

Invited Review

Cohort migration of carcinoma cells: Differentiated colorectal carcinoma cells move as coherent cell clusters or sheets

K. Nabeshima, T. Inoue, Y. Shima, H. Kataoka and M. Kono

Department of Pathology, Miyazaki Medical College, Kihara, Kiyotake, Miyazaki, Japan

Summary. Active migration of tumor cells is usually assessed as single cell locomotion *in vitro* using Boyden chamber-type assays. *In vivo*, however, carcinoma cells, malignant cells of epithelial origin, frequently invade the surrounding tissue as coherent clusters or nests of cells. We have called this type of movement "cohort migration". In our work, the invasion front of colon carcinomas consisted of compact tumor glands, partially resolved glands or markedly resolved glands with scattered tumor cell clusters or single cells lying ahead. In the former two types, which constituted about a half of all cases, cohort migration seems to be the predominant mechanism, whereas both cohort migration and single cell locomotion may be involved in the last one. In this light, it is very advantageous to investigate the mechanisms involved in the cohort migration.

In this review, we present a two-dimensional motility assay as a cohort migration model, in which human colorectal carcinoma cells move outwards from the cell islands mainly as localized coherent sheets of cells when stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA) or hepatocyte growth factor/scatter factor (HGF/SF). Within the migrating cell sheets, wide intercellular gaps occur at the lower portion of the cells to allow the cells to extend leading lamellae forward while close cell-cell contacts remain at the upper portion of the cells. This localized modulation of cell-cell adhesion at the lower portion of the cells is associated with increased tyrosine phosphorylation of the E-cadherin-catenin complex in TPA-induced cohort migration and with reduced α -catenin complexed with E-cadherin in HGF/SF-induced cohort migration. Furthermore, fibronectin deposited by migrating cells is essential for their movement, and on the gelatin-coated substrate even degradation and remodeling of the substrate by matrix metalloproteinases are also needed. Thus, in cohort migration it is likely that cells are released from cell-cell adhesion only at the lower portion

of the cells via modulation of E-cadherin-catenin-based mechanism, and this change allows the cells to extend leading lamellae onto the extracellular matrix substrate remodeled by deposition of fibronectin and organized digestion.

Key words: Carcinoma, Tumor invasion, Motility, E-cadherin-catenin complex, Fibronectin

Introduction

Cell migration plays an essential role in normal development, especially during gastrulation, neural crest cell migration and organogenesis. Even in postnatal life, it occurs in a controlled manner in inflammatory reactions, immune responses and wound healing. Once its control is lost, however, cell migration can also cause harm to hosts. For example, cancer cells can leave the primary tumor, enter the blood and lymphatic vasculature, and disseminate to secondary organs without any physiological control. This ability of tumor cells to infiltrate and disseminate widely is what makes the tumors malignant (Stoker and Gherardi, 1991). *In vitro*, this migratory ability *in vivo* is detectable as (1) stationary motility such as membrane ruffling and pseudopodal extensions (Partin et al., 1989; van Larebeke et al., 1992); and (2) translocative motility which is defined as net translocation of the whole cell and a synonym for locomotion (Haemmerli, 1985). The mechanism of cell migration has been studied predominantly based on the idea of single cell translocation (locomotion), but translocation can also occur *en masse* (Nabeshima et al., 1997b). While the former can be easily visualized and measured by Boyden chamber-type assays *in vitro*, the latter is mainly observed as an *in vivo* phenomenon. In developing embryos, for example, epithelial cells migrate or rearrange collectively during branching morphogenesis and precardiac mesodermal cells also move as sheets of cells during heart formation (Wiens, 1996). These movements are termed "collective cell migration" or

“cell sheet migration”. Neuronal precursor cells also move maintaining cell-cell contact with one another via zonula adherens-like membrane specializations, which is referred to as “chain migration” (Lois et al., 1996). In postnatal life, normal epidermal cells move as cohesive cell sheets during wound healing (Martin, 1997). Since epithelial cells connect with each other by cell-cell adhesion, translocation *en mass* seems to be a characteristic feature of epithelial cell movement *in vivo*.

In histopathological tumor sections, invasion by contiguous sheets or cords of tumor cells is more frequently seen than infiltration by single tumor cells (Sträuli and Weiss, 1977). Carcinoma cells, which are malignant counterparts of epithelial cells, proliferate and preferentially form coherent cell nests, maintaining cell-cell contact with one another, and this mode of growth is also seen even at the invasion front. These findings let us assume that there might be a way by which carcinoma cells move together as coherent cell clusters, and we have called this type of movement “cohort migration” (cohort = tenth part of a legion in the ancient Roman armies; number of persons banded together) (Nabeshima et al., 1995a,b, 1997a,b). Carcinomas constitute 90% of human malignancies, and the extent of carcinoma invasion is one of the most significant prognostic factors especially in the gastrointestinal tract (Astler and Collier, 1954). It is therefore advantageous to have an *in vitro* model showing this cohort migration and to study the mechanism involved in the migration. Additionally, this terminology is applicable not only to *en mass* movement of carcinoma cells but also to that of normal epithelial

cells during wound healing or normal morphogenesis.

For the generation of cell migration, start signals including motogenic ligand-receptor interactions, signal transduction and modification of the cytoskeleton are essential. In addition, there are also signals for directional guidance that preferentially involve cell-cell and cell-substrate interactions (Stoker and Gherardi, 1991). In cohort migration, such cell-cell adhesive interactions should be modulated from the beginning by start signals. Since these adhesive interactions are one of the most prominent features of multicellular life, elucidation of their regulatory mechanisms is critically important in understanding not only cohort migration but also a broad spectrum of biological processes such as tissue morphogenesis and remodeling. In this review, therefore, we will introduce our cohort migration model and discuss the mechanisms of cell migration *en mass* with special reference to the cell-cell and cell-substrate interactions. Furthermore, we will refer to motility factors that can possibly provide start signals for cohort migration and cohort migration-specific regulation of matrix metalloproteinase (MMP) expression.

Clinicopathological significance of cohort migration in tumor pathology

Most of the *in vitro* works regarding tumor cell motility and invasion seem to be based on the idea of single cell locomotion and penetration of ECM by single tumor cells. However, we, as pathologists, feel the presence of discrepancy between this concept of single

