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The tumor antigen epcam: tetraspanins and the tight junction protein claudin-7, new partners, new functions

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1. ABSTRACT

The cell-cell adhesion molecule EpCAM / CD326 has been one of the first tumor-associated antigens and has soon received attention as an antibody target in cancer therapy. However, only recently, progress has been achieved in disclosing the array of functional activities of EpCAM and the underlying molecular mechanisms. This review will particularly focus on cooperative activity of EpCAM with two classes of transmembrane molecules, tetraspanins and claudins. EpCAM can associate with claudin-7 and the tetraspanins CD9 and CO-029. We propose that complex formation of EpCAM with tetraspanins and claudins does not only interfere with EpCAM-mediated homotypic cell-cell adhesion, but importantly, is also associated with a gain of function, like induction of apoptosis resistance.

2. INTRODUCTION

Already for more than 2 decades, the epithelial cell-cell adhesion molecule EpCAM serves as a target structure in cancer therapy (1). Its therapeutic use has been based on overexpression in many types of carcinoma, which as such is surprising, as one would expect tumor progression to be accompanied by downregulation of a cell-cell adhesion molecule, as e.g. described for E-cadherin (2). Surprisingly, too, antibody therapy displayed few side effects on normal epithelium, that also differs for other rather ubiquitously expressed molecules with overexpression in tumor tissue (3). Recent progress in EpCAM-mediated signal transduction and its contribution to gene transcription unravelled its tumor growth promoting activities and provided hints towards an explanation for the discongruent activities in cell-cell

adhesion versus tumor progression and antibody susceptibility of tumor, but not normal epithelial cells (4).

This review will focus on the association of EpCAM with additional transmembrane molecules, which we hypothesize to have major bearing on EpCAM's functional activities. Tetraspanins, in particular CD9 and CO-029, and the tight junction protein claudin-7 have recently been described to associate with EpCAM (5-7). Tetraspanins are known as molecular facilitators that form a net between themselves and other transmembrane molecules in glycolipid-enriched membrane microdomains, which also harbour signal transducing and adaptor molecules, such that a multitude of signaling cascades can be initiated by the tetraspanin complexes (8,9). Claudins have originally been described as the major component of tight junctions that are important for cell polarity and paracellular transport. Only recently it has been observed that at least some members of the claudin family, including claudin-7 are also found basolaterally. The functions claudins exert outside of tight junctions are unknown. It is hypothesized that they may contribute to vesicle stability or vesicle transport (10-12). We propose that EpCAM-tetraspanin-claudin-7 complex formation interferes with EpCAM-mediated homotypic cell-cell adhesion and supports apoptosis resistance.

We will first introduce the EpCAM molecule inasmuch as its structural features are important for the association with other transmembrane molecules. After providing an overview on tetraspanins and claudins with special emphasis on their relation to tumor growth and progression, we will provide our working hypothesis on how EpCAM, tetraspanins and claudin-7 could promote tumor growth and progression in a concerted action.

3. EPCAM

3.1. EpCAM: structure and function

The epithelial cell adhesion molecule EpCAM is a type I transmembrane molecule of 314 AA. It is a panepithelial marker, which is enriched at the basolateral membrane (13,13). Overexpression of EpCAM is frequently observed in various types of carcinomas. Despite its constitutive expression, a blockade of the molecules by EpCAM-specific antibodies can be of therapeutic benefit (15-17). Moreover, self tolerance towards EpCAM can be broken, so that the generation of EpCAM-specific T cells opens an additional therapeutic option (18,19).

EpCAM is a Ca^{++}-independent, homophilic cell-cell adhesion molecule with an EGF-like domain, followed by a thyroglobin repeat domain (20,21), a cysteine poor region, a transmembrane domain and a short cytoplasmic tail (21-25). Both the EGF-like repeat and the thyroglobin domain form a globular structure and are required for the homophilic cell-cell adhesion of EpCAM. The EGF-like domain is required for the reciprocal cell-cell interaction and the thyroglobin-like domain for the lateral interaction of EpCAM molecules. Both domains are also required for the anchoring of actin microfilaments at the cell membrane via α-actinin, a process regulated by the cytoplasmic tail of EpCAM (26). EpCAM has a particular proteolytic cleavage site near the N-terminus, and cleavage has been suggested to be accompanied by conformational changes, which may have impact on the function of the molecule (27). Thyroglobin domains are known to inhibit cathepsins. Whether EpCAM, indeed, serves as a protease inhibitor, to protect the tumor cell from degradation, remains to be explored.

Not much is known on the regulation of EpCAM expression. The EpCAM promoter has no TATA box, and does not contain CCAAT, Ker1 or E-pal transcription factor recognition sequences. Yet, the 687bp proximal promoter region contains transcription factor recognition sequences for Sp-1, AP-1, AP-2, Etss, ESE-1 and E-pal-like (28). It is also known that the EpCAM promoter becomes negatively regulated by NFκB, TNFα and IFNα (29).

The cell adhesion molecule EpCAM might be expected rather to prevent than to support metastasis formation (30-32). However, it has been reported that EpCAM is involved in the abrogation of E-cadherin-mediated cell-cell adhesion by disrupting the link between α-catenin and F-actin (13,33,34). In fact, EpCAM has been shown to be involved in signal transduction and to support cell motility (25,35-40). Overexpression of the EpCAM gene also induces upregulation of the proto-oncogene *c-myc* and supports cell proliferation via upregulated synthesis of cyclin A and E (39,41,42) and regulates E-FABP (epidermal fatty acid binding protein) expression (39). Finally, and likely to be most important for the tumor progression promoting activity of EpCAM, oligomerization of the molecule triggers signals that cleave an intracellular peptide of EpCAM, which requires cooperative activity of TACE (TNF converting enzyme) and PS2-NTF (persenilin 2 N-terminal fragment). This peptide, termed EpIC, forms a complex with ß-catenin and Lef-1. The complex relocates to the nucleus and by binding to Lef consensus sites initiates transcription of c-myc (4, M. Munz and O. Gires, personal communication). EpCAM transgenic mice, where EpCAM is overexpressed in mammary glands, support a role of EpCAM in mitogenic signaling with high level Bcl-2 and Ki67 expression (43). Also, there is evidence that EpCAM may be a stem cell marker in breast, pancreatic and colorectal cancer (44-47).

