrifuged at 1300 rpm for 20 minutes. The supernatants were preabsorbed by incubation with protein A-Sepharose, and incubated with the antibody-coated protein A-Sepharose overnight. The beads were collected by centrifugation, washed 6 times with the extraction buffer, and suspended in SDS sample buffer with 5% 2-mercaptoethanol at 95 °C for 5 minutes. The released materials were used for immunoblotting.

Immunohistochemistry

The resected materials were fixed in 4% paraformaldehyde, and embedded in Tissue-Tek O.C.T. Compound (Miles, Elkhart, IN), and frozen in liquid nitrogen. Details of the experimental procedure for the immunohistochemistry have been described elsewhere (Uchiyama et al., 1994; Hasegawa et al., 1996; Seto et al., 1997). Briefly, sections were cut at 6-µm thickness by Cryostat (Miles, Elkhart, USA) and mounted on poly-L-lysine-coated glass slides. To quench endogenous peroxidase activity, the sections were treated with 3% H_2O_2 in methanol at -20 °C for 20 minutes. To block nonspecific antibodies, the sections were incubated with blocking serum in Tris-buffered saline-Calcium (TBS-Ca) for 20 minutes at room temperature. Negative control sections were treated in the same way omitting the primary antibody. The immunoreaction was visualized by the immunoperoxidase method using biotinylated anti-rat or mouse IgG, avidin-biotinperoxidase complex (Vector Labs, Burlingame, CA), and diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO).

PCR/SSCP analysis for detection of E-cadherin gene variations

DNA was extracted from the schwannomas (n=6) and three samples of normal peripheral blood lymphocytes using the QIAamp Tissue Kit (QIAGEN Inc., Valencia, USA). Screening for mutation in exons 4-16 of the E-cadherin gene, which encode extracellular, transmembrane, and cytoplasmic domains, was carried out by PCR/SSCP compared with the normal leukocytederived DNA. Exons 1-3, which are known as precursor sequence, were not included. PCR primers and PCR/SSCP condition were described by Berx et al. (1997). All primers were labeled with Cy5 (Amersham pharmacia biotech) at the 5' end. Fluorescent PCR products were electrophoretically separated on acrylamide gels (the methylene-bis-acrylamide: acrylamide ratio was 1:49), performed at 40W with a cooling device by water at 16 °C, followed by analysis by an automated DNA sequencer (Amersham Pharmacia biotech Model ALFred) with the fragment analysis program (Amersham Pharmacia Biotech Allele Links version 1.00).

Results

Immunoblot analysis

The immunoblot analysis (Fig. 1) showed that all schwannomas (n=18) as well as normal peripheral nerves (n=6) examined expressed E-cadherin. Meningiomas as positive control (Tohma et al., 1992) showed a similar reaction (n=10). In contrast, the glioma and normal brain did not express E-cadherin. Alpha E-

catenin was expressed in 17 out of 18 schwannomas. Peripheral nerves, brain and meningiomas also expressed alpha E-catenin but the glioma was negative. In contrast, none of the schwannomas nor peripheral nerves expressed alpha N-catenin, whereas the glioma and normal brain expressed alpha N-catenin. All specimens examined expressed beta-catenin.

Immunoprecipitation analysis

Western blotting of alpha E-catenin after immunoprecipitation using anti-E-cadherin antibody showed a band comigrating with alpha E-catenin, indicating that alpha E-catenin forms a complex with Ecadherin in schwannomas (n=2) (Fig. 2).

Immunohistochemical analysis

Immunohistochemically, tumor cells in the schwannomas expressed E-cadherin, alpha E-catenin,



Fig. 1. Representative Western blot analyses of the normal peripheral nerve (lane 1), schwannomas (lanes 2-7), meningioma (lane 8), astrocytoma (lane 9) and normal brain (lane 10). a. E-cadherin is expressed in the normal peripheral nerve, the schwannomas and the meningioma but not in the astrocytoma or normal brain. Molecular weight standards (126, and 71kD) are indicated to the left of the blot. The weaker bands lower than Mr. 100,000 appeared to be degradation products of E-cadherin. b. Alpha E-catenin is expressed in the peripheral nerve, the schwannomas, the meningioma and in the normal brain, but not in the astrocytoma. Molecular weight standards (102kD) are indicated to the left of the blot. c. Alpha N-catenin is expressed in the normal brain and the astrocytoma, but not in the peripheral nerve, schwannomas or meningioma. Molecular weight standards (102kD) are indicated to the left of the blot. d. Beta-catenin is expressed in the schwannomas, the meningioma, the astrocytoma, and the normal brain. Molecular weight standards (126, and 71kD) are indicated to the left of the blot.

and beta-catenin, whereas interstitial tissues and vessels did not (Fig. 3). All molecules examined were not detected intranuclearly. Membranous localization could not be determined on light microscopic level. Normal nerve and meningioma were positive for E-cadherin, whereas glioma and normal brain tissue were negative for E-cadherin.

PCR/SSCP analysis

Exons 4-16 of the E-cadherin gene, which include six putative Ca^{2+} -binding regions of extracellular domain, transmembrane domain and intracellular domain which interact with catenins and cytoskeleton, were analyzed. In our study, however, no alternations in the E-cadherin gene were detected.

