Coordinated Expression of MMPs in Pathfinder Cells and Reorganization of Extracellular Matrix during Cohort Migration

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Abstract: Migration of tumor cells is usually assessed as single cell locomotion in vitro using Boyden chamber type assays. In vivo, however, carcinoma cells frequently invade the surrounding tissue as coherent clusters or nests of cells. We have called this type of movement cohort migration and developed a two-dimensional in vitro cohort migration model, in which human colon carcinoma cells move as coherent cell sheets when stimulated with hepatocyte growth factor/scatter factor (HGF/SF). In this model, we have demonstrated that cohort migration has two unique features: first, temporal disruption of cell-cell adhesion is compartmentalized to the lower portion of cells, which allows the cells to extend leading lamella; second, cell-cell contact in the coherent migrating cell sheets regulates expression and localization of proteins used for migration, such as gelatinase A (MMP-2) and membrane-type I-matrix metalloproteinase (MT1-MMP), to work predominantly at the front of the migrating cell sheets. Gelatin matrix was reorganized to suit cell migration via this leading-lamella-of-pathfinder-cell-specific localization of MT1-MMP and MMP-2 during cohort migration, and this reorganization was essential for this type of migration. These lines of evidence suggest that cohort migration is a specific mode of cell migration, and thus we now consider that not only single-cell locomotion but also cohort migration could be involved in carcinoma-cell invasion: the former mainly in poorly differentiated carcinomas while the latter in well to moderately differentiated carcinomas.

Key words: cell migration, tumor invasion, metastasis, MMP, fibronectin

INTRODUCTION

The ability of tumor cells to infiltrate and disseminate widely is what makes the tumors malignant. In this invasive and metastatic process, cell migration plays an important role. The mechanisms by which cells move have predominantly been investigated using in vitro models in which cells move as single cells. However, in human surgical specimens, pathologists have observed and reported that carcinoma cells, especially those of well to moderately differentiated types, frequently invade the stroma as coherent cell nests or small clusters rather than single cells (Fig. 1), suggesting that there is a way by which carcinoma cells move together as coherent cell clusters. A time-lapse videomicroscopic demonstration of migrating cell clusters in primary tumor explants embedded in collagen gels also supports this concept. We have called this type of movement "cohort migration" and demonstrated that carcinoma cells can actually move en mass keeping cell-cell contact each other in vitro. After our proposal, cell movement in well-differentiated squamous cell carcinoma of the esophagus, malignant melanoma and uterine cervical carcinoma cells has been referred to cohort migration. Being compared with single-cell locomotion (SCL), one characteristic feature of cohort migration is compartmentalized release from cell-cell adhesion: cells extend leading lamellae to move via this release whilst keeping cell-cell contact in other portions. However, the most important feature that clearly differentiates cohort migration from SCL is the fact that migrating cells regulate expression and localization of MMP (matrix metalloproteinases) via cell-cell contact within migrating cell sheets. In our model, membrane-type I-matrix metalloproteinase (MT1-MMP) and gelatinase A (MMP-2) were expressed and localized specifically at the front pathfinder cells of the migrating cell sheets and reorganization of gelatin matrix by these MMPs was essential for this type of migration.

The cohort type migration, cell movement en mass, is not restricted to tumor cells, but also observed in some physiological situations, such as wound healing and fetal development. 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. These movements are termed "collective cell migration" or "cell sheet migration". In this type of migration, pathfinder cells play an important role via cellular interaction with extracellular matrix (ECM) and metalloproteinases. During heart formation, since fibronectin (FN) is arrayed as a localized patch rather than a gradient, only
front cells of collectively migrating precardiac mesodermal cells attach effectively to FN. Thus adhered, their cytoskeletal contractile activity generates force that propagates throughout the migrating cell layer, and efficiently pulls them in the proper direction\(^{13}\). In the nematode Caenorhabditis elegans gonad shape and size is determined by the migration of a leader cell, which is at the tip of the growing gonad arm, and a metalloprotease (GON-1) secreted by the leader cell has recently been found essential for this process, preparing the way ahead for the cell's migration\(^{16}\).

In this paper, first, clinicopathological significance of cohort migration, and then coordinated expression of MMPs in pathfinder cells and reorganization of ECM will be discussed.

