Mini-Review

Submembranous Junctional Plaque Proteins Include Potential Tumor Suppressor Molecules

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THE multistep theory of tumorigenesis is now widely accepted. During the tumor development the genetic alterations are accumulated, oncogenes are activated, and tumor suppressor genes are inactivated. Recently, the interesting idea is emerging that some cell adhesion molecules, integral membrane proteins, may work as tumor suppressors (Hedrick et al., 1993). Most of these cell adhesion molecules are known to be intimately associated with cytoskeletal proteins underlying plasma membranes (peripheral membrane proteins) to form a specialized membrane domain called a junctional apparatus including tight junctions (TJ)[1], adherens junctions (AJ), and desmosomes (DS) (Fig. 1). These peripheral membrane proteins concentrated in a "plaque" are thought to play a role in signal transduction from adhesion receptors (Juliano and Haskill, 1993) and in the regulation of adhesion molecules (Takeichi, 1991). Recently, the application of gene engineering techniques to plaque proteins has generated a wealth of novel observations, leading to the speculation that some of these proteins are required to suppress the development of tumors, i.e., act as a kind of "tumor suppressors." Here we review these findings and discuss some functions of the plaque proteins in normal cells as well as in tumorigenesis.

Tumor Suppressor Genes

The definition of "tumor suppressor gene" is not clear at present. Originally, the existence of tumor suppressor genes was suggested by the genetic studies of tumor formation and suppression in platyfish–swordtail hybrids (Anders, 1967) and in *Drosophila* (Gateff and Schneiderman, 1974). Fusion experiments between normal and tumor cells provided a way to analyze mammalian tumor suppressor genes: normal tumor cell hybrids often display a nonmalignant phenotype, suggesting that "tumor suppressors" derived from the genome of a normal cell normalize the growth patterns of their malignant partners (Harris, 1988). Along this line, some genes were recently identified as "tumor suppressor genes" due to the fact that expression of their products in transformed cells resulted in the suppression of the malignant phenotype, i.e., a reduced ability to grow in soft agar, suppression of the development of tumors in vivo.

Another method for the identification of tumor suppressor genes is to identify the chromosomal gene locus whose defects cause various types of human familial tumors, and to isolate these genes by molecular cloning. For the chromosome assignment, specific losses of heterozygosity (LOH) in tumors are often analyzed. Usually in this case, it is not clear what types of malignant phenotypes (overproliferation, transformation, dedifferentiation, invasion, or metastasis) the alterations of these genes are responsible for, but these genes are called "tumor suppressor genes."

In this mini-review, we use the word "tumor suppressor" in an extended sense. The tumor suppressors are tentatively defined here as proteins which suppress one or several malignant phenotype criteria of tumor formation, including dedifferentiation, invasion, and metastasis.

Tight Junctions

The TJ is an element of epithelial and endothelial junctional complexes (Fig. 1). In polar epithelial cells, it seals the cells together at a subapical position and creates the primary barrier to the diffusion of solutes in the paracellular pathway. It also works as a boundary between the apical and basolateral plasma membrane domains to create cell polarization (Gumbiner, 1987; Stevenson et al., 1988). The molecular organization of TJ is poorly understood; presently, there is no information on the integral membrane proteins of TJ, especially adhesion molecules. So far, by mAb production, Z0-1 (220 kD), cingulin (140 kD), 7H6 (155 kD), and Z0-2 (160 kD) were reported as undercoat-constitutive proteins localized at TJ (Citi, 1993). Among cDNAs encoding these proteins, only the Z0-1 cDNA has been isolated and completely sequenced (Itoh et al., 1991, 1993; see "Note").

Although it was not mentioned in the original paper of Itoh et al. (1993), close comparison of the amino acid Z0-1 sequence with the sequences of other proteins revealed that the amino-terminal half of this protein displays significant similarity to the product of the lethal(1)discs large-1(dlg) gene in *Drosophila* (Fig. 2; see "Note"). In *Drosophila*, so far seven tumor suppressor genes have been identified by isolating recessive lethal mutations in which cell prolifera-

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1. *Abbreviations used in this paper*: AJ, adherens junctions; DS, desmosomes; ERM, ezrin-radixin-moesin; LOH, loss of heterozygosity; NF2, neurofibromatosis 2; TJ, tight junctions.