Discussion

This is the first report that E-cadherin, alpha Ecatenin and beta-catenin are co-expressed, and that Ecadherin forms complex with alpha E-catenin in schwannomas. There are reports of several Schwann cell markers in normal and myelinating Schwann cells, including myelin-related proteins such as myelin basic protein, P0, P2, and others. However, they are not expressed commonly in neoplastic Schwann cells (Clark et al., 1985). The mRNA of proteolipid protein (PLP), the major structural protein of central nervous system myelin, is also expressed in Schwann cells and their tumors, but PLP itself is not present in the tumors at the protein level (Puckett et al., 1987). In this study, normal peripheral nerve tissue and schwannomas were positive for both E-cadherin and alpha E-catenin, whereas normal brain and glioma tissue were not. These data are consistent with those of the normal Schwann cells in



Fig. 2. Western blotting of alpha E-catenin after immunoprecipitation using anti-E-cadherin. A band comigrating with alpha E-catenin is seen in both schwannoma and meningioma. An arrowhead indicates the positions of intact alpha E-catenin bands. A: original tissue; B: after immunoprecipitation; 1, 3: schwannoma; 2, 4: meningioma.

mice (Muramatsu et al., submitted). Among primary brain tumors, only meningiomas (Tohma et al., 1992) and choroid plexus papillomas (Figarella-Branger et al., 1995; Schwechheimer et al., 1998) exclusively express E-cadherin. So far, schwannoma cells have been reported negative for E-cadherin in some reports (Schwechheimer et al., 1998) or positive in others (Roche et al., 1997). In this study, however, E-cadherin immunoreactivity has been uniformly demonstrated in all benign schwannomas. Together with the positive expressions for cadherin-related molecules, alpha E- and beta-catenins, and immunoprecipitation analysis in our series, it is conceivable that benign schwannomas preserve the E-cadherin immunoreactivity. In addition, glioma cells were positive for alpha N-catenin, and negative for alpha E-catenin as well as E-cadherin. Since little is known about expression of the subtypes of catenins in human brain tumors, further study would give us useful information for the differential diagnosis.

Catenins form the link between E-cadherin and the actin cytoskeleton, and beta-catenin (or plakoglobin) is

believed to be the first catenin to interact with Ecadherin (Nagafuchi and Takeichi, 1989; Hinck et al., 1994). Functional defects of cadherin-mediated cell contact are explained by (1) a lack of catenins including alpha (Shimoyama et al., 1992) and beta-type (Oyama et al., 1994) and plakoglobin (Navarro et al., 1993), resulting in invasiveness and metastasis of tumor cells (Kadowaki et al., 1994; Sommers et al., 1994), and (2) functionally inactivating mutations of E-cadherin gene exons 7-16 (Becker et al., 1994; Kanai et al., 1994; Risinger et al., 1994). In this study, however, E-cadherin and catenins were not only co-expressed in schwannomas, but also E-cadherin-catenin complex was detected by immunoprecipitation analysis. Furthermore, no E-cadherin gene alternations were detected in exon 4-16, which includes six putative Ca^{2+} -binding regions of the extracellular domain, the transmembrane domain and the intracellular domain, which interact with catenins and the cytoskeleton. There have been several functional regulators of cadherin-catenin complex reported. For example, tyrosine phosphorylation of the E-



Fig. 3. Immunohistochemistry of E-cadherin, alpha E-catenin and beta-catenin in schwannoma. The reaction products are detected in almost all of the tumor cells, but not in the vessel structure. x 130

cadherin/beta-catenin (Behrens et al., 1993), aberrant CpG island hypermethylation (Graff et al., 1995, 1998), and enlarged cell-associated proteoglycans (Vleminckx et al., 1994) abolish/suppress the function of E-cadherin, whereas exogenous insulin-like growth factor I or Tamoxifen have been reported to activate/restore the function (Bracke et al., 1993, 1994). Further studies are necessary to reveal whether these regulators and others including posttranscriptional modification play a role in schwannomas.

At the conventional ultrastructural level, schwannoma cells are known to have basically the same characteristics as Schwann cells. There are numerous thin undulating cytoplasmic processes coated by electron-dense basement membrane material. Both myelinating and non-myelinating Schwann cells individually have junctional complexes at cell-cell contact areas including inner- and outer mesaxons and Schmidt-Lanterman incisures, where E-cadherin is strongly expressed (Uchiyama et al., 1994, Fannon et al., 1995). In reactive Schwann cells, E-cadherin is distinctively localized at the cell-cell contact areas between adjacent Schwann cells or between processes of a single Schwann cell (Hasegawa et al., 1996). However, compared to these normal and reactive Schwann cells, schwannoma cells have sparse junctional complexes (Dickersin, 1987; Hasegawa et al., 1992). E-cadherin immunoreaction product, in our preliminary study, was diffusely observed along the membrane of the schwannoma cells on the immunoelectron microscope (data not shown). Our previous study in human arachnoid villi and meningiomas using the same anti-Ecadherin antibody reported similar findings (Tohma et al., 1992). E-cadherin was localized at the junctional apparatus in arachnoid cells, while it was detected diffusely at the cell surface of arachnoid cell derivedtumor meningiomas. A similar phenomenon has been reported in pathological skin (Tada and Hashimoto, 1998). In this study, however, we found no evidence of E-cadherin dislocation: SSCP analysis revealed normal sequences including the extracellular, transmembrane, and cytoplasmic domains of E-cadherin gene. Furthermore, the immunoprecipitation method showed that E-cadherin, at least in part, binds to alpha E-catenin. Further studies are mandatory to elucidate the subcellular localization and function of Ecadherin/catenin complex in schwannomas.

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