3.2. EpCAM and cancer

EpCAM is expressed on many epithelia (48). Few exceptions are epidermal keratinocytes, hepatocytes, thymic cortical epithelia, gastric parietal cells and myoepithelial cells (48). Non-epithelial cells, with the exception of plasma cells, lack EpCAM expression (49). According to the distribution in non-transformed tissue, nearly all carcinoma express EpCAM, including cervical, lung, breast, prostate, renal cell, colorectal and cutaneous squamous cell carcinoma, but also myeloma, the malignant counterpart to plasma cells (13,32,48-57). In carcinoma, EpCAM expression is mostly increased as compared to non-transformed tissue and EpCAM overexpression has been found to correlate with the grade and histological type (preferentially lobular) of breast cancer, decreased overall and poor disease free survival (32). In cervical cancer and

squamous cell carcinoma of the lung, EpCAM overexpression also correlates with grading and TNM staging, but not with survival (50,58). In colorectal cancer it appears to correlate with the disease-free survival, but not with grading and staging (59).

3.3. EpCAM-specific monoclonal antibodies in cancer therapy

The therapeutic potential of EpCAM-specific antibody has first been evaluated for colorectal cancer in nude mice (60,61). Several clinical studies have been performed with edrecolomab. Most studies report on benefits considering metastatic progression. With respect to the effect on the local tumor mass, results in different studies are divergent, that has been suggested to be due to the basolateral localization of the molecule, which could hamper access for the antibody in a solid tumor mass (rev. in 15). Yet, the preferential basolateral localization may not account for tumor cells, where EpCAM has been described to become redistributed over the plasma membrane (62). Irrespective of this matter, very few and mostly tolerable side effects have been reported, which is surprising taking the expression of EpCAM on non-transformed tissue (62-64). A new generation of antibodies, either bispecific or coupled to chemotherapeutic drugs is under consideration (65-67). Thus, a trifunctional antibody (anti-EpCAM x anti-CD3) (catumaxomab), where the Fc part of the antibody is taken as the third functionally important domain, is suggested to kill tumor cells via induction of apoptosis, release of cytokines and perforin as well as by antibody-dependent cellular cytototxicity (68,69). The antibody has been efficient in the treatment of patients with malignant ascites of ovarian cancer and peritoneal carcinomatosis from various solid tumors (70). An anti-EpCAM x anti-CD64 bispecific antibody (HEA125x197) has also been most efficient in the treatment of patients with ascites from ovarian cancer (71). EpCAM-specific antibodies also have been explored in non-small cell lung cancer patients, so far with promising results, that need to be further controlled (72). A fully human EpCAM-specific antibody, adecatumumab, is under investigation (73). The efficacy of an anti-erbB2 immunotoxin could also be increased by anti-EpCAM sFv (74).

This list of therapeutically used EpCAM-specific antibodies is by no means complete, as we only wanted to demonstrate that a molecule expressed on non-transformed cells can be efficiently used as a therapeutic target and without severe side effects. This is not only surprising, but makes it a demand to define the molecule's function that could well allow for more directed interference. At present we are still at the stage of a hypothesis, but two lines appear most promising. One line of interest has already been pointed out, the possibility that the cytoplasmic tail of EpCAM (EpIC) functions as part of a transcriptional complex (4,41). The second line of interest has a bias towards the authors field of interest, the modulation of the functional activity of transmembrane molecules by the formation of complexes in membrane subdomains, particularly those generated by the so called tetraspanin web. As EpCAM crossed independently both authors' way in being concerned about tetraspanins, we feel that EpCAM complex formation should receive intense consideration and may further help to elucidate the function of the molecule that already as a "black box" has proven its therapeutic relevance. We also speculate that the observation of EpCAM as part of a complex in glycolipid and tetraspanin enriched membrane microdomains and the discovery of its cytoplasmic domain to account for tumor growth promotion are possibly linked.

To substantiate our hypothesis, we will start to introduce the tetraspanin net and outline the EpCAM complex as far as it is known at present.

4. TETRASPANINS

4.1. Tetraspanins: protein complexes and membrane microdomains

Tetraspanins are a family of 34 proteins. The key feature of tetraspanins is their potential to associate with each other and with a multitude of molecules from other protein families (8,9,75-77). Tetraspanins span the membrane 4 times. The N- and the C-terminal domain and a short inner loop between the 2 extracellular loops of tetraspanins are located in the cytoplasm and are characterized by palmitoylation sites. Polar amino acids in the transmembrane regions are supposed to stabilize the structure and the conformation of the second extracellular loop, also called the large extracellular domain, which is divided into 3 constant and 1 variable regions between the second and the third constant region. This variable region is critical for protein-protein interactions (78-80).

Tetraspanins assemble a multitude of proteins into complexes attached to specific signal transducing molecules. The most prominent tetraspanin partners are integrins. Some integrins (αIIβ3, α3β1, α4β1 and α6β1) are found in tetraspanin complexes with high stoichiometry, whereas others (α2β1, α5β1, β2) are largely excluded (9,75,76,81-85). Many other molecules, besides integrins can associate with tetraspanins and various levels of interaction have been described. These levels of interaction mostly are defined by disruption of the interactions with detergents of increasing stringency. Type I interactions are direct protein-protein interactions, that take place early during biosynthesis, e.g. the association of CD151 with α3β1 and the homo-oligomerization of ROM-1 and peripherin/RDS. Type II interactions occur later during biosynthesis (Golgi or post-Golgi) and might be facilitated by palmitoylation of the tetraspanins. Accordingly, the removal of palmitoylation sites has no effect on primary interactions, but secondary interactions are impaired, that has consequences on cell signaling and cell morphology (86-89). The association of CD151 with other tetraspanins represent examples for this type of association. Type III associations comprise proteins that are only found in tetraspanin complexes when very mild detergents are used for membrane disruption. Signal transducing molecules like PKC or type II PI4K are only detected in tetraspanin complexes under such mild lysis conditions (9,79,88,90-92).