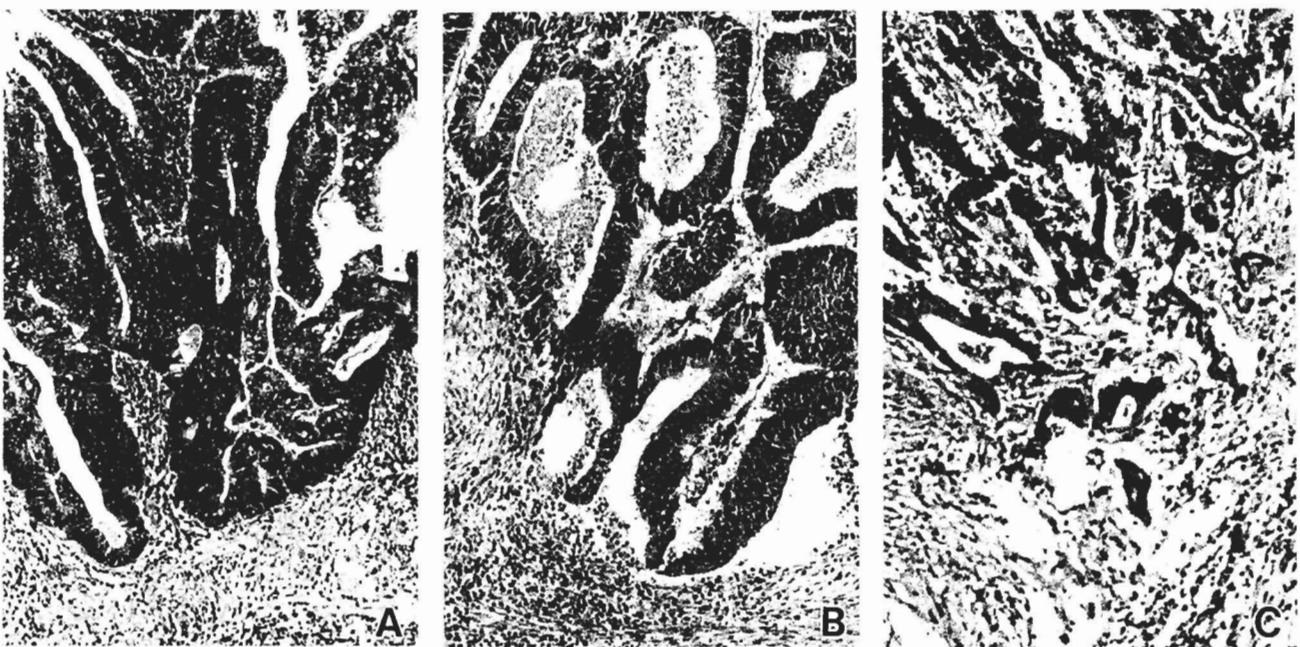


Fig. 1. Invasion fronts of colon carcinomas. Well-circumscribed, compact tumor glands (A, type Ia cohort migration), partially resolved tumor glands (B, type Ib cohort migration), or more markedly resolved tumor glands with small clusters of carcinoma cells lying ahead (C, type II cohort migration) are observed at the invasion front. H.E. x 25

Cohort migration of carcinoma cells

cell locomotion and the mode of carcinoma cell invasion observed in surgical specimens, which has led us to the concept of cohort migration. We discuss here whether this concept has any relevance to tumor invasion *in vivo*. In well to moderately differentiated colon adenocarcinomas, for example, the invasion front consists of well-circumscribed compact tumor glands, partially resolved tumor glands, or more markedly resolved tumor glands with small clusters of carcinoma cells lying ahead (Fig. 1) (Jass et al., 1986; Hase et al., 1993). The former two features are associated predominantly with an expanding pattern of growth (Jass et al., 1986), whereas the latter feature is called tumor budding and is often associated with worse prognosis (Hase et al., 1993). These observations suggest that carcinoma cells at the advancing edge might be able to move as a large unit or dissociate from the primary site and migrate within the host tissue as small coherent aggregates; in both cases cells move together and therefore both are considered as cohort migration. The demonstration of migration of isolated V2 carcinoma cell aggregates as units *in vitro* (Enterline and Coman, 1950) and *in vivo* (Sträuli and Weiss, 1977) supports the concept of cohort migration. Moreover, a recent time-lapse videomicroscopy and computer-assisted cell tracking study of primary neoplastic tissue explants placed in three-dimensional collagen gels has shown that groups of clustered cells comprising 5 to more than 100 cells could detach from the primary tumor lesion and migrate within the adjacent collagen matrix (Friedl et al., 1995). Taken together, these findings suggest that cohort migration could be a mode of carcinoma cell movement, as well as single cell locomotion.

Cohort migration as small aggregates of carcinoma cells, is not a new concept. Imai subdivided the histological growth pattern of gastric and uterine cervical carcinoma into nonsprouting, sprouting and lymphatic-permeating types (1954, 1960). The nonsprouting type was defined as carcinoma cells which spread by forming large cell nests or glandular structures and the sprouting type as carcinoma cells which propagate as more or less anaplastic thin cell cords or as individual cells. The extent of sprouting inversely correlated to prognosis in those carcinomas. Leighton et al. (1960) also described that increasing numbers of aggregates of carcinoma cells were seen as the tumor grew and involved larger volumes of tissue. They thought of an aggregate of cells as a loosely knit, short-lived ecological unit or a supracellular living organism, a distinct individual, and speculated that aggregates of cells function as integrated units in the propagation of similar groups. They also referred to the possibility that there were migrations of multicellular portions of aggregates. This type of cohort migration was also observed in experimental tumors at the invasion front of otherwise expanding, growing tumors (Gabbert, 1985; Carr et al., 1986), and reflected a more aggressive invasive behavior due to active locomotion of the dissociated tumor cells (Gabbert, 1985). This was also

called tumor-cell dissociation (TCD) and its extent at the invasion front inversely correlated to prognosis in human gastric carcinomas (Gabbert et al., 1992) and squamous cell carcinomas of the larynx (Jakobsson et al., 1973) and the oral cavity (Bryne et al., 1989).

To examine how frequently cohort migration occurs *in vivo*, we classified the invasion front (IF) of colon carcinoma into three types on histopathology sections: (i) IF consisting of compact tumor glands (type Ia cohort migration); (ii) IF consisting of partially resolved tumor glands (type Ib cohort migration); and (iii) IF showing tumor budding (type II cohort migration) (Fig. 1). A half of our cases (total 74 cases) showed type I cohort migration and the others type II (Fig. 2A). Type Ia invasion front was observed in carcinomas which were confined to the mucosa or submucosa, whereas type Ib and II were observed in carcinomas showing further invasion. Single cell invasion was noted only in the cases with type II cohort migration, especially in the cases which extended into the subserosa (Fig. 2B). Thus, in cases with tumor budding, both cohort migration and single cell locomotion may be involved in their invasion, while cohort migration seems to be the predominant mechanism in cases with compact or partially resolved tumor glands. Therefore, cohort migration can be involved in more than half of the cases in colon carcinomas. It is likely that when cohort migration is induced as a large unit cancer cells show an expanding pattern of growth, whereas they show more aggressive biological behavior when induced as small clusters of cancer cells lying ahead of the invasive front. In this light, it is important to elucidate the mechanisms involved in cohort migration to control cancer invasion and metastasis.

A cohort migration model *in vitro*

Recently we reported that an enhanced ability to invade Matrigel upon stimulation with 12-O-

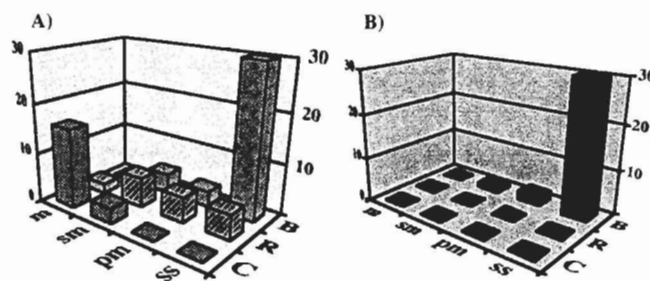


Fig. 2. A. Correlation between histological types of the invasion front and the extents of invasion in colon carcinomas. Histological types of the invasion front are classified into those with compact tumor glands (C), those with partially resolved tumor glands (R) and those showing tumor budding (B). The cases with invading single cells at the invasion front are also shown in the right side panel (B). The extents of invasion: m, mucosa; sm, submucosa; pm, proper muscle layers; ss, subserosa and adventitia.