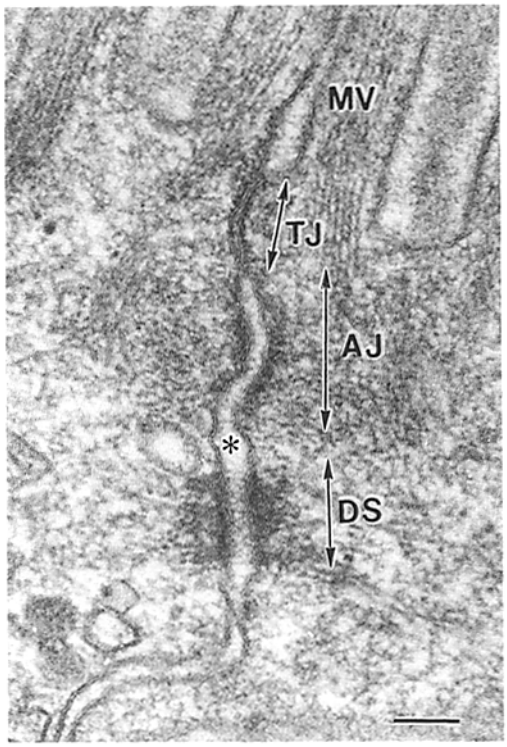

Figure 1. The junctional complex in intestinal epithelial cells. The junctional complex consists of the tight junction (*TJ*), adherens junction (*AJ*), and desmosome (*DS*). Adherens junctions and desmosomes are characterized by a well developed plasmalemmal undercoat, i.e., the plaque structure. (*MV*) microvilli; (*) intercellular space. Bar, 0.1 μm.

tion in imaginal discs continue beyond the normal limits (Gateff and Mechler, 1989). Sequence analysis of the cDNA of dlg, one of the *Drosophila* tumor suppressor genes, reveals that its product contains some putative functional domains: a filamentous domain, an SH3 domain, and the guanylate kinase domain (Woods and Bryant, 1991). As shown in Fig. 2, four regions are conserved between the amino-terminal half of Z0-1 and the dlg gene product, and three of them are included in the filamentous and guanylate kinase domains of the dlg gene product. Interestingly, in *Drosophila* epithelial cells, the dlg gene product was reported to be localized in the undercoat of the septate junctions (Woods and Bryant, 1991), which are thought to be the counterpart of the vertebrate TJ (Noriot-Timothee and Noriot, 1980).

From the above we speculate that Z0-1 can act as a tumor suppressor in vertebrate cells. This speculation, of course must be evaluated by further functional analysis of Z0-1. Furthermore, the structure and function of the other components of TJ should be analyzed to clarify the relationship between TJ and tumorigenesis.

Adherens Junctions

The AJ represent a specialized class of cell-to-cell and cell-to-substrate associations: the major adhesion molecules are cadherins and integrins, respectively (Geiger, 1983; Geiger et al., 1985; Hynes, 1987; Burridge et al., 1988; Takeichi, 1991) (see Fig. 1). This type of junction is characterized by a well-developed undercoat (plaque) on the cytoplasmic surface of the junctional membrane with which bundles of actin filaments are tightly associated. Since AJ are commonly believed to be directly involved in tissue morphogenesis, the molecular architecture of the plaque of both cell-to-cell and cell-to-substrate AJ has attracted interest (Burridge et al., 1988; Tsukita et al., 1992).

The first protein reported to be concentrated at AJ was α-actinin (Lazarides and Burridge, 1975). This protein with a molecular mass of 100 kD is an abundant cytoplasmic actin-binding protein and found along microfilaments and in the plaque of both cell-to-cell and cell-to-substrate AJ. The