Mild detergent conditions also allow tetraspanin complexes to be recovered from lipid rich fractions of isopycnic sucrose gradients. This observation as well as data showing the association of the tetraspanin CD9 with ganglioside GM3 and the demonstration of covalent cross-linking of tetraspanins with cholesterol, have suggested a linkage between tetraspanin complexes and lipid rafts. However, tetraspanin complexes are not located in classical rafts. Instead, tetraspanin complexes initiate their own type of microdomains, called TEMs (tetraspanin-enriched membrane microdomains). The location of tetraspanin complexes in these membrane microdomains enriched for long saturated acyl chains and cholesterol could well provide a signaling platform (86,88,90,93-95).

4.2. Co-operate activity of tetraspanin complexes

Tetraspanins have been described to be involved in a multitude of different functional activities, like B- and T-cell activation, platelet aggregation, migration, proliferation, morphogenesis and tumor cell progression. This divergency of functions can be explained by the organization of the tetraspanin web in lipid-rich membrane microdomains. However, the molecular mechanisms of most of the tetraspanin activities are not yet clarified.

Modulation of cell motility has been the first functional activity ascribed to tetraspanins. Most important for this activity is the association of tetraspanins with integrins (96-98), where the association with tetraspanins is decisive for integrin-mediated cell motility. Thus, mutations in the primary interaction site of CD151 results in loss of α3 and α6 integrin associations, with the consequence that integrins contain their adhesive properties, but loose the capacity to promote spreading and cable-like growth on matrigel (78,84,87). Mutation of the C-terminal tail of CD151 also alters α6 integrin-dependent spreading, cable formation and the strength of adhesion. It is suggested that the C-terminal tetraspanin tail provides a link to an unidentified signaling element that contributes to integrin conformation. Also, the integrin domain associating with tetraspanins, is required for the recruitment of tetraspanin associated PI4K and PKCs. e.g. the α3 tail does not become phosphorylated unless the α chain has associated with the tetraspanin (99). Of special relevance for tumor progression is the finding that several tetraspanins are expressed at high level on endothelial cells and, accordingly, influence endothelial cell motility. Endothelial cell migration can be efficiently inhibited by anti-CD9 and anti-α3ß1. A CD81 - CD151 - α3ß1 complex also is of major importance for endothelial cell motility. Finally, heterotypic interaction between tumor cells and endothelial cells are critical during tumor cell dissemination. Endothelial cell CD9 strikingly localizes towards the contact points between endothelial cells and tumor cells and facilitates tumor cell transmigration (100,101).

Integrin activation via associated tetraspanins is also important during blood coagulation. In resting platelets αIIbß3 is in an inactive conformation. It is converted to a high affinity state by inside-out signaling via G-protein-coupled or tyrosine kinase linked pathways. Activated αIIbß3 binds fibrinogen and vWF. Subsequent outside-in signals lead to clustering and cytoskeletal reorganization, platelet activation, clot retraction and spreading (102). In CD151 knockout mice, CD151 being constitutively associated with αIIbß3, outside-in signaling of αIIbß3 is impaired, which has the consequence of defective platelet aggregation, impaired spreading on fibrinogen and delayed clot retraction (103). There is also evidence for a contribution of endothelial cell derived tetraspanins in platelet activation. Stimulated endothelial cells may shed CD9 in small vesicles, which bind factors IXa and Xa (104).

Tetraspanins also contribute to integrin internalization. Cell motility is accompanied by integrin redistribution to filipodia and lamellipodia (105,106), which could be due to membrane traffic or endocytosis and recycling. Recent evidences point towards a major contribution of integrin recycling, which is guided by associated tetraspanins (107). In this context it is important to mention that tetraspanins are also enriched in fused endocytic vesicles, called multivesicular bodies (108-110). Multivesicular bodies fuse with the plasma membrane and are released as 50-90nm particles, called exosomes (111). Exosomes are claimed to function as intercellular communication vesicles (112-115). It has been suggested that tetraspanin enriched microdomains may be particularly adapted to facilitate vesicular fusion and/or fission. However, the proteins that associate with tetraspanins in membrane fusion, exosome formation, shedding and uptake have not yet been identified.

The integrin tetraspanin association also modulates the strength of adhesion. This is surprising in as far as tetraspanins have little effect on integrin-ligand binding (116). Nonetheless, tetraspanins strengthen integrin-mediated adhesion. To give a few examples, CD151 regulates post-ligand-binding events (84), including retraction of platelet clots (102) and CD81 enhanced α4β1 adhesion under shear flow (117). The mechanistic that underlies the tetraspanin-mediated strengthening of integrin binding is poorly understood. There is evidence that CD9 and CD82 can regulate actin organization (87) via PKC recruitment (99); Alternatively, recognition of typeIII or typeI PDZ domains by the 3 C-terminal residues of CD81 and CD151 could support strengthening of tetraspanin-associated integrin adhesion (118).

Finally, via their association with integrins, tetraspanins are supposed to exert morphogenic features. CD82 might attenuate cellular morphogenesis through down-regulation of α6-mediated cell adhesion, likely by CD82-mediated integrin internalization. Furthermore, network formation of NIH3T3, which is mediated by α6ß1 essentially depends on CD151 (119,120). CO-029 (the rat synonym is D6.1A, for convenience we will use the term CO-029 throughout) overexpression also strongly supports network formation of carcinoma cells. However, different to CD151, cable like growth was inhibited by anti-α3β1,

but not by anti-α6β1 (121). The mechansims underlying these differences in morphogenic features of CD151 and CO-029 remain to be explored. It is, however, conceivable that differences in the cytoskeletal elements of the fibroblast-derived NIH cells versus the epithelial tumor cells are decisive.