tetradecanoylphorbol-13-acetate (TPA) was one of the major properties of a highly metastatic variant (L-10) of the human colon adenocarcinoma cell line RCM-1 (Komada et al., 1993). This TPA-enhanced invasion of Matrigel was associated with augmentation of cell motility but not metalloproteinase activity in conditioned medium (Nabeshima et al., 1993). To elucidate the mechanisms of this enhanced L-10 cell motility we used a two-dimensional simple Lab-Tek chamber motility assay, which meets the requirements for morphological observation (Nabeshima et al., 1995a,b, 1997a). In this assay, L-10 cells were seeded into compartments of an eight-well Lab-Tek tissue culture chamber slide (1.2 x 10⁵ cells in 0.4 ml growth medium) and allowed to attach for 24 h at standard culture conditions (37 °C, 5% CO₂ in air, 100% humidity). L-10 cells formed interlinked and piled-up cell islands on the tissue culture glass substrate of the Lab-Tek chamber slide (Fig. 3a). Then, the cells were exposed to test medium containing TPA for one hour. The test medium was then discarded and the cells were rinsed lightly and further incubated without TPA for various times up to 24 h. This TPA treatment induced cell motility with characteristic morphology: the cells migrated as localized coherent cell sheets at intervals along the margin of the cell islands (Fig. 3b). We call this type of cell motility "cohort migration" as described above. The cells at the edges of the migrating cell sheets showed motile cell features such as fan-shaped leading lamellae, while the following cells had cell contact with one another. The same type of migration was also induced by a naturally occurring motogenic factor, hepatocyte growth factor/scatter factor (HGF/SF) in L-10 cells and several other human colon carcinoma cell lines (Nabeshima et al., 1998b). Since the migrating cells did not overlap one another in either TPA or HGF/SF-induced cases, the quantitation was available by counting their number. The quantitative studies showed that the migration was induced with the peak response at 20 ng/ml of TPA (Nabeshima et al., 1993) and 10 to 50 ng/ml of HGF/SF (Nabeshima et al., 1998b). The migration progressed in a time-dependent manner for up to 24 h. Approximately 80% of the maximum cell translocation was observed after 6 h incubation with TPA but around 20 h with HGF/SF. Involvement of passive cell movement due to cell growth was examined, but there was no apparent contribution of cell proliferation to this L-10 cell translocation since neither TPA nor HGF/SF stimulated L-10 cell proliferation during the assay period compared to control. Hydroxyurea, an agent blocking DNA synthesis, did not significantly reduce the extent of migration in both cases, either. The morphological characteristics of TPA and HGF/SF-induced L-10 cell movement are similar to those previously reported for normal epithelial cell movement (Vaughan and Trinkaus, 1966). Thus, we reasoned that these TPA and HGF/SF-L-10-cell systems could be two-dimensional models of well differentiated adenocarcinoma cell movement.

Cohort-type migration of epithelial cells can be

observed in several three-dimensional *in vitro* culture models. Raft cultures of human epidermal cells on a gelled lattice of collagen containing fibroblasts differentiate and exhibit morphological and biochemical features of human skin *in vivo*. When stimulated with transforming growth factor- α (TGF- α), the organization of cells within the epidermis changed and keratinocytes started invading the collagen matrix as coherent islands of cells (Turksen et al., 1991). Well differentiated squamous carcinoma cell line OSC-19 also formed multi-layers when cultured on 3T3 fibroblast-embedded collagen gel, and invaded the collagen gel matrix as wedge-shaped coherent cell nests after more than 8 days

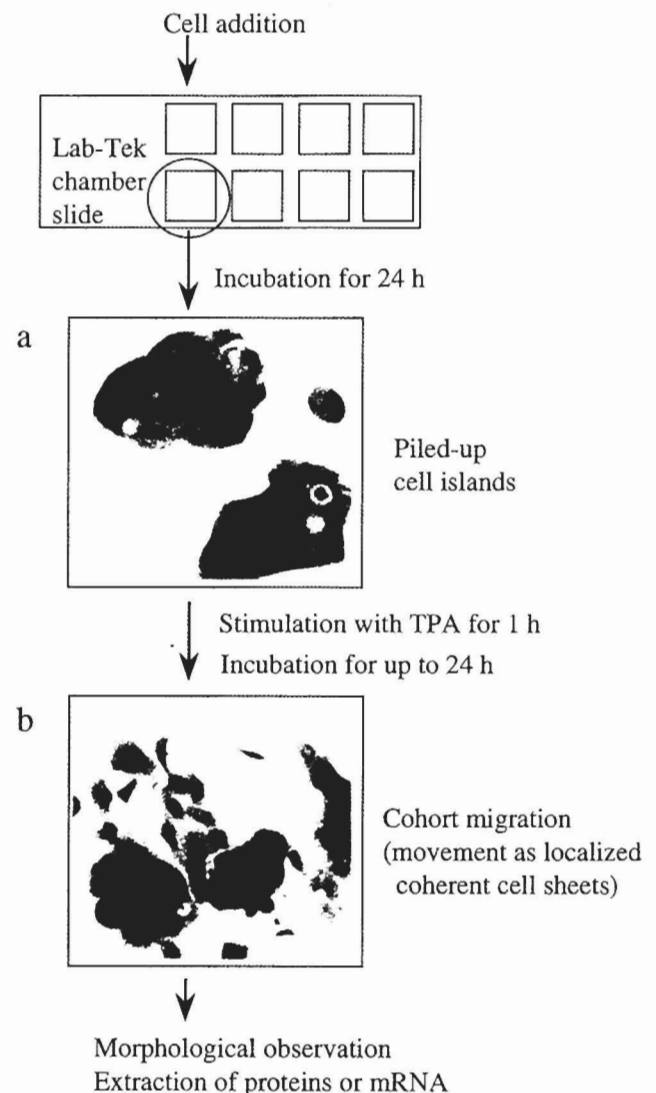


Fig. 3. The cohort migration assay. L-10 cells seeded into compartments of a Lab-Tek tissue culture chamber slide form piled-up cell islands and few cells emerge from the islands in the absence of TPA treatment (a). In the presence of TPA, cells move outwards from the islands forming localized coherent sheets (cohort migration) (5 h after TPA treatment) (b). H.E. (Nabeshima et al., 1995a).

of culture (Kawahara et al., 1993). A human gallbladder cancer cell line, which formed the spherical clusters in collagen gels, was also transformed by the fibroblast-conditioned medium to arborizing colonies with tubular projections (Shimura et al., 1995). All these features of *in vitro* invasions mimic an invasion of carcinoma cells into the surrounding tissue *in vivo*. Additionally, since our RCM-1 cells also invaded the gel as organized clusters with tubular structure when cultured on reconstituted collagen gels (Nabeshima et al., 1988), the manner of locomotion in the two-dimensional model described above appears to reflect the locomotion in a three-dimensional system.

Regulation of cell-cell adhesion during cohort migration

Disruption of cell-cell contacts is generally thought to be important for epithelial cell movement. This was well demonstrated in 5T4 works (Carsberg et al., 1996). The 5T4 oncofetal antigen is a 72 kDa transmembrane glycoprotein, and its expression correlates with disease progression, metastasis and clinical outcome in colorectal, gastric and ovarian tumors. 5T4 over-expressing MDCK canine kidney epithelial cells showed increased motility only when cell-cell contacts were disrupted by addition of HGF/SF. Since 5T4 over-expressing murine mammary cells showed both disruption of cell-cell contacts and increased motility without HGF/SF, even among epithelial cells, response to motogenic factors may vary depending on cell types and possibly their own cell adhesive nature.