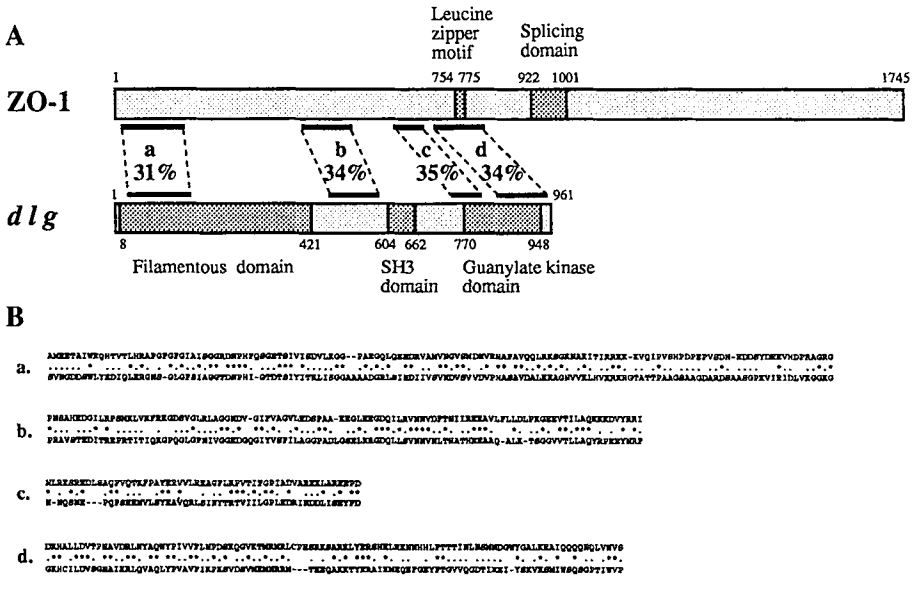

Figure 2. Comparison of mouse ZO-1 with *Drosophila* dlg gene product. (*A*) Schematic structures of mouse ZO-1 and *Drosophila* dlg gene product. The dlg gene product contains three putative functional domains; filamentous domain, SH3 domain, and guanylate kinase domain. Boundaries of each domain are shown by the number of boundary residues. Four regions (*a–d*) are conserved between the amino-terminal half of ZO-1 and the dlg gene product. Respective regions correspond to amino acids 12–144, 409–510, 613–667, 701–801 in ZO-1 and to amino acids 29–163, 472–573, 743–793, 859–955 in the dlg gene product. The SH3 domain in dlg shows 38% identity to that in crk, whereas the corresponding region in mouse ZO-1 is not so similar to the crk SH3 (28%), restraining us from concluding that mouse ZO-1 includes an SH3 domain. (*B*) Comparison of amino acid sequences between mouse ZO-1 (*upper sequence*) and *Drosophila* dlg gene product (*lower sequence*). The amino acid sequences of the four homologous domains (*a–d*) are aligned. Asterisks and dots denote identity and homology, respectively, and dashes indicate gaps introduced to optimize the alignment. The sequences were initially aligned by means of the MAXMH computer program.

second major protein was vinculin (molecular mass 116 kD) which is associated with the plaques of both cell-to-cell and cell-to-substrate AJ (Geiger, 1979). The complete amino acid sequences of these proteins have been determined by cDNA cloning (Arimura et al., 1988; Baron et al., 1987; Coutu and Craig, 1988; Price et al., 1989). Using these cDNAs, in cell transfection experiments Ben-Ze'ev's group has shown that both α-actinin and vinculin have a suppressive effect on the transformed phenotype (Glück et al., 1993; Rodriguez Fernández et al., 1992). In their experiments, human α-actinin cDNA was transfected into highly malignant SV-40-transformed mouse fibroblasts expressing six-fold lower levels of endogenous α-actinin, and chicken vinculin cDNA was introduced into two mouse tumor cell lines expressing diminished levels of the endogenous vinculin. Restoration of α-actinin and vinculin in these cells, up to the level of normal cells, resulted in an apparent increase in substrate adhesiveness, a decrease in the ability to grow in soft agar, and suppression of tumor development and metastases after injection into syngeneic hosts or nude mice. These findings indicate that α-actinin and vinculin can act as tumor suppressors, at least in the experimental mouse system.

Radixin is a barbed end-capping, actin-modulating protein first identified as one of the major plaque proteins isolated from cell-to-cell AJ in rat liver (Tsukita et al., 1989). Sequence analysis of the radixin cDNA demonstrated that radixin is highly homologous to ezrin and moesin, pointing to the existence of the ezrin-radixin-moesin (ERM) family (Funayama et al., 1991; Sato et al., 1992). Ezrin was first identified as one of the components of intestinal microvilli (Bretscher, 1983; Gould et al., 1989; Turunen et al., 1989), and moesin originally thought to be an extracellular protein, was later found to be an intracellular protein (Lankes and Furthmayr, 1991). As shown in Fig. 3, among the ERM family members, amino acid substitutions in the amino-terminal half of each member are highly conservative: showing ~83% identity in the amino-terminal half and ~65% in the carboxy-terminal half. The amino-terminal half domain also shows some similarity to the amino-terminal half of the band 4.1 protein, one of the undercoat-constituent proteins of erythrocyte membranes. Recent detailed immunolocalization has also clarified that ERM family members are concentrated at the plaque of the cell-to-cell and cell-to-substrate AJ, at cleavage furrows, microvilli, and ruffling membranes (Sato et al., 1992). The most prominent feature shared by these sites is the tight and dense association of actin filaments with plasma membranes, leading us to conclude that ERM family members play a crucial role in the actin filament/plasma membrane association in general. Surprisingly, these ERM family members were recently found to have a close relationship to the tumor suppressor protein of neurofibromatosis 2 (NF2), one of the two major human genetic disorders both of which display autosomal dominant inheritance and involve tumors of the nervous system (Trofatter et al., 1993; Rouleau et al., 1993). Genetic linkage studies of NF2 had revealed that this familial tumor is caused by inactivation of a tumor suppressor gene in chromosome 22q12 (Rouleau et al., 1987), and recently, a candidate for the NF2 tumor suppressor gene was cloned. The candidate gene was shown to encode a cytoplasmic protein with a striking similarity to ERM family members, and was named "merlin" (moesin-ezrin-radixin-like protein) or "schwannomin". Clusters of amino acids identical in merlin and the ERM family is concentrated in the amino-terminal half of each molecule (~62% identity) (Fig. 3). Therefore, it is tempting to speculate that merlin, and probably other ERM family members, act as tumor suppressors by affecting the actin filament/plasma membrane association.