Tetraspanins are also invovled in hematopoiesis, leukocyte activation and apoptosis (rev in 76,77). This is mainly due to cooperations and associations with intracellular signaling molecules, like phosphatases, PKCs and type II PI4K (81), and cytoskeletal components. Thus, CD82 can act as a co-stimulator by tyrosine phosphorylation of the Rho GTPase guanosine exchange factor Vav1 and the adaptor protein SLP76 or phosphorylation of ZAP70 and LAT. Depending on the palmitoylation state, tetraspanins also regulate integrin signaling via PI3K. Tetraspanins also promote proliferation by stimulating MAP kinases and tyrosine phosphorylation of p46 and p52 Shc. The phenomenon is linked to an increased association with a type II PI4K and is integrin-independent. CD81 and CD9 modulate apoptosis resistance by signal transduction via JNK and p38 MAPK (122-124). Furthermore, tetraspanins can associate with G-protein-coupled receptors (GPCR), which transmit signals to associated heterotrimeric G proteins. CD9, CD81 and CO-029 selectively associate with an orphan G-protein coupled receptor (GPCR) (GPR56) and Gαq, Gα11 and Gß, where the tetraspanins function as selective scaffolding proteins for GPCRs (87,99,125,126). Tetraspanins have also been shown to modulate growth factor signaling. CD9 associates with and modulates the function of pro-TGFα, pro-HB-EGF and pro-amphiregulin by increasing the potency of these ligands during juxtacrine signaling (127), that might involve prevention of ligand cleavage and/or concentration of the ligand in CD9 microdomains (128,129). Also the tyrosine kinase receptor c-kit forms a complex with CD9, CD63 and CD81 such that basal tyrosine phosphorylation is increased, but steel factor-stimulated tyrosine phosphorylation is decreased (130).

To summarize the most essential features, tetraspanins associate with other tetraspanins, integrins and additional transmembrane molecules in membrane microdomains. They function as specific membrane docks that cluster their associated membrane proteins with intra-cellular membrane-proximal signaling proteins. The clustering depends on the palmitoylation state of the interacting proteins. The reversibility of palmitoylation facilitates local and temporal rearrangements of the tetras-panin web and the associated signaling molecules. This so called tetraspanin web allows a single class of molecules to play an important role in several fundamental biological processes, like activation, proliferation, apoptosis and migration, morphogenesis and cell and vesicular membrane fusion. The latter two features are less well defined. Nonetheless, they likely are of major relevance in tumor progression.

4.3. Tumor growth promoting and metastasis suppressing activity of tetraspanins

There is undoubtedly evidence that tetraspanins contribute to tumor progression (131-133). Yet, taking into account that tetraspanins function as molecular facilitators, it becomes most likely that the very same tetraspanin may exert opposing effects on tumor progression depending on the associating molecules. This, in fact. has been observed in several instances. Nonetheless, two tetraspanins, CD151 and CO-029 are mostly associated with tumor progression, while CD82 has originally been described as the metastasis suppressor gene Kai1, where high expression is associated with a favourable prognosis. High CD9 expression has also mostly been associated with a favourable prognosis. The mechanisms accounting for tetraspanin-mediated metastasis promotion or suppression are not fully explored. However, there are some most interesting observations that deserve further exploration.

The metastasis suppressor gene KAI1 / CD82 (134-138) is expressed in many tissues and cells, but frequently is downregulated in tumors (139). Recent results enhance our understanding of how CD82 might inhibit invasiveness. Ectopic expression of CD82 leads to down-regulation of p130Cas, thereby suppressing the p30Cas-CRKII coupling, which triggers DOCK180, a guanine nucleotide exchange factor for Rac1, that is important for membrane ruffling and directional migration (140). Because the p130Cas-CRKII complex functions as the molecular switch for directional cell migration, it is suggested that the metastasis suppressor activity of CD82 relies mainly on modulating this complex formation (140,141). Additional mechanisms are also discussed. EWI2, a member of a new subgroup of the Ig superfamily also suppresses ruffling and migration (80,142) and was found to associate with CD82 (143), that was, however not confirmed by another group (144). Finally, CD82 might attenuate signaling via the EGFR and/or ERBB2 inasmuch as its association with these recpetors diminishes ligand-in-duced dimerization and endocytosis (129,131,145,146). It also has been described that kitenin, a metastasis-supporting four transmembrane protein, that does not be-long to the tetraspanin family, bind the C-terminal tail of CD82 whereby its metastasing promoting activity becomes inhibited (139,147). Finally, CD82 may regulate the prote-olytic activity of the uPA/uPAR system such that in the presence of CD82 uPAR co-localizes with α5β1 in focal adhesions. By the stable association between uPAR and α5β1 binding of uPA to its receptor was prevented and the pericellular proteolysis was reduced by 50-fold (148).

Two tetraspanins, CD151 and CO-029, have been associated with tumor progression. CD151 expression is upregulated in lung, prostate, pancreatic and colon cancer and high level expression has been found to correlate with poor prognosis (149-152). Also, transfection of tumor cells with CD151 cDNA promoted their motility and invasiveness (133,153). *In vivo* metastasis formation could be inhibited by a CD151 antibody blockade (150). Transfection of tumor cells with recombinant adenoviral vectors containing sense and anti-sense CD151 sig-nificantly promoted, respectively, inhibited tumor cell mi-gration (154). The following mechanisms are discussed: i. CD151 supports tumor cell migration (133). CD151 is closely associated with laminin receptors and overex-

pression enhances Rac and Cdc42 activation (155). By transfection of CD151 into focal adhesion (FAK) competent and deficient cells and antibody inhibition, it could be demonstrated that CD151 contributes to integrin-mediated tumor cell motility also via FAK activation (153); ii. MMP-7 associates with CD151. MMP7 becomes activated by this association and is captured at the cell membrane, thus allowing for pericellular lysis (156); iii. The association of CD151 with pro-growth factors (157) and growth factor receptors (145) may also contribute to its metastasis promoting activity; iv. The morphogenic features of CD151 (120,143) as well as its involvement in platelet activation (102) have not yet been explored for their relevance with tumor progression. Nonetheless, those features could make a considerable contribution to tumor progression.