The cell-cell adhesion of epithelial cells is mediated predominantly by a transmembrane glycoprotein, E-cadherin (also known as Arc-1, uvomorulin and cell CAM 120/80) (Takeichi, 1993). E-cadherin is, however, only part of a complex cell adhesion cascade, and other components include a set of cytoplasmic cadherin-associated molecules collectively called the catenins (including α -, β -, γ - (plakoglobin) and p120^{cas} catenins) (Tsukita et al., 1992; Aberle et al., 1996). The catenins complex with E-cadherin via its specific cytoplasmic binding domain, and this complex formation mediates the linkage of E-cadherin to the actin cytoskeleton and thus is essential for E-cadherin to express its full adhesive function. This cadherin-catenin complex-based cell-cell adhesion, which corresponds to the adherens junction structurally, is possibly regulated via genetic and epigenetic alterations. Although mutations and deletions in E-cadherin, α - and β -catenin genes have been reported (Shimoyama et al., 1992; Becker et al., 1994; Oyama et al., 1994; Guilford et al., 1998), in the gastrointestinal tract those abnormalities are confined predominantly to poorly differentiated adenocarcinomas, most of which show scattered histological phenotypes. In contrast, only few mutations of the E-cadherin gene have been found in well to moderately differentiated adenocarcinomas (Becker et al., 1994), which show characteristic epithelial morphology both *in vivo* and *in*

vitro. In these better differentiated cases, temporal and spacial regulation of the cadherin-catenin complex seems to be more important for their movement (Takeichi, 1993; Vermeulen et al., 1996; Nabeshima et al., 1997a,b). The temporal regulation possibly includes proteolysis and transient reduction in the expression of E-cadherin (Takeichi, 1993), inappropriate expression of a nonepithelial cadherin (N-cadherin) instead of E-cadherin (Islam et al., 1996), and tyrosine phosphorylation of the E-cadherin-catenin complex, especially β -catenin and p120^{cas} (Tsukita et al., 1992; Takeichi, 1993; Kinch et al., 1995; Aberle et al., 1996). Among these mechanisms, tyrosine phosphorylation has been studied in most detail. The suppression of cadherin-mediated cell-cell adhesion correlated with the elevated tyrosine phosphorylation at the adherens junction (Volberg et al., 1991). The adherens junction molecules which underwent increased tyrosine phosphorylation were β -catenin in human breast cancer cells (Sommers et al., 1994), β - and α -catenin in Rous sarcoma virus-transformed fibroblasts (Hamaguchi et al., 1993) and β - and γ -catenin in epidermal growth factor (EGF)-treated human epidermoid carcinoma cells (Hoschuetzky et al., 1994). Since specific proto-oncogenic tyrosine kinases of the src-family (c-yes and c-src) were enriched to work as signal mediators in the adherens junction (Tsukita et al., 1992) and that tyrosine phosphorylation of β -catenin was stimulated in v-src-transformed 3Y1 cells (Matsuyoshi et al., 1992), c-yes and c-src are possible candidate kinases responsible for the elevation of tyrosine phosphorylation at the adherens junction. In addition, two receptor tyrosine kinases, the c-erbB-2 gene product and the EGF receptor (EGFR), were shown to associate directly with β -catenin, and EGFR induced tyrosine phosphorylation of β -catenin *in vitro* (Hoschuetzky et al., 1994; Ochiai et al., 1994). The increased tyrosine phosphorylation, however, did not disrupt the physical interaction of catenins with E-cadherin, but instead, dispersed complete E-cadherin-catenin complexes around the cell membrane. This suggests that tyrosine phosphorylation causes the dissociation of the whole E-cadherin-catenin complex from the actin cytoskeleton (Hoschuetzky et al., 1994). Not only protein kinases but also protein tyrosine phosphatases (PTP) can associate with the cadherin-catenin complex. A member of the leukocyte antigen-related protein (LAR)-related transmembrane PTP family was demonstrated to associate with the cadherin-catenin complex via the N-terminal domain of β -catenin in PC12 cells (Kypta et al., 1996). LAR-PTPs were phosphorylated on tyrosine in a TrkA-dependent manner, and their association with the cadherin-catenin complex was reduced in cells treated with nerve growth factor (NGF), suggesting that changes in tyrosine phosphorylation of β -catenin mediated by TrkA and LAR-PTPs control cadherin adhesive function during neurite outgrowth. Thus, both tyrosine kinases and PTPs may coordinately participate in the regulation of the dynamic tyrosine phosphorylation of the cadherin-

catenin complex.

In our TPA and HGF/SF-induced cohort migration models, ultrastructural study clearly demonstrated that the cells of the migrating cell sheets were in a state of active locomotion whilst maintaining cell-cell contact. This was performed via localized modulation of cell-cell adhesion such as the occurrence of wide intercellular gaps in the lower portion of the cells to allow the cells to extend tapering cytoplasmic processes and leading lamellae forward while cell-cell contacts remained close in the upper portion of the cells with desmosomes (Fig. 4) (Nabeshima et al., 1995b). Thus, complete disruption of cell-cell contacts, which is critical in scattering or single cell locomotion, is not needed in cohort migration; only partial disruption is sufficient. The mechanism involved in this localized modulation of cell-cell adhesion between migrating cells was investigated with special reference to the expression and phosphorylation of the E-cadherin-catenin complex. E-cadherin immunostaining, which was demonstrated

using an anti-E-cadherin monoclonal antibody, HECD-1, decreased in migrating L-10 cell sheets, but E-cadherin was involved in sheet formation of the migrating cells since simultaneous or sequential treatment with TPA and HECD-1 inhibited sheet formation and caused scattering of migrating cells. Furthermore, immunoelectron microscopical study revealed that E-cadherin immunoreactivity was confined to the upper portion of the migrating cells but was lost at the lower portion, where wide intercellular gaps appeared. In TPA-induced cohort migration, these morphological changes were associated with increased tyrosine phosphorylation of the E-cadherin-catenin complex, including β -catenin (Nabeshima et al., 1997a). In HGF/SF-induced cohort migration, however, the tyrosine phosphorylation of the E-cadherin-catenin complex was not increased (Nabeshima et al., 1998b). Since the amount of α -catenin complexed with E-cadherin was reduced, the linkage of E-cadherin to actin cytoskeleton via α -catenin might have been partly disrupted. In both cases, E-