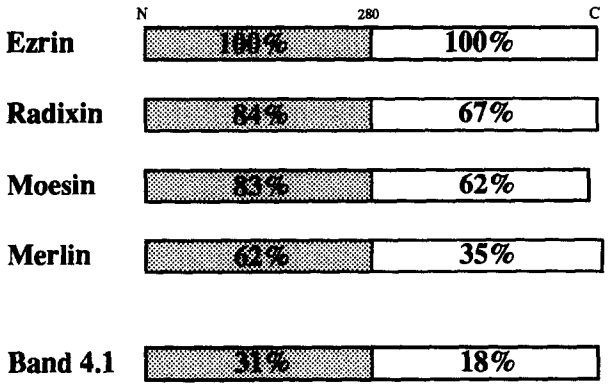

Figure 3. ERM family and merlin. The structures of mouse ezrin, mouse radixin, mouse moesin, human merlin, and human band 4.1 protein are compared. These proteins have a homologous domain (▩) in their amino-terminal half. The identity of the amino-terminal and carboxyl-terminal halves of each protein with those of ezrin was calculated and shown in each box.

Three cytoplasmic proteins were reported to be co-precipitated with cadherin molecules in immunoprecipitation experiments with anti-cadherin antibodies (Ozawa et al., 1989; Vestweber and Kemler, 1984; Peyrieras et al., 1985): α-(102 kD), β-(88 kD) and γ-(80 kD) catenin. Deletion and mutagenesis experiments with cadherin molecules clearly revealed that cadherin molecules lacking the catenin binding sites cannot bind to the cytoskeleton or connect cells to each other (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989).

Recently, α-catenin cDNA was isolated and its deduced amino acid sequence revealed that α-catenin resembles vinculin (Nagafuchi et al., 1991; Herrenknecht et al., 1991). Using a mAb called α-18 which clearly recognizes human α-catenin (Nagafuchi, A., and Sh. Tsukita, manuscript submitted for publication), Hirohashi's group has examined the expression level of α-catenin in a human lung cancer cell line, PC9. PC9 cells had been known to strongly express normal E-cadherin molecules on their cell surface, but to show reduced cadherin-mediated aggregation activity, resulting in poorly differentiated round-shaped cells. Immunoprecipitation, immunoblot and PCR analyses have shown that PC9 cells lacked the expression of intact α-catenin due to the homologous deletion of its gene (Shimoyama et al., 1992; Oda et al., 1993). Furthermore, transfection of the cDNA encoding α-catenin into PC9 cells brought about a drastic restoration of cell aggregation activity, resulting in cultures of a well-differentiated epithelial cell (Hirano et al., 1992). Most recently, using mAb α18, the loss of α-catenin expression was also observed in a human prostate cancer cell line, PC3, which shows a high expression level of E-cadherin but lacks cell-cell adhesion ability (Morton et al., 1993). Therefore, we are led to conclude that, at least in PC9 and PC3 cells, α-catenin is a key protein not only to exert the cadherin-

dependent cell adhesion but also to 'suppress' dedifferentiation phenomena. The expression of α-catenin in human adenocarcinomas was then examined, especially those of the scirrhous-type, in which cancer cells are characteristically detached from each other to form single cells that infiltrate the connective tissue in a scattered manner (Ochiai et al., 1994; Kadowaki et al., 1994). These scattered cancer cells were reported occasionally to express E-cadherin at a normal level, leading us to speculate that, like PC9 cells, these scattered cancer cells also lack α-catenin.

