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Molecular mechanisms leading to loss of differentiation and gain of invasiveness in epithelial cells

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SUMMARY

It has been realized for some time that the loss of epithelial differentiation in carcinomas, which is accompanied by higher mobility and invasiveness of the tumor cells, is a consequence of reduced intercellular adhesion. A variety of recent reports have indicated that the primary cause for the 'scattering' of the cells in invasive carcinomas is a loss of the integrity of intercellular junctions. Thus, defects in expression or structure of several components of the epithelial adherens junctions (e.g. E-cadherin, α-catenin) can occur, and our increased knowledge about the molecules of the junctions allows an explanation of these defects in molecular terms in some of the cases. Furthermore, tyrosine phosphorylation of junctional components (e.g. β-catenin) appears to play a role in the assembly and disassembly of cell-cell contacts. Some of the effectors of epithelial junction formation are tyrosine protein kinases, e.g. the scatter factor/hepatocyte growth factor receptor c-Met, the FGF receptors and the pp60^{src} kinase. The importance of tyrosine phosphorylation in junctions during tumor development is becoming increasingly evident.

Key words: tumor differentiation, adherens junctions, catenins, E-cadherin promoter, scatter factor, hepatocyte growth factor

INVASIVE CARCINOMA CELLS EXHIBIT REDUCED INTERCELLULAR ADHESION

Pathologists have observed for some decades that invasive carcinomas generally show reduced epithelial differentiation, and that this is particularly prominent at invasion fronts where carcinoma cells break into surrounding mesenchymal tissue (Weinstein et al., 1976; Gabbert et al., 1985). The invading cells lose their epithelial appearance (they become amorphic, spindle-shaped, fibroblastoid, more mobile) and in the electron microscope a reduced number of intercellular junctions are observed. Desmosomes (spot desmosomes) represent the intercellular junctions which are most easily detected by electron microscopy in intact tissue. Together with adherens junctions (belt desmosomes), they constitute the main adhering junctions of epithelial cells. Other known junctions (tight, gap) do not primarily function in intercellular adhesion; the hemidesmosomes (and the focal contacts) play a specific role in cell-basement membrane contacts.

In recent years, our laboratory and others have set out to examine molecular components responsible for the generation of invasive, dedifferentiated epithelial (carcinoma) cells (Imhof et al., 1983; Behrens et al., 1985, 1989, 1993; Frixen et al., 1991; Weidner et al., 1991). We were not simply interested in markers which would be differentially expressed in differentiated and dedifferentiated epithelial cells. Instead we concentrated on the identification of molecular components which are directly responsible for the loss or maintenance of epithelial differentiation and junction formation.

THE ROLE OF THE CELL ADHESION MOLECULE E-CADHERIN

It became apparent by the work of our and other laboratories that the epithelial cell adhesion molecule E-cadherin is one potent regulator of the epithelial phenotype and of junction formation. For instance, forced expression of E-cadherin in nonepithelial cells by cDNA transfection induces junction formation, and disturbance of E-cadherin function in epithelial cells by specific antibodies leads to loss of junctions and to a fibroblast-like morphology (Behrens et al., 1985; Gumbiner and Simons, 1986; Nagafuchi et al., 1987; Matsuzaki et al., 1990; McNeill et al., 1990). E-cadherin, a 120 kDa transmembrane glycoprotein, is enriched in the adherens junctions of epithelial cells and interacts with the cytoskeleton via associated cytoplasmic molecules, the catenins (Boller et al., 1985; Behrens et al., 1985; Nagafuchi and Takeichi, 1988; Ozawa et al., 1989, 1990b; Ozawa and Kemler 1992). The cDNAs for α- and β-catenin have recently been characterized; they show sequence similarities to the cDNAs of other well known junction-asso-

ciated proteins, i.e. vinculin and plakoglobin, respectively (Nagafuchi et al., 1991; Herrenknecht et al., 1991; McCrea and Gumbiner, 1991; McCrea et al., 1991). β-catenin is homologous to the armadillo gene product of Drosophila (McCrea et al., 1991). E-cadherin expression is frequently downregulated in highly invasive, poorly differentiated carcinomas (Behrens et al., 1989; Schipper et al., 1991; Frixen et al., 1991; Shiozaki et al., 1991; Shimoyama and Hirohashi, 1991a,b; Mayer et al., 1993; Moll et al., 1993), and re-expression of E-cadherin by cDNA transfection in poorly differentiated carcinoma cell lines inhibits invasiveness (Frixen et al., 1991; Vleminckx et al., 1991; Chen and Öbrink 1991; Navarro et al., 1991). Thus, E-cadherin seems to act as a kind of master molecule for maintaining the differentiation of normal epithelial cells, and loss of expression or function of E-cadherin in transformed epithelial cells appears to be a key step in the progression of the cells to a malignant phenotype (see Behrens et al., 1992, for a review).