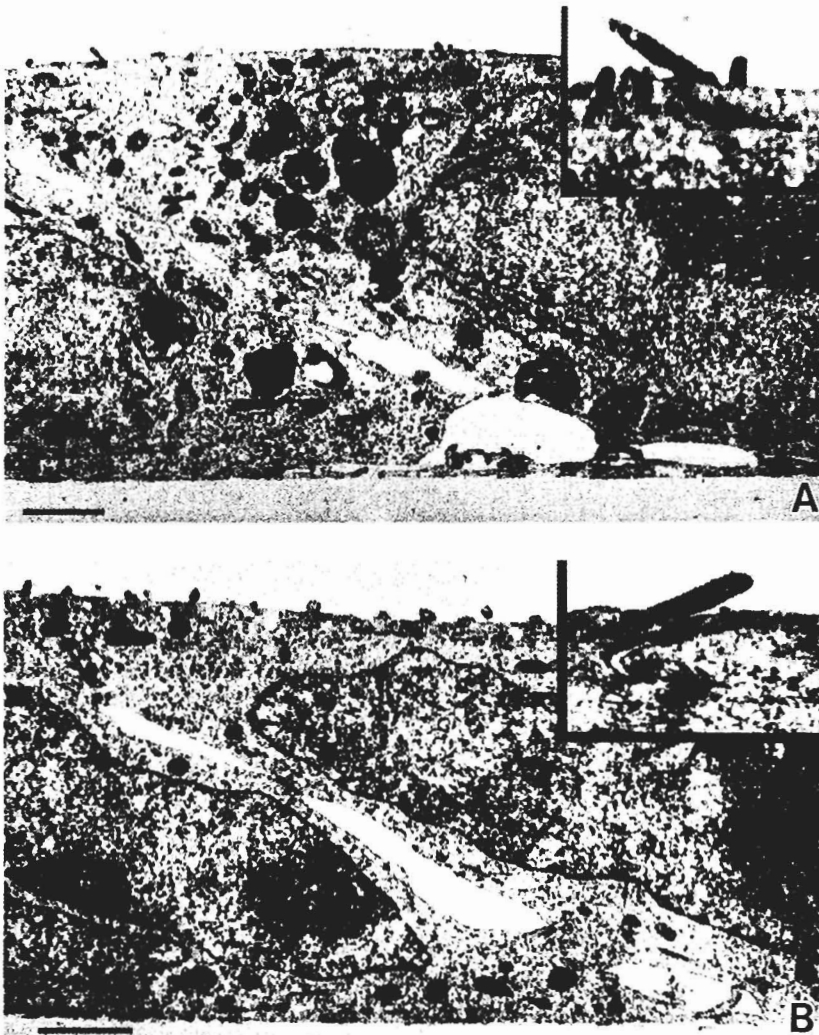


Fig. 4. Ultrastructural appearance of migrating cell sheets. In both TPA- (A, Nabeshima et al., 1995b) and HGF/SF-induced cohort migration (B, Nabeshima et al., 1998b), wide intercellular gaps occur in the lower portion of the cells to allow the cells to extend tapering cytoplasmic processes and leading lamellae forward whilst maintaining close cell-cell contacts in the upper portion of the cells with desmosomes (insets). Bars: 1 μ m.

cadherin and catenin expression levels were not altered. Thus, in L-10 cells the localized modulation of cell-cell adhesion can be achieved in a different manner depending on the stimulatory agents; via increased tyrosine phosphorylation of β -catenin in TPA-stimulated cases and via another unknown mechanism leading to reduction of α -catenin complexed with E-cadherin in HGF/SF-stimulated cases (Fig. 5), which is an example of redundancy frequently observed in living cells. MDCK cells also show similar findings: in temperature sensitive-src-transfected MDCK cells, scattering of cells was associated with increased phosphorylation of E-cadherin and β -catenin (Behrens et al., 1993), whereas no alteration of phosphorylation levels was found in HGF/SF-induced scattering (Weidner et al., 1990). The alterations of E-cadherin and catenins during HGF/SF-induced scattering are variable and seem to depend on cell types: HGF/SF induced increased phosphorylation of β - and γ -catenins in MKN7 and HT29 cells (Shibamoto et al., 1994), reduced E-cadherin expression at the protein level in TMK-1 cells (Tannapfel et al., 1994) and temporal reduction in phosphorylation of p120^{cas} in HT29 cells (Shibamoto et al., 1995), while no apparent detectable alterations in the protein and phosphorylation levels were described in MDCK cells (Weidner et al., 1990).

The precise mechanism underlying the reduction in complexed α -catenin in HGF/SF-induced cohort migration is currently unknown. Recently it was suggested that tyrosine phosphorylation of junctional proteins other than β -catenin was required for the weakening of cadherin-based cell adhesion in L fibroblasts. Although these fibroblasts expressed the cadherin- α -catenin fusion protein and did not require β -catenin for cell adhesion, the introduction of v-src into these cells shifted their adhesion from a strong to a weak state (Takeda et al., 1995). On the other hand, Rac and Cdc42 subfamilies of the Rho small G protein family also regulate the formation of the cadherin-based cell-cell adhesion (Takaishi et al., 1997; Kuroda et al., 1998). MDCK cells expressing dominant active mutants of Rac1 made tight contact with each other throughout the lateral membranes associated with increased E-cadherin, β -catenin and actin filaments at the cell-cell adhesion sites, whereas those adherens junctional proteins decreased in MDCK cells expressing dominant negative mutants of Rac1, compared with those in wild-type MDCK cells (Takaishi et al., 1997). Moreover, IQGAP1, a target of Cdc42 and Rac1, interacted with E-cadherin and β -catenin at sites of cell-cell adhesion in mouse L fibroblasts expressing E-cadherin and induced the dissociation of α -catenin from the cadherin-catenin complex, resulting in a decrease in E-cadherin-based cell-cell adhesion (Kuroda et al., 1998). Since HGF/SF-induced scattering of MDCK cells was dependent on Rac activation (Ridley et al., 1995), Rac and IQGAP1 might be involved in the mechanisms leading to a reduction in complexed α -catenin in HGF/SF-induced L-10 cell cohort migration.

E-cadherin-catenin complex and carcinoma: the rule and exceptions

Although the exact function of E-cadherin and catenins in tumor progression remains to be determined, it is likely that all are somehow related to the potential invasiveness of epithelial tumors (Rimm et al., 1995). Several experimental studies showed an invasion-suppressor role of E-cadherin (Behrens et al., 1989; Chen and Öbrink, 1991; Vlemingckx et al., 1991; Doki et al., 1993) and catenins (Sommers et al., 1994), and a number of immunostaining studies of different human carcinomas revealed an inverse correlation between expression of E-cadherin (Schipper et al., 1991; Shimoyama and Hirohashi, 1991; Oka et al., 1992, 1993; Umbas et al., 1992; Dorudi et al., 1993) or catenins (Rimm et al., 1995; Takayama et al., 1996) and tumor grade, invasiveness and metastasis. However, there are exceptional observations: 1) In gastric cancers, carcinomas maintaining high levels of E-cadherin expression can metastasize to the liver and E-cadherin expression is preserved in more than half of the lymph node metastasis (Oka et al., 1992; Takeichi, 1993); 2) E-cadherin levels are higher in the intravascular tumor compartment than in the adjacent extravascular tumor compartment in stomach, breast and colon cancers (Cowley and Smith, 1995); and 3) in pancreatic cancers, there is a greater tendency for liver metastasis in cases in which the integrity of the E-cadherin-catenin-mediated cell-cell adhesion system is intact (Gunji et al., 1998). The concept of cohort migration may give an explanation for these findings: better differentiated adenocarcinomas cells are released from intercellular adhesion transiently via modulation of the function of

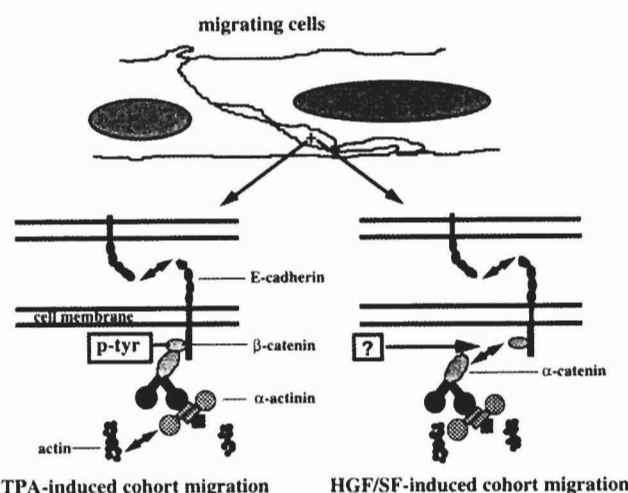


Fig. 5. Possible mechanisms involved in cohort migration. Focal disruption of cell-cell adhesion at the lower portion of cells is associated with increased tyrosine phosphorylation of β -catenin in TPA-induced cohort migration and another unknown mechanism leading to reduction of α -catenin complexed with E-cadherin in HGF/SF-induced cohort migration.

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Table 1. Motogenic and morphogenetic factors/proteins that can possibly modulate epithelial cell-cell adhesion.

