Compensation mechanism in tumor cell migration: mesenchymal–amoeboid transition after blocking of pericellular proteolysis

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Invasive tumor dissemination in vitro and in vivo involves the proteolytic degradation of ECM barriers. This process, however, is only incompletely attenuated by protease inhibitor–based treatment, suggesting the existence of migratory compensation strategies. In three-dimensional collagen matrices, spindle-shaped proteolytically potent HT-1080 fibrosarcoma and MDA-MB-231 carcinoma cells exhibited a constitutive mesenchymal-type movement including the coclustering of β1 integrins and MT1–matrix metalloproteinase (MMP) at fiber binding sites and the generation of tube-like proteolytic degradation tracks. Near-total inhibition of MMPs, serine proteases, cathepsins, and other proteases, however, induced a conversion toward spherical morphology at near undiminished migration rates. Sustained protease-independent migration resulted from a flexible amoeba-like shape change, i.e., propulsive squeezing through preexisting matrix gaps and formation of constriction rings in the absence of matrix degradation, concomitant loss of clustered β1 integrins and MT1-MMP from fiber binding sites, and a diffuse cortical distribution of the actin cytoskeleton. Acquisition of protease-independent amoeboid dissemination was confirmed for HT-1080 cells injected into the mouse dermis monitored by intravitral multiphoton microscopy. In conclusion, the transition from proteolytic mesenchymal toward nonproteolytic amoeboid movement highlights a supramolecular plasticity mechanism in cell migration and further represents a putative escape mechanism in tumor cell dissemination after abrogation of pericellular proteolysis.

Introduction

The invasion and migration of tumor cells involves coordinated adhesion as well as proteolytic interaction with the ECM substrate, resulting in the degradation and remodeling of interstitial tissue barriers (Stetler-Stevenson et al., 1993). Upon tumor progression, multiple classes of ECM-degrading enzymes are up-regulated and activated, including matrix metalloproteinases (MMPs),* serine proteases, and cathepsins (Birkedal-Hansen, 1995). Fibrillar collagen, the main constituent of the connective tissue matrix is degraded by several proteases, including MMP-1, -2, -8, and -13, membrane-anchored MT1-MMP, MT3-MMP, and cathepsin B, K, and L (Montcourrier et al., 1990; Aimes and Quigley, 1995; Ohuchi et al., 1997; Sassi et al., 2000). Accordingly, tumor cells expressing MMPs and other proteases generate proteolytic substrate degradation along their migration tracks on gelatin, matrigel, or fibronectin (Nakahara et al., 1997; d’Ortho et al., 1998). Blocking of MMPs and other proteases by pharmacological compounds impairs invasive tumor cell behavior in different migration and invasion models in vitro (Mignatti et al., 1986; Kurschat et al., 1999; Ntayi et al., 2001) as well as experimental metastasis in vivo (Coussens et al., 2002; and references therein). Together, these findings support a concept on proteolytic path generation favoring tumor cell dissemination within the tissues (Murphy and Gavrilovic, 1999; Friedl and Bröcker, 2000).

*Abbreviations used in this paper: 2-, 3-, and 4D, two, three, and four dimensional; MAT, mesenchymal–amoeboid transition; MMP, matrix metalloproteinase; uPA, urokinase-type plasminogen activator; uPAR, receptor for uPA.

Key words: cell migration; invasion; fibrosarcoma cells; carcinoma cells; matrix proteases

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Conversely, accumulating evidence suggests that, despite their established proinvasive function, ECM-degrading enzymes could be dispensable for tumor cell motility and dissemination. After blocking of MMPs or serine proteases, significant residual migration of individual cells is observed in different migration models (Deryugina et al., 1997; Hiraoka et al., 1998; Kurschat et al., 1999; Ntayi et al., 2001). In vivo, protease inhibitor–based targeting of MMPs and serine proteases has prompted an unexpectedly weak benefit in some animal tumor models as well as clinical trials in humans, suggesting that a principal dissemination capacity remained intact (Della et al., 1999; Zucker et al., 2000; Kruger et al., 2001; Coussens et al., 2002). These studies, however, leave unresolved by which mechanisms cells may maintain migratory dissemination in the absence of ECM-degrading capacity. As one possibility, proteolytic compensation could be provided by enzymes not inhibited in these studies; alternatively, cells could sustain motility via unknown protease-independent compensation strategies.