CO-029 was originally described as tumor-associated antigen expressed by several human carcinoma, including astrocytoma and colorectal cancer (158). High CO-029 expression is associated with a poor prognosis and pronounced tumor progression (140,159). Rat CO-029 has been suggested to be involved in cell proliferation and differentiation (160) and to support hematogenous spread, where an interaction with platelets and leukocytes may provide tumor cells with a survival advantage in the hostile environment encountered during metastatic spread (149,160-162). Alternatively, CO-029 could well support migration of metastatic tumor cells by its association with integrins and similar mechanisms as described for CD151. Though CO-029 is not constitutively associated with α6β4, it does so after stimulation and disassembly of hemidesmosomes (85), which is accompanied by transient internalisation of α6β4 – CD151 / CO-029 complexes, changes in cell shape towards a migratory phenotype and increased motility (151). Another possible mechanistic basis of pro-metastatic functions of CO-029 relies on its involvement in cancer thrombosis. When rats received a CO-029 overexpressing rat pancreatic carcinoma line, animals developed disseminated intravascular coagulation, which could be prevented by a CO-029-specific antibodies (132). First studies to unravel the underlying mechanism provided evidence that CO-029 is an utmost strong angiogenesis inducer that contributes to a systemic angiogenic switch by shedded CO-029 that is found abundantly in tumor-derived exosomes (121).

5. CLAUDINS

5.1. Claudins and tight junctions

Tight junctions represent sites of close contact between the outer leaflets of plasma membranes of adjacent cells. At the so-called "kissing points" the intercellular space is completely obliterated (163-165). Tight junctions are found in epithelia and endothelia and provide a barrier to the paracellular diffusion of solutes. They also separate apical from basolateral membrane domains (166,167). The major components of tight junctions are transmembrane proteins such as occludin and tricellulin (168,169), claudins and the junctional adhesion molecule and cytoplasmic plaque proteins such as ZO-1, ZO-2, ZO-3, cingulin, symplekin and others (170,171). The family of claudins meanwhile comprises 24 members with molecular weights between 20 kDa to 33 kDa. They are integral membrane proteins with four hydrophobic transmembrane domains and two extracellular loops which appear to be involved in homophilic and/or heterophilic interactions implicated in tight junction formation (172). Claudins share these features with tetraspanins. However the 2 protein families are not related. The internal N-terminal sequence is very short. The first extracellular loop contains a set of highly conserved amino acids, W-GLW-C-C, where the 2 cysteins are supposed to form a loop. This loop is important for paracellular charge selectivity (173). The second extracellular loop is smaller and can be a receptor for bacterial toxins (174). The c-terminal tail is most diverse, varies in length from 21-63 AA, contains, with the exception of claudin-12, a PDZ motif (175-178) and has several potential phosphorylation sites (171,176,177).

The tight junction proteins interact with cytosolic scaffold proteins, which creates a platform for the recruitment of signal transducing molecules and linkage to the cytoskeleton (179). Important partner molecules in these complexes are PA3, PAR6, aPKC and the PAT-J/Pals-1/Crb-3 protein complex (180,181). Mutations in this complex, e.g. of aPKC does not affect localization of the complex in tight junctions, but disrupts the physical continuity (182). The sequential interaction of the proteins during the assembly of junctions is incompletely defined, but there is evidence that components of the barrier and polarity complexes are reciprocally regulated and interdependent (183-185). Tight junctions Proteins are also linked to the cytoskeleton, where direct interactions have been described for ZO-1 and ZO-3, but not for the claudins. ZO-1 and ZO-3 bind F-actin (186), ZO-3 also binds AF-6 and p120 catenin. Intriguingly, claudins have conserved dicysteine palmitoylation motifs (187) similar to tetraspanins (9,77). Palmitoylated claudins, like palmitoylated tetraspanins, are partitioned into glycolipid-enriched membrane microdomains, that harbour signal transducing molecules, facilitate complex formation and may contribute to the tight junction assembly (9,77,187-191), although the roles of these microdomains in the supposed oligomerization of claudins (178,192) are not well understood. Another aspect of claudins / tight junctions is important to mention. Tight junctions are constantly remodelled by endocytosis (193). One major pathway appears to be a cell-eat-cell model (194), where the intact tight junction complex is internalized by the adjacent cell. It is not yet known, whether clathrin-mediated (195) or caveolar (196) endocytosis of claudins are independent pathways or are part of the cell-eat-cell internalization process. Internalized tight junction proteins enter early endosomes, but are not recovered from late and recycling endosomes or the Golgi. Thus, there seems to be a special storage compartment that colocalizes with syntaxin 4. It is supposed that a better understanding of the recycling of claudins will provide important insight into mechanisms of altered barriere function e.g. in inflammatory bowel disease (197). The internalization of claudins is of physiological importance, e.g. internalization becomes strengthened by IFNγ, which is accompanied by

increased permeability (198). Claudin endocytosis becomes enhanced by claudin phosphorylation (199) and claudins are direct targets for PKA, PKC and myosin light chain kinase (199). It also has been noted that claudin phosphorylation may negatively regulate claudin integration into the tight junctions (200). Finally, EphA2 and ephrin-B1 bind to the first extracellular loop of claudin-7 (201). The tyrosine kinase EphA2 phosphorylates a conserved C-terminal residue of claudin-4, which is accompanied by increased paracellular permeability. Because the Eph-ephrin axis is important in controlling the epithelial-mesenchymal transition (EMT) (202), it is tempting to speculate that EMT is accompanied and facilitated by downregulation of claudins (203,204). Yet, it also has been described that claudin-1 regulates EMT by signaling through Wnt / ß-catenin (205).