FACTORS	MW (kDa)	TARGET CELLS	RECEPTORS	MOTILITY	E-CADHERIN/CATENINS	REFERENCES
HGF/SF	90	keratinocytes, MDCK cells TMK-1 human gastric & colon carcinomas human colorectal carcinomas	c-Met	SC, EMT SC	no alteration down regulation (E-cad) phosphorylation (Ecc)	Weidner et al., 1990 Tannapfel et al., 1994 Shibamoto et al., 1994 Nabeshima et al., 1998b
EGF	6	human epidermal cells HSC-1 (human SCC)	EGFR	CMD SC	n.d. phosphorylation (Ecc)	Zeigler et al., 1996 Fujii et al., 1996
aFGF	16	NBT-II bladder carcinoma	FGFR	SC, EMT	no alteration	Boyer et al., 1992
TGF- α	18	human epidermal cells NBT-II bladder carcinoma	EGFR	CMCG SC, EMT	n.d. n.d. (*-1)	Turksen et al., 1991 Gavrilovic et al., 1990
TGF- β 1	25	t-mouse epidermal cells	TGF- β R	SC, EMT	down regulation (E-cad)	Caulin et al., 1995
TNF- α	17	carcinomas	TNFR	SC	n.d.	Rosen et al., 1991
IL-6	26	human ductal breast carcinoma	IL-6R	SC	n.d. (*-2)	Tamm et al., 1989
AMF-like	55	HOC313 (human SCC)	n.d.	SC	down regulation (E-cad)	Ishisaki et al., 1994
5T4	72	murine mammary cells	-	SC	down regulation (E-cad)	Carsberg et al., 1996
epimorphin	60-70/150	murine epidermal cells	n.d.	ME	n.d.	Hirai et al., 1992

SC: scattering; CM: cohort migration; CMCG: cohort type migration into collagen gels; CMD: cohort type migration into human dermis; EMT: epithelial-mesenchymal transition; ME: morphogenesis of epithelia; n.d.: not determined; E-cad: E-cadherin; Ecc: E-cadherin/catenin complex; cat: catenin; *-1: internalization of desmosomal components was detected; *-2: intercellular desmosomal attachments were decreased; SCC: squamous cell carcinoma; t: transformed; HGF/SF: hepatocyte growth factor/scatter factor; EGF: epidermal growth factor; aFGF: acidic fibroblast growth factor; TGF: transforming growth factor; TNF: tumor necrosis factor; IL: interleukin; AMF-like: autocrine motility factor-like factor; R: receptor.

the E-cadherin-catenin complex, which usually takes place focally around the cell and enables cells to extend leading lamella to move as coherent cell nests. Since the above exceptions are all related to blood-borne metastasis, this movement as coherent cell nests might be advantageous for intravasation, survival within vasculature and lodgement at distant sites. In fact, in histopathology sections, carcinoma cells invading blood vessels (Talbot et al., 1981) and lymphatics (Carr et al., 1986) as coherent clusters of cells are frequently observed. Moreover, this cohort migration model may also explain the heterogeneous cadherin immunostaining in many tumors; the sites of tight cell-cell adhesion stain intensely while the sites released from the adhesion via phosphorylation or other mechanisms may stain negatively. Since at the site of cadherin-based cell-cell adhesion, α -catenin molecules possibly associate with each other or bind to vinculin through their fifth domain and these associations or bindings result in formation of aggregated patches of cadherin molecules (Tsukita et al., 1992), immunostaining might be able to detect only these aggregated patches of cadherins formed at the tight adhesion site. This seems to be the case for E-cadherin immunostaining in migrating cell sheets in our cohort migration model.

Motogenic factors which can possibly modulate cell-cell adhesion

Recently, significant progress has been achieved in the analysis of the factors that stimulate motility of tumor cells, including tissue and serum proteins, ECM

proteins and their degradation products, motility factors and growth factors (Goldberg, 1990). This depends on development of very convenient motility assays. These days the most frequently used cell motility assays are Boyden chamber-type assays and scattering assays (Stoker and Gherardi, 1991; Nabeshima et al., 1997b). Although both detect single cell locomotion, the mechanisms involved in these assays are not the same. Disruption of cell-cell contacts is one of the important steps involved in the latter, while it may not be included in the former, since single cell suspensions are usually added simultaneously with motility factors. Since disruption of cell-cell contacts, irrespective of whether they are complete or incomplete, is critical for epithelial cell movement, only motogenic factors that can possibly modulate cell-cell adhesion are reviewed here (Table 1). These motogenic factors are predominantly scattering-inducible motogenic growth factors or cytokines, some (HGF/SF, EGF and TGF- α) of which can also induce cohort type migration in two-dimensional assays or in three-dimensional collagen gels. Cell-cell contacts can be disrupted via downregulation of E-cadherin caused by HGF/SF and TGF- β 1 (Tannapfel et al., 1994; Caulin et al., 1995), via tyrosine phosphorylation of the E-cadherin/catenin complex induced by HGF/SF and EGF (Shibamoto et al., 1994; Fujii et al., 1996) or via reduction in amount of α -catenin complexed with E-cadherin caused by HGF/SF (Nabeshima et al., 1998b). Epithelial cell movement induced by HGF/SF, acidic fibroblast growth factor (aFGF), TGF- α and TGF- β are also considered as *in vitro* models of epithelial-mesenchymal transition (EMT) (Birchemier and

Birchmeier 1993; Boyer et al., 1996). During normal development, transitions of epithelia to mesenchyme can occur, and mesenchyme can differentiate into new epithelia (Birchemier and Birchmeier, 1993). Such transitions are not confined to development, but also occur in carcinoma cell invasion. EMT is, however, often referred to conversion of epithelial cells into individual mesenchymal or fibroblast-like cells, capable of migrating freely throughout the connective tissue (Hay, 1995; Boyer et al., 1996). As described above, carcinoma cells, especially those maintaining epithelial nature, can move as cohesive clusters of cells, and almost the same mode of movement of mesodermal cells is also seen during heart formation (Wiens, 1996). Therefore, we would like to consider the cohort type migration as a form of EMT. As for induction of EMT, not only soluble factors but also cell surface- or ECM-linked proteins, such as epimorphin (Hirai et al., 1992) and 5T4 (Carsberg et al., 1996), can be involved. Epimorphin is a mesenchymal protein, which is present on the mesenchymal cell surface or bound to ECM, especially type IV collagen, and controls epithelial morphogenesis, such as hair follicle formation (Hirai et al., 1992, 1993). Thus, it may not strictly be a motogenic factor, but morphogenesis includes cell movement as its important component. In this light, epimorphin can be considered as an EMT inducer, possibly of cohort-type EMT. Since epimorphin was shown to be contained in the Engelbreth-Himes-Swarm (EHS) sarcoma-derived basement membrane matrix (Matrigel) (Kleinman et al., 1986), it may be involved in carcinoma cell movement in a paracrine fashion. On the other hand, 5T4 is a transmembrane glycoprotein which is expressed on carcinoma cells and stimulates their own motility as described above (in the section of "Regulation of cell-cell adhesion during cohort migration"). A cell surface molecule which plays a role in maintaining epithelial morphology and restricts cell motility is also known. Syndecan-1, the cell surface heparan sulfate proteoglycan, is expressed at high levels in normal stratified epithelia, but following malignant transformation its expression is reduced or lost (Jalkanen et al., 1991; Inki et al., 1994). Transfection of anti-sense syndecan-1 cDNA into epithelial cells resulted in a more fibroblastic morphology accompanied by a disorganized actin microfilament cytoskeleton, reduced expression levels of E-cadherin and increased cell motility (Kato et al., 1995). The specific motility factor, autocrine motility factor (AMF), has not been directly shown to modulate E-cadherin-based cell-cell adhesion as far as we know, although the AMF-like factor produced by human squamous carcinoma cells caused downregulation of E-cadherin (Ishisaki et al., 1994). The expression of the cellular receptor for AMF (AMF-R or gp78) was increased in human bladder carcinomas and also in Moloney sarcoma virus-transformed MDCK cells associated with the concomitant decreased expression of E-cadherin (Otto et al., 1994; Simard and Nabi, 1996).