EXCEPTIONS TO THE RULE

It has recently become clear, however, that loss of differentiation of epithelial (carcinoma) cells in vitro and in vivo can also occur while E-cadherin expression is maintained. Scatter factor/hepatocyte growth factor is a motility and growth factor for various cell types and has multiple effects on MDCK epithelial cells. It leads to the dissociation of the cells, to increased cell motility, to a change from an epithelial to a fibroblastoid shape, to invasiveness into collagen gels, but not to a loss of E-cadherin expression (Weidner et al., 1990, 1991; see also below). Similar results were observed in dissociation experiments of rat bladder carcinoma cells with acidic fibroblast growth factor (Jouanneau et al., 1991). Transformation of MDCK epithelial cells with a temperature-sensitive v-*src* gene leads to morphology changes and invasiveness at the permissive temperature, but not to a loss of E-cadherin expression (Behrens et al., 1993; see also below). Similarly, expression of an estrogen receptor-*fos* hybrid gene in breast epithelial cells results in a hormone-inducible, rapid loss of differentiation of the cells, but E-cadherin expression is only affected after prolonged hormone exposure (Reichmann et al., 1992). Furthermore, in a number of 'scattered' gastric carcinomas, E-cadherin was found to be present (Shimoyama and Hirohashi, 1991a). In one case of a poorly differentiated lung carcinoma cell line (PC 9), E-cadherin expression was unchanged, but the E-cadherin-associated protein α-catenin was not expressed, probably due to homozygous mutation in the α-catenin gene (Hirano et al., 1992; Shimoyama et al., 1992). Other cases in which E-cadherin expression is observed in poorly differentiated carcinomas have been reported by Eidelman et al. (1989) and Moll et al. (1993). However, in all of these studies E-cadherin was analyzed by immunological techniques only. Whether the molecule is functionally intact in all of these examples is unkown.

Abberations that result in direct junction defects have been studied recently in molecular terms. For instance, expression of E-cadherin without a cytoplasmic domain resulted in defective cell-cell adhesion. Apparently, the truncated E-cadherin is unable to interact with the catenins and, therefore, proper junctions cannot be formed (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990b). E-cadherin without the entire extracellular domain acted in a dominant-negative fashion. It is likely that these truncated molecules collect catenins which are thus not available to form proper complexes with intact E-cadherin (Kintner, 1992). Furthermore, a point mutation generated in the Ca^{2+}-binding region of the first extracellular repeat of E-cadherin also resulted in non-functional cell adhesion molecules (Ozawa et al., 1990a). Similar mutations in the extracellular portions of E-cadherin have recently been found in two non-adhesive cell lines from gastric carcinomas (S. Hirohashi, Tokyo, personal communication). The adhesive properties of PC 9 lung cancer cells, which harbor a null mutation in the α-catenin gene, could be restored by transfection with α-catenin cDNA (Hirano et al., 1992; Shimoyama et al., 1992).

In our lab, we have recently found that the v-*src* oncogene is a potent effector of epithelial differentiation and invasiveness without affecting E-cadherin expression. MDCK epithelial cells transformed with a temperature-sensitive mutant of v-*src* exhibit a strictly epithelial phenotype at the nonpermissive temeprature for $pp60^{v\text{-}src}$ activity (40.5°C), but rapidly lose cell-to-cell contacts and acquire a fibroblast-like morphology after culture at the permissive temperature (35°C). Furthermore, the invasiveness of the cells into collagen gels or into chick heart fragments was increased at the permissive temperature (Behrens et al., 1993). The profound effects of v-*src* on intercellular adhesion were not linked to changes in the levels of expression of E-cadherin. Rather, we observed an increase in tyrosine phosphorylation of β-catenin. Similar data were reported by Matsuyoshi et al. (1992). These results suggest a mechanism by which v-*src* counteracts junctional assembly and thereby promotes invasiveness and dedifferentiation of epithelial cells through phosphorylation of the E-cadherin/catenin complex.

CONTROL OF EXPRESSION OF JUNCTIONAL COMPONENTS: THE E-CADHERIN PROMOTER

In addition to mutation and post-translational modification, adherens junction integrity could also be affected by changes in the regulation of gene expression of particular junctional constituents. Thus, any variation in the battery of the many transcription factors which regulate the promoters of the adherens junction proteins might lead to changes in the invasiveness of carcinoma cells. These studies are still at the beginning stage since the promoters of most of the involved genes have not been characterized.