5.2. Regulation of claudin expression

Not much is known on the regulation of claudin expression. For the transcriptional regulation, Snail, that triggers EMT, has been described to repress besides cadherin and occludin (203,204,206), also claudin-3, -4 and -7 expression (207). Snail may suppress claudin-1 expression also on the translational level (206). GATA-4 together with HNF (hepatocyte nuclear factor)-1α promotes claudin-2 expression (207). HNF-4α provokes expression of tight junction proteins, including claudin-7, and modulates the subcellular distribution resulting in junction formation and supports claudin-6 and claudin-7 transcription (208). Hormones and cytokines, like EGF, HGF, IL-1ß, IL-17, IFN, TNF and oncostatin have all been described to contribute to the regulation of claudin expression (209-216). However, the same signaling molecules may exert opposing effects, e.g. IL-1ß induces claudin-2 expression via p38 and PI3K (214), while in Ras or Raf-1 transfected cells claudin-2 become downregulated through the MAPK pathway (217,218), respectively becomes upregulated by downregulation of MAPK and Akt (219).

5.3. Claudin-7

Claudin-7 has first been described to form a paracellular barrier to Cl^- and a paracellular channel to Na^+ (220), whereby the first extracellular domain affects paracellular permeability (221). Notably, though it is meanwhile well established that claudins in general may not only be found in tight junctions, this has been become particularly obvious with claudin-7. While claudin-7 together with claudin-1, -3, -4 and -14 is located in crypts of palatine tonsils, distinct to the other claudins, claudin-7 does not become downregulated in tonsillitis, that was suggested to indicate that it may serve different functions (222). Also, claudin-7 and claudin-8 show different subcellular localization in Henle's loop and collecting tubes of the kidney, where claudin-7 is localized in the cytosol and basolaterally and claudin-8 is found at the cell border and in tight junctions (223). In the mouse intestine, too, claudin-7 is located in tight junctions, as well as basolateral (224). Thus, claudin-7 serves additional function besides providing a paracellular barriere.

5.4. Claudins in cancer progression

Tumor cells frequently exhibit abnormal tight junction functions as well as decreased differentiation and loss of cell polarity (225,226). The loss of tight junctions may be important to allow diffusion of nutritients and other survival factors to promote tumor cells growth and survival (227). Decreased polarity may facilitate EMT (228).

In fact several claudins have been found to be downregulated in cancer. Downregulation of claudin-1 has been observed in breast cancer (229,230), colon cancer (231), glioblastoma multiforme (224), and prostate cancer (225). Expression of claudin-7, too, is reduced in breast cancer (234,235). In primary breast cancer reduced E-cadherin and claudin-7 expression correlate with poor prognosis. However, claudin-7 was found to be re-expresed in lymph node metastasis (236). Other studies describe loss of claudin-7 expression in ductal mammary carcinoma *in situ*, that remains stable in invasive breast cancer (234). In the mouse, it has been noted that claudin-7 is expressed in mammary epithelium as well as in mammary carcinoma, but in tumors it was found to be punctated in the cytoplasma and in the basolateral membranes and has been suggested to be involved in stabilizing cytoplasmic vesicles (237). Reduced claudin-7 expression has also been observed in head and neck cancer (238). In the oesophagus, claudin-7 expression is confined to membranes of differentiated keratinocytes, while in squamous cell carcinoma of the oesophagus claudin-7 expression was reduced or completely lost. Downregulation was accompanied by decreased E-cadherin expression, increased proliferation and enhanced invasiveness (239,240). However, in other studies, a gradual increase of claudin-1 and claudin-7 during progression of oesophageal cancer has been reported (241) and has been defined as an early event in carcinogenesis (242). In high grade prostate cancer claudin-7 expression was also described to be reduced (233).

On the other hand, claudins may become upregulated in cancer. This accounts for claudin-3 and -4 in ovarian cancer (243-247), breast (248), prostate (233,249), pancreatic cancer (250-254) and squamous cell carcinoma of the oesophagus (242). Considering claudin-7 expression, we already mentioned that different expression profiles are reported for breast and oesophageal cancer. Upregulated claudin-7 expression has also been seen in chromophobe renal cell carcinoma and renal oncocytoma (255), giant cell tumors of the bone (256) and hepatocellular carcinoma (257). Claudin-7 expression also becomes upregulated early during gastric tumorigenesis and remains high in intestinal type gastric adenocarcinoma (258). Finally, claudin-7 regulates expression of the prostate-cancer specific antigen, but the pathway has not yet been clarified (259).

Besides the expression level, also the phosphorylation state of claudins can be important for their functional activity in tumor progression. Several kinases have been described to be involved in the phosphorylation of claudins. Claudin-1 becomes phosphorylated by MAP kinases (260) and PKC (200), claudin-5 by cAMP-dependent kinase (261,262) and claudin-3 by WNK4 (263). For claudin-3 and -4 it has been demonstrated in ovarian cancer that their phosphorylation is accompanied by disruption of tight junctions (264).

Taken together, there is evidence that tight junction-located claudins interfere with tumor progression and are downregulated in most cancer type. However and in line with supposed additional functional activities of claudins located outside of tight junctions, expression of claudins located in the cytoplasm or basolaterally is either not affected by oncogenic transformation and tumor progression or becomes strengthened. We hypothesize that claudins, particularly claudin-7, located outside of tight junctions form a complex with EpCAM, that becomes recruited into TEM. Within this membrane microenvironment, both EpCAM and claudin-7 likely fullfill different functions, that, however, have not yet been defined.