The scirrhous-type adenocarcinomas with scattered cell growth are most commonly found in the stomach and the breast. Interestingly, close immunohistochemical analysis showed apparently complete loss of α-catenin expression in gastric and breast scirrhous adenocarcinomas in ~55% and ~75% of the cases studied, respectively. Most recently, the human α-catenin gene was assigned to chromosome 5q21 and/or 31, for which a LOH had frequently been reported (Furukawa et al., 1993; Morton, R. A., C. M. Ewing, J. J. Wasmuth, J. D. McPherson, J. Overhauser, A. Nagafuchi, Sh. Tsukita, and W. B. Isaacs, manuscript submitted for publication): the APC gene, a tumor suppressor gene in the familial adenomatous polyposis, is also located in this chromosome region (Kinzler et al., 1991). And all these findings lead us to suggest that the loss of α-catenin may be a key step for the scattered phenotype of the scirrhous-type adenocarcinomas, i.e., that α-catenin is a suppressor of tumor cell invasion as well as of cell dedifferentiation. Therefore, α-catenin is a good candidate for the "suppressor" in scirrhous-type adenocarcinoma with scattered cell growth.

Desmosomes

The DS, another cell-to-cell junctional apparatus, is characterized by a prominent plaque, through which intermediate-sized filaments are tightly and densely associated with the plasma membrane (see Fig. 1). The adhesion molecules working in DS are called desmogleins and desmocolins, which belong to the cadherin superfamily (Koch et al., 1990; Buxton et al., 1993). Since DS can be isolated from bovine muzzle epidermis, most of the major plaque proteins have already been identified (Schwarz et al., 1990) and cDNAs encoding some plaque proteins such as desmoplakin I & II, plakoglobin etc., have been sequenced (Green et al., 1988, 1990; Franke et al., 1989). So far, only one plaque protein, desmoyokin, was suggested to have something to do with the tumor suppression, but this relationship remains to be further evaluated in detail (Hieda et al., 1989; Hashimoto et al., 1993).

Conclusions

The results discussed above favor the idea that junctional plaque proteins play a crucial role in suppression of the malignant phenotypes of tumors such as overproliferation, transformation, dedifferentiation, invasion, and metastasis. Recently, some proto-oncogenic tyrosine kinases such as c-yes and c-src kinases were found to be concentrated at the cadherin-based AJ plaque (Tsukita et al., 1991), and a unique tyrosine kinase called FAK to be involved in the signal transduction in the integrin-based AJ plaque (Juliano and Haskill, 1993). Therefore, the junctional plaque structure appears to have an intimate relationship with both tumor suppressor gene and oncogene products.

The question is then how junctional plaque proteins are involved in the suppression of the malignant phenotypes of tumors. As to the overproliferation, two explanations are possible. The first explanation is that the major task of junctional plaque proteins is to bring together adhesion receptors and signal-transducers involved in the negative regulation of cell growth at a specialized membrane domain (junctional apparatus). This mechanism is expected to drastically upregulate the efficiency of the signal transduction. Alternatively, the junctional plaque protein itself may act as a signal transducer to transmit the information from adhesion receptors to the cell's interior, although at present how these cytoskeletal proteins underlying the plasma membrane are directly involved in the signal transduction system remains unknown. As to the other malignant phenotypes, the explanation may be simple: the junctional plaque proteins may be directly involved in the regulation of the organization of cytoskeletons, cell adhesion, and cell motility.

In future, genetic and biochemical experiments with *Drosophila* tumor suppressor genes will shed light on this field. In this connection, interestingly, the lethal giant larvae (l[2]gl) protein, one of the *Drosophila* tumor suppressor genes, was recently reported to be localized at the undercoat of the cell-to-cell adhesion sites, just like dlg products (Török et al., 1993). The technique of gene targeting in mice will also be powerful in this field. The findings reviewed here indicate that in "normal cells" the submembranous plaque proteins play crucial roles in the negative regulation of cell growth, the determination of cell shape and differentiation, the regulation of cell adhesion and cell motility. If we continue to further investigate the relationship between these proteins and tumorigenesis, we may soon be able to understand the role of the submembranous junctional plaque proteins in normal cells.

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Note: Since submission of this mini-review, we received a preprint of the article entitled "The tight junction protein ZO-1 is homologous to the *Drosophila* discs-large tumor suppressor protein of septate junctions" (Willott, E., M. S. Balda, A. S. Fanning, B. Jameson, C. V. Itallie, and J. M. Anderson. 1993. *Proc. Natl. Acad. Sci. USA.* In press), in which the cDNA encoding "human" ZO-1 protein was cloned and sequenced. Comparison between this "human" ZO-1 and "mouse" ZO-1 (Ito et al., 1993) reveals that they are highly homologous not only within the coding regions (~90% identity) but also in 3'- and 5'-noncoding regions.

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