Clinicopathological Significance of Cohort Migration Observed in Tumor Histopathology

Invasion as small aggregates or clusters of carcinoma cells itself is not a new finding. In '50s to '60s, nonsprouting and sprouting types of tumor growth were reported in gastric and uterine cervical carcinoma\(^{5}\). 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. In '70s to '90s, the sprouting feature was repeatedly paid attention clinicopathologically under the names of tumor-cell dissociation\(^{17}\) and tumor budding\(^{18}\), because these were associated with worse prognosis in gastric\(^{17}\), colorectal\(^{19}\), laryngeal\(^{19}\) and oral\(^{20}\) carcinomas. Furthermore in '60s, interestingly, Leighton et al. speculated based on their morphological observations of tumor pathology that aggregates of cells function as integrated units and propagate and migrate as a whole\(^{6}\).

To examine how frequently this cohort-type 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 (markedly resolved tumor glands with small clusters of carcinoma cells lying ahead) (type II cohort migration)\(^{4}\). A half of our cases (total 74 cases) showed type I cohort migration and the others type II (Fig. 2). Type Ia invasion front was observed in carcinomas which were confined to the mucosa (m) or submucosa (sm), 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 (ss) (m + sm, 1/4 cases; mp (muscularis propria), 2/3 cases; ss, 33/33 cases). Thus, in cases with tumor budding, both cohort migration and SCL 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 invasive growth and aggressive biological behavior when it is induced as small clusters of cancer cells lying ahead of the invasive front.

Cell movement as groups of cells affects not only invasion but also hematogenous metastasis. Tumor cell clumps that enter the circulation play a significant role in the hematogenous metastatic process. Intravenous injection of tumor cells in clumps of 6 to 7 cells produces a significantly greater number of metastatic foci than do a similar number of single tumor cells. Moreover, using the tumor transplantation system, it has been shown that tumor cell clumps arise locally within the vascular bed of the tumor and that a majority of the metastatic foci seen in the hematogenous metastatic process are a result of tumor cells in clump form. In accordance with this, carcinoma cells invading blood vessels and lymphatics as coherent clusters of cells are frequently observed in histopathology sections. Furthermore, there are reports that better differentiated adenocarcinomas, which maintain coherent tubular structures via the E-cadherin-catenin-mediated cell-cell adhesion system, show a relatively higher tendency for hematogenous metastasis to the liver in gastric and pancreatic cancers. These lines of evidence suggest that movement as coherent cell nests is advantageous for intravasation, survival within vasculature and lodgment at distant sites. In this light, it is important to elucidate the mechanisms involved in cohort migration to control cancer invasion and metastasis.

**Cohort Migration-Inducible Motogenic Factors**

To find out a naturally occurring motogenic factor(s) that can induce cohort migration of human colon carcinoma cells *in vitro*, we screened all the commercially available factors that had been reported to stimulate SCL of tumor cells. Among the factors examined, only hepatocyte growth factor/scatter factor (HGF/SF) clearly induced cohort migration of colon carcinoma cells in a Lab-Tek chamber slide assay. Carcinoma cells moved as coherent cell sheets one-cell thick in response to HGF/SF (Fig. 3a) with a peak response at 10-20 ng/ml, and this migration was specifically inhibited by anti-HGF/SF antibody in a dose dependent manner. Several human colorectal carcinoma cell lines showed this type of migration in response to HGF/SF, while yet others showed scattering type motility (dispersion of coherent clustered cells into single cells). Electron-microscope study of the migrating cell sheets demonstrated that migrating cells maintained cell-cell contact with one another in the upper portion of cells, with tight junctions and desmosomes. At the same time, the migrating cells formed wide-open intercellular spaces in the lower portions that enabled cells to extend leading lamella to move (Fig. 3b). Induction of cohort migration with HGF/SF in uterine cervical carcinoma cells is also recently reported.

**HGF/SF** is a mesenchymal-derived pleiotropic cytokine that shows mitogenic, motogenic and morphogenic activities on various types of cells. As a motogenic factor, it is widely accepted that HGF/SF induces scattering and SCL of various tumor cells *in vitro*. The cell-surface receptor for HGF/SF is c-Met (c-met proto-oncogene product), which is a 190-kD transmembrane tyrosine kinase. The serum level of HGF/SF is elevated in patients with gastric cancers, which is upregulated in colorectal cancers, synergistically stimulated HGF/SF-induced cohort migration of colon carcinoma cells, although TGF-β1 alone did not induce cohort migration.