6. EPCAM-TETRASPANIN-CLAUDIN-7 COMPLEXES

6.1. EpCAM and tetraspanins

Tetraspanin complexes have been analyzed by Maldi-TOF and MS and LC-MS/MS in human, mouse and rat lymphoid and epithelial cells (5,89,265,266). Two of these analysis were particularly concerned about tetraspanin microdomains in tumors and metastases of the colorectum using two different models of tumor lines derived from primary colorectal cancer, liver metastasis and, in one instance, peritoneal metastasis (5,265). Both studies searched for CD9 associated proteins. Notably, in all 3 metastasis derived lines EpCAM was associated with CD9. It was also associated with CD9 in one of the primary tumor derived lines. CO-029 co-immunoprecipitated with EpCAM only in metastasis-derived lines from one of the tumors (8).

The emergence of mass spectrometry in biology has opened new avenues for the characterization of tetraspanin complexes. Several studies combined immuno-affinity purification using mAbs directed against tetraspanins (CD9 or CD81) or associated molecules with gel-based protein separation followed by MALDI-TOF mass spectrometry or LC-MS/MS. Studies were performed in different cell types including epithelial cells or T- or B-lymphoid cells (5,89,265,266). Two of these analyses were devoted to the composition of tetraspanin microdomains in tumors and metastases (5,265). These reports were both focused on colon cancer using two different cellular models. The models were constituted of cell lines derived from primary colon tumors and metastases (liver, lymph node or peritoneal) from the same patients. These studies were based on the biochemical properties of the tetraspanin complexes. Therefore, cells were lysed with the mild detergent Brij97 followed by immunoprecipitation experiments of the CD9-containing complexes. The associated proteins were further eluted using the more stringent detergent Triton X-100, which dissociates tetraspanin-tetraspanin associations. Proteomics has revealed the presence of different categories of membrane proteins in tetraspanin complexes, including adhesion molecules, membrane proteases, receptors and signaling molecules and proteins involved in membrane fusion process as well as poorly characterized proteins. Among the newly identified proteins by mass spectrometry, EpCAM was observed associated with CD9 in all models. The interaction of EpCAM with CD9 can be visualized under conditions where tetraspanin to tetraspanin interactions are not observed or strongly diminished (using digitonin for cell lysis). In addition, the association was stabilized by chemical cross-linking. Therefore, it has been suggested that CD9/EpCAM constitutes a new primary complex in the tetraspanin web. To gain further information about the potential relevance of CD9/EpCAM complexes, the distributions of these molecules in normal and cancer colon were compared by confocal microscopy. There was a substantial colocalization of these two molecules in the normal colon and a lower level of colocalization in primary tumor and metastasis. Interestingly, immunoprecipitation experiments with CD9, CO-029 or EpCAM mAbs led to observe a 20 kDa protein that may correspond to a claudin family member.

A study on a metastasizing rat pancreatic adenocarcinoma also revealed co-immunoprecipitation of EpCAM with CD9 and CO-029 (6,7). Overexpression of EpCAM in the non-mestasizing subline of the same tumor, that does not express CO-029, provided clear-cut evidence for a strong increased in homophilic cell-cell adhesion. Instead, overexpression of EpCAM by itself had only a minor impact on tumor progression (268). This finding suggested that the association of EpCAM with tetraspanins might be important for its tumor promoting activity. Indeed, an analysis of EpCAM ascribed activities in the metastasizing versus the EpCAM cDNA transfected non-metastasizing subline revealed functional differences, which support our hypothesis. Thus, methyl-ß-cyclodextrin treatment, which destroys TEM, had a significant impact on apoptosis resistance of the metastasizing subline and a minor impact on the low apoptosis resistance of the EpCAM cDNA transfected non-metastasizing subline. Also, methyl-ß-cyclodextrin treatment strongly interfered with cell-cell adhesion of the metastasizing subline, but rather strengthened cell-cell adhesion and agglomeration of the EpCAM cDNA transfected non-metastasizing subline (6). Finally, the TEM located EpCAM-CO-029 complex contained an additional, phosphorylated 20kDa protein that was identified as claudin-7 (6,7). Methyl-ß-cyclodextrin treatment did not destroy the CO-029-EpCAM-claudin-7 complex, but prevented claudin-7 phosphorylation (6,7). Because methyl-ß-cyclodextrin treatment of the metastasizing subline was accompanied by loss of cell-cell adhesion and apoptosis resistance, it became tempting to speculate that the metastasis promoting activities of EpCAM do not only rely on the association between CO-029, EpCAM and claudin-7, but also on the location of this complex in TEM.

6.2. EpCAM and claudin-7

To support our assumption that a TEM located CO-029-EpCAM-claudin-7 complex is promoting tumor progression, we performed a screening of primary colorectal cancer tissue and liver metastasis derived thereof. This study confirmed co-expression of CO-029, EpCAM and claudin-7 as well as of CD44 variant isoforms in a high percentage the primary tumors and liver metastasis. Adjacent normal liver tissue does neither express CO-029 nor EpCAM. In the colonic mucosa

EpCAM and claudin-7 expression was low and distinct CO-029 expression was only seen in 3%. Co-immunoprecipitation of EpCAM and CO-029 in selected tissue samples has been noted in all instances, provided the cells expressed, in addition, claudin-7 (59). The EpCAM – claudin-7 association is not restriced to tumor tissue, but is also observed in normal epithelium of the gastointestinal tract, albeit in the non-transformed tissue comparably few EpCAM molecules are associated with claudin-7. Notably, too, even in the polarized normal mucosa, co-localization of EpCAM with claudin-7 is found in the basolateral region (7), which is in line with several reports on the localization of claudin-7 outside of tigh junctions (222-224). With respect to the CO-029-EpCAM-claudin-7 association in primary cancer and metastatic tissue it is important to note that neither CO-029, nor EpCAM nor claudin-7 expression by itself appeared to be of prognostic relevance. However, co-expression and complex formation of the molecules was accompanied by a significantly decreased disease free survival (59). Thus, at least, in colorectal carcinoma, EpCAM and claudin-7 are found in association with the tetraspanins CD9 and/or CO-029 in TEM and promote tumor progression (8,59).