Cell-ECM interactions involved in cohort migration

A role of ECM in induction and regulation of tumor cell motility can be considered in two different aspects: 1) ECM proteins and proteoglycans themselves can function as motility-start signals and also as directional guidance for tumor cells; and 2) ECM, especially proteoglycans, can be a reservoir of motogenic growth factors and cytokines (Zetter and Brightman, 1990; Stoker and Gherardi, 1991; Nabeshima et al., 1997b). By protecting the motogenic factors from proteases ECM may also be their physiological concentrator. Upon degradation of ECM, the motogenic factors are released from the matrix and induce or enhance migration of tumor cells. Motogenic factors that bind to heparin or heparan sulfate include FGF, platelet-derived growth factor (PDGF), granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor- α (TNF- α) and HGF/SF (Ruoslahti and Yamaguchi, 1991; Mizuno et al., 1994). Thus, cohort migration can also be induced by this mechanism via released HGF/SF (Fig. 6). Moreover, although HGF/SF remains as an inactive

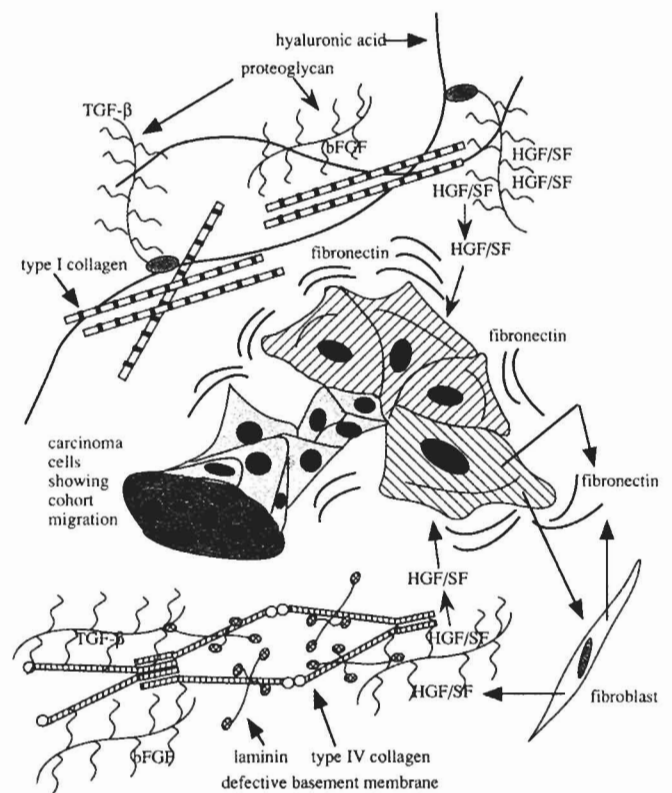


Fig. 6. Interaction between carcinoma cells showing cohort migration and ECM. Upon degradation of ECM, entrapped motogenic factors, including HGF/SF, are released from the matrix and induce cohort migration of carcinoma cells. This migration is guided by ECM components, especially fibronectin (FN) in the case of cohort-type migration. FN, which is predominantly EDA+FN, is produced by carcinoma cells themselves and also by fibroblasts via stimulation by carcinoma-derived soluble factors.

single-chain form in the normal state, it is converted to an active heterodimeric form at the site of tissue injury (Miyazawa et al., 1996), which is frequently caused by tumor invasion. A protease responsible for this conversion is HGF/SF activator, which can be activated by thrombin generated at the injured site.

ECM proteins, including type I and IV collagens, fibronectin (FN), laminin, vitronectin, elastin and thrombospondin, and their degradation products have chemotactic (when they are in solution) or haptotactic (when they are insoluble or substratum-bound) activities to tumor cells *in vitro* when measured by single cell locomotion assays (Zetter and Brightman, 1990; Levine et al., 1995; Nabeshima et al., 1997b). In cohort type migration, FN is known to play an important role. During sea urchin and amphibian gastrulation, the primary mesodermal cells, once inside the blastocoele, appear to move collectively along the path lined with FN (Boucaut et al., 1984; Katow and Hayashi, 1985). An injection of Arg-Gly-Asp (RGD)-containing FN peptides completely blocked this migration. The fact that FN gene-knockout mice died from developmental gastrulation defects (Schlaepfer and Hunter, 1998) supports the involvement of FN in the above collective cell migration. Involvement of FN is also described in cohort type migration of precardiac mesodermal cells during heart formation (Wiens, 1996). In this study, an attractive model suggesting directional guidance and generation of a migratory force by interactions between front cells and FN is proposed. Since FN is arrayed as a localized patch on the adjacent endoderm rather than a gradient, only front cells of collectively migrating mesodermal cells attach effectively to FN. Thus adhered, their cytoskeletal contractile activity generates a force which propagates throughout the migrating cell layer, and efficiently pulls them in the proper direction. At a single cell level, localized activation of motility receptors, such as integrins and focal adhesion-associated proteins, by discrete clusters of ligands (a guidepost) might serve to direct cell migration by activating leading-edge extension, which permits the cell to turn towards the guidepost (Sheetz et al., 1998). The same can occur at the front cells in cohort-type migration, and this interaction between the ligand (FN) and front cells might guide the direction of not just one cell but the whole sheets of cells via cell-cell contacts. In this mode of movement, E-cadherin-mediated cell-cell adhesion, which is generally thought to be one of invasion-suppression mechanisms, could help the directional guidance of migration provided by ECM proteins once migration starts; the cells easily follow the preceding cells by cell-cell contact.

In our study, TPA-induced L-10 cell cohort migration on glass substratum was almost completely inhibited by addition of RGD peptides into the medium, and thus this is RGD-dependent (Nabeshima et al., 1998a). Cohort migration was stimulated on type I and IV collagens, FN and laminin-coated substratum, but was inhibited by RGD only on the FN-coated surface. By using immunofluorescent techniques, FN was

demonstrated preferentially around migrating cells, and a protein synthesis inhibitor, cycloheximide, inhibited the migration by about 75%. FN produced by L-10 cells were found to be mostly EDA+ FN when analysed by RT-PCR. Moreover, anti-FN antibody, but not anti-vitronectin antibody, inhibited the TPA-induced cohort migration almost completely. Thus, it is likely that L-10 cells produce and deposit FN into pericellular spaces and this enables cells to move in an RGD-dependent manner when stimulated (Fig. 6). Also in a three-dimensional culture model with OSC-19 squamous carcinoma cells, FN secreted and deposited by carcinoma cells was associated with cohort-type invasion into the collagen gel (Kawahara et al., 1993). As it provides directed guidance for collective cell migration during development, FN also appears to favor carcinoma cell cohort migration.