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Fig. 3. HGF/SF-induced cohort migration of colorectal carcinoma cells (L-10). a. In response to HGF/SF treatment (10 ng/ml), the cells move outwards from the cell islands as coherent sheets one-cell thick. The front cells of the migrating cell sheets show motile cell morphologies with leading lamellae (arrows). The remaining piled-up cell islands are shown by arrowheads. b. Ultrastructural appearance of migrating cell sheets. Wide intercellular gaps occur in the lower portion of the cells to allow the cells to extend leading lamellae forward whilst maintaining close cell-cell contacts in the upper portion of the cells with desmosomes (inset, arrow). Bar=1 μm. (from reference 27)
Coordinated Expression of MMPs

Coordinated Expression of MMP and Reorganization of ECM during Cohort Migration

Degradation of ECM by MMP is assumed to be a prerequisite for the cells to migrate into native or provisional tissue matrix. This proteolytic degradation of ECM suggests two possibilities: one is simply a path clearing mechanism, and the other is a way of reorganizing the matrix to facilitate cellular interaction. We

Fig. 4. Expression and localization of MT1-MMP and matrix degradation during migration.

a-c. Immunolocalization of MT1-MMP. When cohort migration of L-10 carcinoma cells is induced with HGF/SF treatment (20 ng/ml, 24 h) (a, b), only the frontmost cells stain positive for MT1-MMP (a, arrows), whereas the following cells in the migrating cell sheets are negative (**). The remaining piled-up cell islands are indicated by single asterisk (*). Distribution of MT1-MMP in the leading edges of the frontmost cells is shown in b. Nuclei are indicated as "n".

When scattering is induced with HGF/SF treatment in the presence of anti-E-cadherin antibody (c), MT1-MMP is demonstrated in each scattering cells. d, e. In situ hybridization with MT1-MMP RNA probe. When cohort migration is induced, the frontmost cells of migrating cell sheets specifically express MT1-MMP mRNA (d), while all single or small clusters of cells express the message when scattering is induced (e). f. Organized degradation of coated gelatin substrate by migrating L-10 cells. When cohort migration is induced on FITC-labelled gelatin matrix, dark areas caused by lysis of gelatin is seen at the sites corresponding to leading edges. The lysis of gelatin is caused in an organized manner: radially arrayed gelatinolysis (arrows) is observed at the frontmost portion of the leading edges. A portion of migrating cell sheets is indicated by two asterisks (**). The cells were photographed at a magnification of ×50 (a), ×100 (c, d, e, f), or ×250 (b). (from reference 14)
would prefer the latter possibility based on our findings, which we show hereafter. Additionally, this reorganization is done via coordinated expression and localization of MMPs at the cell surface; they are preferentially localized in leading edges of each migrating cell in SCL36 and front pathfinder cells of migrating cell sheets in cohort migration10.

In our two-dimensional cohort migration assay, when migration was induced with HGF/SF, MT1-MMP and MMP-2 were expressed only at the front cells of migrating cell sheets, especially in their leading edges, with the following migrating cells being negative (Fig. 4a, b)14. In contrast, when cell scattering was induced by stimulating cell migration in the presence of anti-E-cadherin antibody, the front cell-specific pattern of expression observed during cohort migration was lost: individual scattering cells expressed both MT1-MMP and MMP-2 in their leading edges and cytoplasm (Fig. 4c). The same findings were observed in both of the two human colon carcinoma cell lines examined, L-10 and SW837. This pattern, expression in front cells during cohort migration and in individual cells during scattering, was also the case in MT1-MMP mRNA expression that was demonstrated by in situ hybridization (Fig. 4d, e), suggesting the regulation at the gene expression level. When cohort migration was induced on gelatin-coated substratum, these MMPs expressed in the front cells degraded the gelatin matrix in a very organized manner, leaving radially arrayed gelatin matrix at the sites of leading edges (Fig. 4f). Inhibition of this organized lysis with BB94, a synthetic inhibitor specific to MMPs, caused inhibition of migration in both L-10 and SW837 cells in a dose-dependent manner (Fig. 5). Additionally, tissue inhibitor of metalloproteinases (TIMP)-1 and -2, and the COOH-terminal hemopexin-like domain of MMP-2 (GelA PEX) inhibited the migration on gelatin matrix, indicating that MMP-2 activated on the cell surface by MT1-MMP plays a major role in cohort migration on gelatin matrix14.