We have recently identified the E-cadherin promoter in order to study the mechanisms that regulate epithelial-specific expression of E-cadherin and possible downregulation of expression during dedifferentiation of carcinomas (Behrens et al., 1991). We found that a −178 to +92 bp upstream fragment of the E-cadherin gene induced specific expression of a CAT reporter gene in epithelial cells, whereas in nonepithelial cells this fragment was inactive, indicating the presence of cell type-specific regulatory ele-

ments in this region. A further deletion fragment (from −58 bp to +92 bp) resulted in fivefold-reduced activity that was still epithelium-specific. Importantly, both the −178 bp and the −58 bp fragments were highly active in differentiated breast carcinoma cells, but were inactive in a line of dedifferentiated carcinoma cells. In these dedifferentiated breast carcinoma cells we detected a change in the activity of transcription factors responsible for the epithelial specificity of E-cadherin expression. By DNase footprinting and gel retardation analysis we could identify binding of nuclear factors to three regions within the −178 to +92 bp E-cadherin promoter fragment: first, to a GC-rich region at −58 to −25, second, to a palindromic sequence centered at −86 (named E-pal) which harbors two copies of the CANNTG consensus sequence for binding of helix-loop-helix transcription factors, and third, to the CAAT box. Identification of factors binding to and regulating the E-cadherin promoter is currently being pursued.

EPITHELIAL MOTILITY FACTORS: THE ROLE OF SCATTER FACTOR/HEPATOCYTE GROWTH FACTOR

Cell motility factors have been described previously as a group of proteins that selectively stimulate cell motility with little or no effect on cell proliferation. This group of proteins includes scatter factor (SF), which is secreted by mesenchymal cells and increases the motility of epithelial and endothelial cells in a paracrine fashion (Stoker et al., 1987; Rosen et al., 1990; Weidner et al., 1990, 1991), autocrine motility factor, which is derived from melanoma and breast carcinoma cells (Liotta et al., 1986), and migration-stimulating factor, which affects fibroblasts in an autocrine way (Grey et al., 1989). However, in the course of more thorough investigations of the phenomena of cell motility and growth, it became clear that motility factors often also act as growth factors, and vice versa. For instance, SF was shown to be identical to hepatocyte growth factor (HGF; Weidner et al., 1991) and vascular permeability factor (VPF) was identified as vascular endothelial growth factor (VEGF; Leung et al., 1989). Furthermore, many well known growth factors such as PDGF, FGF, EGF, II-6, NGF or IGF-1 were also found to affect cell motility (see Stoker and Gherardi, 1991, for a review). We have examined the effect of SF/HGF on the invasiveness of various epithelial cell lines into extracellular matrices. SF/HGF induces the invasion of MDCK epithelial cells into collagen in a dose-dependent manner. Some human carcinoma cell lines respond to SF/HGF by movement whereas others are insensitive to the factor (Weidner et al., 1990).

The SF/HGF cDNA, recently isolated from several sources such as placenta, liver, leukocytes or fibroblasts (Miyazawa et al., 1989; Nakamura et al., 1989; Seki et al., 1990; Rubin et al., 1991; Weidner et al., 1991), encodes a 83 kDa protein with sequence and overall structure similar to plasminogen and other enzymes involved in blood clotting, fibrinolysis and complement activation. The primary translation product is an inactive precursor (Hartmann et al., 1992; Lokker et al., 1992) which is proteolytically processed into the active, disulfide-linked heterodimer. The heavy (H) chain is composed of a short N-terminal region followed by four kringle modules, and the light (L) chain exhibits sequence similarity to serine proteases, but SF/HGF has no detectable proteolytic activity, possibly due to alterations of two of three amino acid residues forming the catalytic site (serine and histidine residues are replaced by tyrosine and glutamine, respectively). The light chain is produced by proteolytic processing of the precursor at a dibasic cleavage site.

The receptor for SF/HGF has recently been identified as the c-Met protooncogene product (Bottaro et al., 1991; Naldini et al., 1991), which is a transmembrane protein with tyrosine kinase activity. The MET gene is expressed in a variety of epithelial cells as well as in a number of tumors of epithelial origin (Di Renzo et al., 1991). The Met receptor is a 190 kDa heterodimer made up of a 50 kDa α-subunit disulphide-linked to a 145 kDa β-subunit which is synthesized as a single-chain precursor and then proteolyzed to yield the mature two-chain protein (Giordano et al., 1989). The α chain and the N-terminal portion of the β chain are located in the cytoplasm and contain the tyrosine kinase domain as well as phosphorylation sites involved in the regulation of the activity of the protein and in signal transduction. Binding of SF/HGF to the Met receptor was demonstrated by coprecipitation in immunocomplexes, by chemical crosslinking to the β-subunit of the Met protein and by ligand-induced tyrosine phosphorylation of the receptor (Naldini et al., 1991). In addition to the specific, high affinity receptor Met, low affinity/high capacity binding sites of SF/HGF have been detected in binding experiments (Naldini et al., 1991). Since it is known that SF/HGF binds to heparin, it is likely that heparan sulfate proteoglycans of the extracellular matrix and the cell surface provide these additional binding sites.