To understand how proteases contribute to cell migration, the concept of pericellular proteolysis has to be integrated into the current paradigm on cell migration as a supramolecular process (Lauffenburger and Horwitz, 1996; Friedl and Bröcker, 2000). Cell migration results from a three-step cycle of polarized cell extension and substrate binding through leading pseudopod(s), followed by actin-based contraction of the cell body and release of adhesive bonds at the trailing edge (Palecek et al., 1997). Adhesive interaction and traction toward the ECM substrate are generated by adhesion receptors predominantly of the β1 and β3 integrin families (Brooks et al., 1996; Palecek et al., 1997; Sheetz et al., 1998). After binding to extracellular scaffolds, integrins cluster and couple to the actin cytoskeleton to further strengthen cell–ECM linkages, which generates bipolar traction between anterior and posterior cell edges to elongate the cell body toward a spindle-shaped morphology (Sheetz et al., 1998; Maaser et al., 1999). For focalized degradation of ECM substrata, integrins cooperate with cell surface proteases. In invasive tumor cells, αvβ3 integrin colocalizes with MMP-2 (Brooks et al., 1996) and β1 integrins cocluster with MT1-MMP (Belkin et al., 2001), as well as the receptor for urokinase-type plasminogen activator (uPAR) (Wei et al., 1996). Accordingly, overexpression of uPAR and MT-MMPs favor integrin-mediated motility and invasion in two-dimensional (2D) and three-dimensional (3D) ECM models, respectively (Wei et al., 1996; Hiraoka et al., 1998). Consistent with migration-associated proteolysis, integrin-dependent migration of tumor cells results in the formation of tube-like matrix defects that represent paths of least resistance for the advancing cell body (Friedl et al., 1997). However, although proteolytic ECM degradation accompanies and augments tumor invasion and motility, it remains to be clarified whether pericellular proteolysis is indeed indispensable for the penetration of interstitial matrix barriers.
In the present study, using proteolytic tumor cells migrating through 3D fibrillar collagen-rich matrix in vitro and in vivo, we have investigated to what extent abrogation of ECM-degrading capacity, including the blocking of MMPs, serine proteases, and cathepsins, impairs the migratory action. By means of time-lapse videomicroscopy and four-dimensional (4D) confocal and multiphoton microscopy, we have particularly focused on the mechanisms of residual tumor cell movement after protease function was antagonized.

**Results**

HT-1080 fibrosarcoma cells overexpressing the membrane-localized MT1-MMP (Deryugina et al., 1997, 1998) and MDA-MB-231 mammary carcinoma cells (Ishibashi et al., 1999; Bachmeier et al., 2001) were used as metastatic tumor cell lines capable of efficiently degrading and invading the ECM.

**A mesenchymal type of tumor cell migration**

In 3D collagen matrices, the migration of HT-1080/MT1 cells was characterized by polarized binding of the leading edge to collagen fibers, generating traction and spindle-shaped elongation of the cell body (Fig. 1 A; Video 1 A, available online at http://www.jcb.org/cgi/content/full/jcb.200209006/DC1). Both polarization and migration were abrogated by adhesion-perturbing anti-β1 integrin mAb 4B4 (Fig. 1 B; Video 1 B), confirming β1 integrin-mediated migratory force generation. MT1-MMP and β1 integrins were coclustered at interaction sites to collagen fibers (Fig. 1 C), which represented the location of initial fiber binding, traction, and bundling toward the leading edge (Fig. 1, D and E, white arrowheads; Video 2, available online at http://www.jcb.org/cgi/content/full/jcb.200209006/DC1). Upon forward movement of the cell body, the detachment of the trailing edge generated a circumscribed matrix defect that was bordered by aligned multifibrillar collagen bundles (Fig. 1 E, asterisks; Video 2). Cell detachment was further accompanied by the shedding of surface determinants and cell fragments along the migration track (Fig. 1 D, black arrowhead). This mesenchymal migration type of spindle-shaped cells that produce matrix defects is consistent with the integrin-dependent haptokinetic migration reported for fibroblasts (Doane and Birk, 1991; Palecek et al., 1997) and further supports the concept of focalized pericellular proteolysis for the generation of migration tracks (Murphy and Gavrilovic, 1999).