What is the contribution of claudin-7 to the formation of the complex and the functional activity of EpCAM within the complex? These questions are not yet answered. However, ongoing studies in the above mentioned metastasizing rat pancreatic adenocarcinoma line revealed the following: i. Claudin-7 is essentially required for the TEM localization and the tetraspanin-association of EpCAM. In cell lines overexpressing EpCAM and CO-029, but missing claudin-7 expression, EpCAM does not associated with CO-029 and is not located in TEM, while claudin-7 associates with CO-029 even in the absence of EpCAM and is, by not yet defined mechanisms, recruited into TEM (59). Thus, it is the claudin-7 that is essentially required for complex formation and TEM localization. ii. TEM-associated claudin-7 is serine phosphorylated, that is not the case for claudin-7 located outside of TEM (6,7).

The finding that EpCAM associates with a tight junction protein and that the complex of these molecules is recruited into TEM was unexpected, but suggested that the EpCAM-claudin-7 complex might exert different functions besides cell-cell adhesion. In fact, there is evidence that the association of claudin-7 with EpCAM prevents EpCAM oligomerization. By transfection of stably EpCAM expressing HEK293 cells with increasing amounts of claudin-7 cDNA, decreasing amounts of EpCAM oligomers were detected and at about a 1:1 ratio of claudin-7 to EpCAM, only monomeric EpCAM was detected by Western blot (unpublished finding). Thus, one could speculate that complex formation of EpCAM with claudin-7 promotes tumor progression by hindering EpCAM oligomerization, that is essentially required for EpCAM-mediated cell-cell adhesion. However, this loss of function may also be accompanied by a gain of function(s).

The supposed gain of function by the association between EpCAM and claudin-7 essentially depends on the co-localization of the EpCAM-claudin-7 complex with tetraspanins in TEM and the claudin-7 phosphorylation, which is only observed in the TEM localized complex (6,7). This assumption is supported by the above outlined findings that destruction of TEM by partial cholesterol depletion with methyl-ß-cyclodextrin suffices for strongly decreased cell-cell and cell matrix adhesion and a striking loss in apoptosis resistance (6). The latter observation also accounts for human colorectal cancer lines. Irrespective of whether either EpCAM or claudin-7 expression was significantly downregulated by transient siRNA transfection, resistance towards cisplation and γ-irradiation becomes strongly decreased (59).

7. PERSPECTIVE

Three lines of evidence suggest that the basic feature of EpCAM as a homophilic cell-cell adhesion molecule on most epithelial cells may not be of relevance for its tumor growth promoting activity. First, antibody-mediated EpCAM cross-linking triggers proteolytic enzymes, such that not only the extracellular part of EpCAM becomes cleaved, but concomitantly part of the cytoplasmic tail. This is important, because the deliberated cytoplasmic tail of EpCAM, EpIC, associates with additional transcription factors, which move to the nucleus, where the complex promotes upregulation of c-myc as well as cyclin A and E (4, M. Munz and O. Gires, personal communication). Second, EpCAM has a strong association for claudin-7, with which it forms a direct protein-protein complex (7). Claudin-7-associated EpCAM can no longer form tetramers (unpublished finding). Thus, the cell-cell adhesion activity of the molecule is ablated. Third, via its association with claudin-7, EpCAM becomes recruited into specialized membrane microdomains, which are rich in tetraspanins (6,7) and, accordingly, are termed TEM (9). TEM, similar to rafts, are rich in glycolipids and long un-satturated fatty acids and serve as a scaffold for signal transducing molecules, which mostly attach to the inner side of the membrane via palmitoylation and or myristoylation (8,80). The tetraspanins themselves harbour additional transmembrane molecules, mostly integrins (9,75,76,81), but also G-protein coupled receptors (128) and peptidases, like CD13 (C.Claas, personal communica-tion) and CD26, TADG-15/matriptase and ADAM10 (265), that could well contribute to the digestion of EpCAM after it has been recruited via claudin-7. It will be of special interest to determine whether EpCAM could be a substrate of any of these membrane proteases and whether tetraspanins could regulate such a cleavage thus yielding EpICS.

Thus, the homophilic cell-cell adhesion molecule EpCAM has found new partners within the membrane and on its way from the membrane into the nucleus, which convert the cell-cell adhesion molecule towards a signal transducing and transcription initiating unit. We propose that the relocation of claudin-7 from tigh junctions to the basolateral region may be one of the very early events, that accounts for loss of cell-cell adhesion. The EpCAM–claudin-7 complex may then become recruited towards TEM, where tetraspanin-associated peptidases arrange the

release of EpIC. Concomitantly, claudin-7 may become phosphorylated by tetraspanin-associated PKC that could be involved in activation of the PI3K/Akt survival pathway. The initiating trigger for the EpCAM-claudin-7 complex formation remain to be explored. This also accounts for the recruitment of the complex into TEM and the particular scaffolding activities that are responsible for the generation of EpIC and the activation of anti-apoptotic proteins.

Though knowledge on functional activity of EpCAM was scarce for a long time, it still served as a valuable target in cancer therapy for the last two decades. Recent insights into its involvement in gene transcription as well as on its embedding in microdomains, that promote signal transduction, will greatly facilitate to elucidate the underlying mechanisms. Beyond this, unraveling in a comprehensive manner the initiating signals as well as their targets, most likely will lay a solid ground for new, highly efficient therapeutic concepts. Last, not least, EpCAM has been defined as a cancer stem cell marker (44-47) and it is well accepted that targeting cancer stem cells provides a most promising therapeutic option.

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