Expression of naturally occurring splice variants and in vitro-mutagenized cDNAs of SF/HGF allowed us to define the biological activities of different SF/HGF isoforms. The recombinant molecules also enabled us to delineate the protein domains of SF/HGF necessary for Met receptor interaction and activation (Hartmann et al., 1992). The results were as follows. (1) A single-chain SF/HGF resulting from the destruction of the protease cleavage site between heavy and light chain (Arg-494 + Gln) was largely inactive, indicating that proteolytic cleavage is essential for acquisition of the biologically active conformation. (2) A SF/HGF splice variant encoding a protein with a 5-amino acid deletion in the first kringle domain was as highly active as the wild-type molecule. (3) The separately expressed light chain (with serine protease homology) was inactive in all assays tested. (4) The separate heavy chain as well as a naturally occurring splice variant consisting of the N terminus and the first two kringle domains bound the c-Met receptor, stimulated tyrosine autophosphorylation, and induced scattering of epithelial cells but not mitogenesis. These data indicate that a functional domain in the N terminus/first two kringle regions of SF/HGF is sufficient for binding to the Met receptor and that this leads to the activation of the downstream signal cascade involved in the motility response. However, the complete SF/HGF protein seems to be required for mitogenic activity.

We have also addressed the question of whether all bio-

logical signals of SF/HGF are actually transmitted into the cell by the Met receptor. For this purpose, we have introduced into epithelial cells a cDNA expression vector encoding a hybrid tyrosine kinase receptor, which consists of the membrane-spanning and cytoplasmic domains of Met fused to the extracellular domain of the NGF receptor (trk) (Weidner et al., 1993). It has previously been shown that the extracellular domains of such hybrid receptors provide the ligand specificity, whereas the tyrosine kinase domain is responsible for the signalling specificity (Riedel et al., 1989; Bernhanu et al., 1990). We then asked which of the biological activities normally induced by SF/HGF could now be observed in response to NGF. We could demonstrate that all biological effects of SF/HGF upon epithelial cells such as the induction of cell motility, proliferation, invasiveness and tubular morphogenesis (Montesano et al., 1991) are now triggered by the addition of NGF. Thus, it is likely that all known biological signals of SF/HGF are transduced through the receptor tyrosine kinase encoded by the c-Met protooncogene (Weidner et al., 1993).

Coordinated movement, growth and differentiation of cells are essential prerequisites for embryonal development and tissue regeneration in vivo. A recurring picture in development is that certain groups of cells influence motility, growth and differentiation of other cells in their vicinity. In particular, exchange of signals between epithelial and mesenchymal cell compartments are major driving forces in development (cf. Ekblom 1989; Gumbiner 1992, for reviews). Recently, several receptor tyrosine kinases expressed in epithelia and their mesenchymally derived ligands have been implicated in these mesenchymal-epithelial interactions (see Stern et al., 1990; Montesano et al., 1991; Sonnenberg et al., 1991, 1992; Wen et al., 1992; Holmes et al., 1992; cf. Birchmeier and Birchmeier, 1993, for a review). The best characterized of these receptor/ligand systems involved in mesenchymal-epithelial interactions is SF/HGF/c-Met. Studies of SF/HGF and Met expression in cell culture show that the ligand is generally not produced by the same cells which respond to it and which express the receptor (Stoker et al., 1987; Weidner et al., 1991; Naldini et al., 1991). Furthermore, during mouse embryogenesis the cells expressing SF/HGF are also different but often in close vicinity to cells which transcribe the c-Met gene, suggesting a paracrine mode of action in vivo as well. For example, during kidney development c-Met is expressed in the epithelia of the ureter buds and of the proximal and distal tubules; transcripts for SF/HGF are found in the surrounding nephrogenic mesenchyme (Sonnenberg et al., 1992). Our present data suggest that the paracrine effects of SF/HGF in vivo leading to changes in cell motility, migration, differentiation and proliferation, are also mediated through the protooncogene product c-Met.

How could signal transduction through the Met receptor result in disturbance of intercellular junctions? The Met tyrosine kinase could directly phosphorylate junctional components, as seen in the case of the v-*src* gene product. Alternatively, the Met kinase could activate downstream targets such as c-*src* and thus indirectly induce phosphorylation of junctional complexes. No such reactions have so far been discovered. However, c-*src* seems to be involved in downstream signalling of other receptor tyrosine kinases. Lastly, activation of Met could lead to changes in gene expression, which might indirectly affect junctional complexes.

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