**Inhibition of collagenolysis by protease inhibitor cocktail**

Pericellular degradation of native or denatured collagens can be directly or indirectly provided by proteases from different classes, including MMPs, serine proteases (e.g., plasmin and urokinase-type plasminogen activator [uPA]), as well as cysteine and aspartic proteases (e.g., cathepsins) (Montcourrier et al., 1990; Aimes and Quigley, 1995; Ohuchi et al., 1997; Sassi et al., 2000). In addition to MT1-MMP, many of these proteases, including up to five other collagenases toward native collagen (Fig. 2 A, asterisks), were expressed by HT-1080/MT1 cells, as detected by RT-PCR. Because collagenolytic redundancy was anticipated, we used a cocktail of broad-spectrum protease inhibitors to simultaneously target a wide spectrum of endoproteolytic activity (Table I). To maintain sufficient inhibitory activity within the matrix, the employed concentrations of protease inhibitors were orders of magnitude higher than the known maximum inhibitory values, unless cell viability was dose limiting (Table I). Because most of the proteases detectable by RT-PCR escape vi-
visualization by zymography (Deryugina et al., 1997, 1998; Ntayi et al., 2001), inhibition of cell-dependent collagenolysis in situ was measured using the fibrillar collagen migration substrate as read-out. On a qualitative basis, structural breakdown of the matrix fibers caused by HT-1080/MT1 cells (Fig. 2 B, 1 and 3) was fully abrogated in the presence of protease inhibitor cocktail (Fig. 2 B, 2). This strong inhibitory effect was confirmed by a quantitative 3D fluorometric fluorescein (FITC) release assay, detecting the degradation of FITC-labeled collagen fibers (Fig. 2 C). Upon migration within FITC–collagen, HT-1080/MT1 cells released 20% of the total FITC content (Fig. 2 D), confirming the observed macroscopic (Fig. 2 B, 1) and microscopic collagen fiber degradation (Fig. 1 E; Fig. 2 B, 3). Protease inhibitor cocktail inhibited 95% of the cell-mediated FITC release generated by HT-1080/MT1 cells (Fig. 2 D) and to the same residual level in vector-transfected HT-1080/neo cells (unpublished data). Broad-range MMP inhibitor BB-2516 (marimastat) alone abrogated /H11022/80% of the FITC release in HT-1080/MT1 cells, suggesting a major contribution of MMPs to the degradation of collagen (Fig. 2 D).

Unexpectedly, although inhibition of collagenolysis by protease inhibitor cocktail was near complete, the migration efficiency of HT-1080/MT1 cells within 3D collagen lattices was barely reduced (Fig. 3). Persistent migration was monitored by sensitive real-time analysis of time-dependent population speed (Fig. 3 A) and the median speed derived from single cell analysis (Fig. 3 B). Similar high migration rates were obtained by BB-2516 alone as well as from HT-1080/neo and wild-type cells in the presence of inhibitor cocktail (unpublished data). Because the capacity to move, i.e., to generate traction and overcome physical matrix constraints appeared intact after blocking of collagenolysis, we hypothesized the existence of compensation strategies to counterbalance the loss of pericellular proteolysis.