Possible Regulation of MMP Expression via Cell-cell Contact among Migrating Cells

Although the precise mechanisms involved in the front cell-specific localization of MT1-MMP and MMP-2 are currently unknown, at least that of MT1-MMP is regulated at the gene expression level. Based on its different expression patterns in cohort migration and scattering, gene expression of MT1-MMP in the following cells of migrating cell sheets appears to be down-regulated via cell-cell contact among migrating cell sheets. Similarly, gene expression of MT1-MMP is reported to decrease in confluent cultures of mouse mammary gland epithelial cells37. They also suggested the presence of translational control mechanisms for MT1-MMP expression because MT1-MMP immunoreactivity disappeared very early in confluence whereas MT1-MMP mRNA levels started to decline a few days later when cell cultures reached confluence. The cellular binding of MMP-2 also reduces in confluent cultures compared with that in sparse cultures of breast carcinoma cells39. On the other hand, expression of MT1-MMP and MMP-2 in the front pathfinder cells may be related to abundant interaction with ECM there. FN is one of the candidate ECM components, since FN is preferentially produced and deposited extracellularly by migrating cells during cohort migration43,39 and that culturing of L-10 colon carcinoma cells on FN substratum stimulates production and activation of MMP-2 (unpublished data). Taken together, the presence of abundant cell-cell contact within migrating cell sheets as in confluent cultures seems to suppress MT1-MMP expression and MMP-2 binding, whereas specialized cell-ECM contact may facilitate expression of these enzymes in the front pathfinder cells.

Localization of MT1-MMP and MMP-2 to Leading Edges within Pathfinder Cells

Preferential localization of MT1-MMP and MMP-2 to leading edges within pathfinder cells suggests the presence of regulation mechanisms at the protein level. The mechanisms consist of two parts: i) a mechanism for cell surface association of MMPs, and ii) that for localization of MMPs to leading edges. The first mechanism that focus MMP activity at the cell surface would appear to be the most logical mechanism to efficiently effect and regulate cell movement through ECM40. A relatively major function for the cell-bound enzyme in comparison to soluble MMP has been shown40. In human glioma and fibrosarcoma cells transfected with cDNA encoding MT1-MMP, their ability to contract collagen lattices was shown to be dependent on the MT1-MMP-mediated activation of proMMP-2 and cell surface association of activated MMP-2. Soluble MMP-2 failed to affect gel contraction. In our cohort migration

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**Fig. 5.** Effect of BB-94 on HGF/SF-induced cohort migration on gelatin-coated substratum. Cells were treated with BB-94 (10 or 50 μM) for 1 h, and then cohort migration was induced with HGF/SF treatment (20 ng/ml, 24 h) on gelatin-coated substratum in the presence of the above inhibitors. *; statistically significant compared with control, p<0.01.
model, this cell surface association mechanism depends on MT1-MMP. In our study, GelA PEX effectively inhibited cohort migration on gelatin matrix. GelA PEX, the carboxyl-end domain of MMP-2, is involved in the trimolecular complex formation of MT1-MMP, TIMP-2 and MMP-2 on the cell surfaces\(^{41,42}\) and competitively inhibits MMP-2 activation by MT1-MMP\(^{40,41}\). Thus, MMP-2 that is bound to and activated by MT1-MMP on the cell surfaces seems to play a major role during the cohort migration. Effective inhibition by both TIMP-1 and TIMP-2 also supports this hypothesis because MMP-2 is known to be inhibited by these TIMPs whereas MT1-MMP is inhibited by TIMP-2 but not by TIMP-1\(^{19}\). In human melanoma cells, however, the presence of active MMP-2 just on the cell surfaces was not enough but more specialized localization of MT1-MMP and MMP-2 to the invasion front of cells (invadopodia, specialized membrane extensions into the FN-coated gelatin matrix) was essential for their invasion\(^{41}\). Similarly, localization of MMP to leading edges of migrating cells in two-dimensional systems is suitable for cell migration\(^{30}\). Cytoplasmic docking systems supporting these localizations are not yet fully understood, but recently binding of MT1-MMP to CD44 by its extracellular domain has been reported\(^{40}\). CD44 and MT1-MMP colocalize at the leading edge of the motile cells, and MT1-MMP acts as a processing enzyme for CD44 there, shedding CD44H from the cell surface. This processing event stimulates cell motility. Since CD44 are connected to actin cytoskeleton of cells, CD44 may link the MT1-MMP to the cytoskeleton. Since accumulation of gelatinase B (MMP-9) through an actin-dependent pathway to the leading edges is observed in migrating airway epithelial cells\(^{40}\), a similar transportation system may be present also for MT1-MMP. In our cohort migration model, radial array of linear gelatinolytic zones beneath the leading edges of front cells was similar to the arrangement of actin in the leading edges\(^{44}\). Localization of the MT1-MMP/MMP-2 complex might somehow be related to actin distribution. Since MMP-2 binds to \(\alpha_v\beta_3\) integrins\(^{11}\), association of MT1-MMP with MMP-2 captured onto \(\alpha_v\beta_3\) integrins is another possibility.