Recently increased deposition of FN in various cancer tissues has been reported by several investigators (Carnemolla et al., 1989; Oyama et al., 1993; Kaczmarek et al., 1994; Hauptmann et al., 1995). We also demonstrated that carcinoma cells could stimulate fibroblasts to produce more FN via TGF- β and other soluble factors (Fig. 6) (Inoue et al., 1996). FNs from different sources are slightly different from each other with respect to subunit sizes, and the heterogeneity arises mainly from alternative splicing of a primary transcript at three distinct regions termed EDA, EDB, and IIICS (Schwarzbauer et al., 1987). FN expressed in fetal and tumor tissues contains a greater percentage of EDA and EDB segments than that expressed in normal adult tissues (Carnemolla et al., 1989; Oyama et al., 1993; Kaczmarek et al., 1994; Hauptmann et al., 1995). We also showed that carcinoma-derived factors stimulated the alternative splicing at the EDA region in cancer-associated fibroblasts and their effect depended on cell density of fibroblasts (Inoue et al., 1996, 1998). The enhancing activity of the EDA segment in cell adhesion and motility was clearly demonstrated recently (Manabe et al., 1997): recombinant FNs containing the EDA segment were approximately twice as potent as those lacking EDA in their abilities to promote cell adhesion and migration, irrespective of the presence or absence of EDB. Since this activity was abolished by antibodies against integrin $\alpha 5$ and $\beta 1$ subunits and by RGD-containing peptides, the EDA segment seemed to enhance the cell-adhesive activity of FN by potentiating the interaction of FN with integrin $\alpha 5\beta 1$. In our study, FN expressed by L-10 cells was mainly EDA+ and EDB-. This seems suitable for carcinoma cell migration. Since L-10 cell cohort migration was RGD-dependent and L-10 cells expressed integrin $\alpha 5\beta 1$, L-10 cells possibly interact with FN via integrin $\alpha 5\beta 1$ during cohort migration. Similarly, the increased EDA+ FN in cancer tissue may favor cohort migration of carcinoma cells *in vivo*.

Cross talk between cell-cell and cell-ECM adhesion

In cohort-type migration, whether regulations of

cell-substrate and cell-cell adhesion coordinately occur is a very interesting topic. Coordinate regulation of expressions of cell-cell and cell-ECM adhesion molecules has recently been described. In general, expression levels of FN, integrins and integrin-binding proteins are inversely correlated to those of cell-cell adhesion molecules, such as E-cadherin. For example, overexpression of integrin-linked kinase (ILK), a serine/threonine kinase that binds to the integrin $\beta 1$ cytoplasmic domain, stimulated FN matrix assembly in epithelial cells, and this was accompanied by a reduction in the E-cadherin expression (Wu et al., 1998). Xenopus XTC cells stably transfected with E-cadherin showed downregulation of FN and $\alpha 3\beta 1$ integrin expression, associated with impaired adhesion to FN and laminin (Finnemann et al., 1995). Similarly, in mouse mammary epithelial cells, transition from epithelial to fibroblastic phenotype by TGF- $\beta 1$ correlated with decreased expression of E-cadherin, ZO-1 (a tight junction molecule) and desmoplakin I and II (desmosomal molecules) and increased expression of FN (Miettinen et al., 1994). Furthermore, localization and function of cell-cell adhesion molecules are also coordinately regulated according to changes in cell-ECM adhesion in migrating neural crest cells. Treatment of cells with RGD peptides or antibodies to FN and to $\beta 1$ and $\beta 3$ integrins blocked cell migration and at the same time caused rapid N-cadherin-mediated cell clustering. This cell clustering was induced via recruitment of an intracellular pool of N-cadherin molecules that accumulated into adherens junctions in tight association with the cytoskeleton (Monier-Gavelle and Duband, 1997). Thus, inhibition of the activity of $\beta 1$ and $\beta 3$ integrins appears to stimulate intracellular signaling events, which result in control of the surface distribution and activity of N-cadherin.

In our study with the TPA-induced cohort migration model, FN synthesis was mildly stimulated by TPA treatment, but expression levels of $\beta 1$ integrin, E-cadherin and catenins showed no alteration. Moreover, stimulation of cohort migration by type I collagen-coating and inhibition by RGD treatment did not affect the level of tyrosine phosphorylation of the E-cadherin-catenin complex induced by TPA. This indicates that cell-cell interactions are adjusted to suit cell migration directly by TPA, irrespective of the condition of cell-ECM adhesion, during TPA-induced cohort migration (Nabeshima et al., 1998a). Cross talk involved in HGF/SF-induced cohort migration might be different from that in the TPA-induced one.

Regulation of MMP expression during cohort migration

We investigated whether there is any specific way of regulation of MMP expression during cohort migration, since degradation of ECM is as important as active migration of cancer cells in tumor invasion. A membrane-type MMP (MT1-MMP) was expressed on L-10 cell surface in non-treated piled-up cell islands, while

in migrating cell sheets induced by HGF/SF, immunoreactive MT1-MMP is specifically demonstrated only at the leading edges of the front cells but not in the following cells, suggesting the presence of the regulation of MT1-MMP expression by cell-cell interactions within the migrating cell sheets (Nabeshima et al., 1998c). MMP-2, which can be activated on the cell surface by MT1-MMP, showed almost the same distribution as MT1-MMP in migrating cell sheets. When the migration was induced on gelatin-coated substratum, the gelatin matrix was degraded by these MMPs, but in a very organized manner, leaving radially arrayed gelatin matrix at the sites of leading edges. This organized gelatin degradation was necessary for cells to migrate on the gelatin matrix because migration ceased in the presence of a synthetic inhibitor specific to MMPs, BB94. Thus, gelatin matrix seems to be reorganized to suit cell migration beneath the leading edges, and this appears to be done via leading-edge-of-front-cell-specific localization of MT1-MMP and MMP-2 during cohort migration.

Conclusion and perspectives

Cell motility consists of stationary motility and translocative motility (Haemmerli, 1985). The translocative motility usually means single cell locomotion. In our series of studies, however, we have shown that (i) cohort type migration is observed in histopathology, especially of colon cancer; (ii) a naturally occurring motogenic factor, HGF/SF, can induce cohort migration in several human colorectal carcinoma cell lines; and (iii) during the cohort migration there is a specific way of regulation of MMPs, possibly via cell-cell interactions. Thus, we would like to propose cohort migration as an important mode of cell translocation, as well as single cell locomotion (Table 2). In this cohort-type migration, the most characteristic feature is that only partial disruption of epithelial cell-cell adhesion is sufficient for movement.

Cohort-type migration is a type of movement observed in better-differentiated carcinomas, and in colon cancer, well- to moderately-differentiated adenocarcinomas constitute more than 80%. In this light, control of cohort-type migration is worth considering. In poorly differentiated carcinomas, which show scattered

Table 2. Cell Motility.

a) Stationary motility
membrane ruffling
pseudopodal extensions
(blebs, filopodia, leading lamellae etc.)
b) Translocation
single cell translocation
(Boyden chamber, phagokinetic tracks and scattering assays, etc)
cohort migration
(cell translocation as coherent cell sheets)
(Lab-Tek chamber slide assay)

phenotype due to genetical abnormalities of the E-cadherin-catenin complex molecules, isolated carcinoma cells may be easy to move in response to motility-stimulating factors, whereas in differentiated carcinomas maintaining epithelial cell-cell adhesion, the concerted action of cell-cell adhesion-modulators and motility-stimulators may be necessary. Synergistic action of multiple factors may be needed to modulate cell-cell adhesion of well-differentiated carcinoma cells, although it is just partial disruption, compared with poorly differentiated ones. At the same time this means, however, that more steps are available to control cohort type migration. Thus, elucidation of the networks of motogenic factors (or combination of cell-cell adhesion-modulators and motility-stimulators) which can accelerate cohort migration is very important. These networks may involve cell-cell (for example, carcinoma cell-fibroblast) and cell-ECM interactions, and therefore their elucidation leads not only to control of cohort migration but also to the understanding of an aspect of multicellular life.

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