**Induced amoeboid migration in vitro**

As it became apparent from the video recordings (Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200209006/DC1), the presence of protease inhibitor cocktail changed several aspects of migratory behavior in HT-1080/MT1 cells (Fig. 4). Whereas spontaneously moving cells maintained a spindle-shaped, elongated morphology (Fig. 4 A, left), this mesenchymal migration type was converted to less polarized, more spherical morphodynamics in the presence of protease inhibitor cocktail (Fig. 4 A, right; Video 3). Spherical-shaped cells sustained migration (Fig. 4 B) and exhibited a significant reduction in median axis length compared with control cells (Fig. 4 C). In the presence of protease inhibitors, spherical morphodynamics included flexible shape change and forward propulsion guided by multiple anterior and outward ruffling filopodia, as detected by high resolution video microscopy (Video 4, available online at http://www.jcb.org/cgi/content/full/jcb.200209006/DC1). Very similar observations were obtained for invasive

### Table I. Protease inhibitor cocktail

| Inhibitor | Target protease | IC50 values | Concentration | Reference |
|-----------|-----------------|-------------|---------------|-----------|
| BB-2516   | MMPs<sup>a</sup> | 0.002–0.2  | 100           | Bedi and Williams, 1994 |
| E-64      | Cysteine proteases (cathepsin B, H, L, and K) | 0.001–20  | 250           |          |
| Pepstatin A | Aspartatic proteases (including cathepsin D) | 0.0001–5 | 100           | Laurent and Salzet, 1995; Nisbet and Billingsley, 1999 |
| Leupeptin  | Cathepsin D     | 0.1–10      | 2<sup>b</sup> | Bedi and Williams, 1994 |
| Aprotinin  | Serine proteases (uPA and PA) | 0.15–3.5 | 2.2<sup>b</sup> | Callas et al., 1994 |

<sup>a</sup>Guidelines for the clinical use of marimastat were from British Biotech Inc. (1998).

<sup>b</sup>Highest nontoxic concentration (for details see Materials and methods).

Figure 3. **Sustained migration in the presence of protease inhibitor cocktail.** (A) Cell tracking analysis of the steady-state population speed ± SD and (B) mean speed for each individual cell (time, 20 h; n = 3 experiments, 120 cells).
MDA-MB-231 mammary carcinoma cells, expressing soluble MMPs, MT-MMPs, serine proteases, and cathepsins (Ishibashi et al., 1999; Bachmeier et al., 2001). Inhibition of proteolysis in MDA-MB-231 cells by protease inhibitor cocktail (Fig. 4 D), albeit slightly less efficient than in HT-1080 cells, resulted in a similar transition from constitutive spindle shape to less polarized, elliptoid morphodynamics (Fig. 4 E; Video 5, available online at http://www.jcb.org/cgi/content/full/jcb.200209006/DC1), including a significantly shortened median axis length (Fig. 4 F).

This novel nonproteolytic phenotype in HT-1080/MT1 and MDA-MB-231 cells was reminiscent of Dictyostelium amoeba migrating across 2D surfaces (Yumura et al., 1984; Killich et al., 1993) as well as leukocytes crawling through 3D ECM substrata (Gunzer et al., 2000; Friedl et al., 2001; for comparison, see migrating T lymphocytes in Gunzer et al. [2000] supplemental material). Morphometric analysis showed a reduced number in mesenchymally moving cells in the presence of protease inhibitor cocktail (HT-1080/MT1 cells, n = 3; 200 cells; ***, P < 0.0001). (G) Frequency of mesenchymal and amoeboid shape in actually migrating cells in the absence (■; and presence (□) of protease inhibitor cocktail (HT-1080/MT1 cells, n = 3; 100 cells; **, P < 0.001 for difference to untreated control; two-tailed t test for independent means). Cells of indeterminate morphology (15–40%; for details see the Materials and methods) were excluded from analysis. Bars: (A and E) 100 μm; (B) 20 μm.

Mechanisms of induced nonproteolytic migration

Consistent with impaired collagenolysis, amoeboid moving HT-1080/MT1 cells did not cause structural remodeling of collagen fibers (Video 6, available online at http://www.jcb.org/cgi/content/full/jcb.200209006/DC1). Induced protease-independent migration resulted from adaptation and alignment of the cell body along preformed fiber strands (Fig. 5 A, white arrowheads) and consecutive migratory guidance along fibrillar scaffolds (Fig. 5 B, black arrowheads). Upon cell detachment, no remodeling, bundling, or destruction of the collagen network was generated, leaving behind the intact reticular texture of individual fibrils at their original position (Fig. 5 A, black arrowheads; Video 6).