**Reorganization of ECM by Pathfinder Cells**

In general, ECM is thought to be a kind of barrier for tumor cell invasion, and more or less its cleavage and removal are necessary for tumor cells to migrate\(^{30}\). At the same time, however, ECM components provide cells with good substrate to move on. Recent studies demonstrated an essential role of enzymatic modification of the basal lamina of the endoneurium for peripheral nerve regeneration following injury. The neurite-promoting activity of endoneurial laminin may be inhibited by a Schwann cell-derived chondroitin sulfate proteoglycans (CSPG)s. Treatment of peripheral nerve sections with MMP-2 resulted in the removal of CSPG$s and exposure of epitopes of laminin permissive for neurite extension\(^{49}\).

In our cohort migration study, removal of gelatin matrix at the leading edges of the front cells of migrating cell sheets was not random or complete. Instead, it was performed in a very coordinate and organized manner, leaving radially arrayed gelatin matrix at the frontmost part. This limited and organized clearing of gelatin matrix was essential for cell migration because MMP inhibitors efficiently inhibited migration. Thus, an important role of MMP is not just to remove ECM but to rearrange it to suit cell migration. Similarly, in three-dimensional matrix-based models, fibroblastic type tumor cells are reported to cause initial fiber traction at the leading edge followed by radial fiber alignment towards the cell which then favors persistent migration in direction of maximal traction\(^{19}\). Moreover, it is suggested that binding of integrins to this kind of pre-stressed ECM fibers would strengthen the linkage between those receptors and the force-generating cytoskeleton at that side of cells, thereby causing the cell to migrate along the direction of the rigid substrate\(^{50}\).

We previously showed that synthesis and deposition of FN, especially EDA+FN (EDA-containing FN), by migrating carcinoma cells were important for cohort-type migration: cells migrate on this substrate in a RGD and \(\beta_1\)-integrin dependent manner\(^{29}\). EDA+FN is one of variants which are produced by alternative splicing of its primary transcripts, and its expression increases during morphogenesis and tissue remodeling that need cell migration\(^{51}\). Selective expression of this EDA+FN variant was stimulated by HGF/SF treatment\(^{29}\) especially in the migrating cells\(^{51}\), and HGF/SF-stimulated expression was further enhanced during cohort migration by the presence of fibroblasts via TGF-\(\beta\) generated by interactions between carcinoma cells and fibroblasts\(^{44}\). Because EDA+FN deposition occurs around and beneath the leading edges of front migrating cells\(^{39}\) and gelatin is removed orderly, rearrangement of ECM by cells for cell migration may include both partial removal of pre-existing matrix and the addition of newly synthesized matrix components. MMP may play a role in the reorganization of the pre-existing and newly deposited matrix components to suit cell migration.

**Conclusions**

In this paper we presented reorganization of pericellular ECM via coordinated expression and localization of cell surface MMPs during cohort migration. Coordinated expression of MMPs was regulated by cell-cell contact among migrating cells. The reorganization of ECM was essential for this type of cell movement, and thus inhibition of MMP activities will lead to suppression of tumor invasion that includes cohort migration. However, it is known that MMP inhibitors cause some side effects at the same time. In this light, regulation mechanisms for MMP localization at the cell surface, specifically to leading edges of pathfinder cells, will be another target. In addition, investigation of new roles of cell surface MMPs other than cleavage of ECM may give
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