To overcome regions of narrow space by changing shape, an initial pseudopod elongation through a preformed matrix gap (4 μm pore diameter from the central section, 18 μm cell diameter; Fig. 5 C, black arrowhead) was followed by propulsion of the cell body and the development of a narrow region confined by matrix fibers (constriction ring; Lewis, 1934; Fig. 5 C, black arrowhead). Constriction rings persisted until the cell body had squeezed or pulled forward, while no matrix defect was apparent after cell detachment (Fig. 5 C; Video 7, available at http://www.jcb.org/cgi/content/full/jcb.200209006/DC1).

Because the structure and location of adhesive cell–matrix contacts control cell shape and migration dynamics (Palecek et al., 1997; Sheetz et al., 1998), we investigated the cytoskeletal structure and integrin distribution in amoeboid HT-1080/MT1 cells. Induced amoeboid morphology was characterized by diffuse cortical actin rims and small actin-rich spots at interactions with collagen fibers (Fig. 5 D). In amoeboid HT1080/MT1 cells, β1 integrins showed non-clustered, linear surface distribution at contacts to collagen...
fibers (Fig. 5, D and E) deviating from clustered β1 integrins and prominent actin nucleation zones in spindle-shaped control cells (Fig. 1 C; Fig. 5 F). MT1-MMP was consistently dissociated from fiber binding sites but accumulated intracellularly (Fig. 5 E). Because of constriction caused by a perpendicular collagen fiber, this cell contains a lobulated main body. Black arrowhead, uropod. (E) Loss of clustered MT1-MMP and β1 integrins from interactions with fibers in induced amoeboid migration. Arrowheads indicate two simultaneous constriction rings bordered by perpendicular collagen fibers.

Mesenchymal–amoeboid transition in vivo
Although collagen lattices provide a complex 3D ECM scaffold and a barrier for moving cells, a putatively different spacing and molecular composition of life connective tissue may impose additional physical and molecular constraints, putatively yielding in distinct migration mechanisms. Therefore, the in vivo migration of HT-1080/MT1 cells within the mouse dermis was investigated by multiphoton microscopy. 3 h after the injection of HT-1080/MT1 cells into the loose connective tissue of the mouse dermis (Fig. 6 A), both nontreated control cells (green) and cells pretreated with protease inhibitor cocktail (red) were detected at and passively scattered around the injection site (Fig. 6 A, asterisk), or located within multicellular cords (Fig. 6 A, black arrowheads). In wash-out experiments in vitro, preincubation of cells with protease inhibitor cocktail for 6 h resulted in stable amoeboid movement for at least 10 h before reversion toward mesenchymal migration occurred (unpublished data), indicating both relatively slow turnover of the target proteases after inhibition and full reversibility of inhibitor-induced phenotypic change. In the dermis, ortotopic 3D reconstruction of the injection site 3 and 8 h after injection identified considerable position changes for both control cells (Fig. 6 B, top) and inhibitor-pretreated HT-1080/MT1 cells (Fig. 6 B, bottom). This position change corresponded to a translocation of 4–10 μm/hr (Fig. 6 B, white arrowheads), in part approximating migration velocities present in collagen matrices in vitro (6–36 μm/h; compare Fig. 3 B). At high-resolution reconstruction, cells pretreated with protease inhibitors developed a significantly reduced elongation, reminiscent of amoeboid morphology (Fig. 6 C, red cells), and a near-round median shape (Fig. 6 D), whereas control cells retained their constitutive spindle-like elongation (Fig. 6 C, green cells; Video 8, available at http://www.jcb.org/cgi/content/full/jcb.200209006/DC1). In summary, similar to 3D collagen matrices, abrogation of matrix protease function in HT-1080/MT1 cells was followed by cell movement and concomitant induction of amoeboid morphology.
Discussion

The transition from proteolytic and “mesenchymal” to protease independent and “amoeboid” movement reveals a novel cellular and molecular adaptation pathway in sustaining cell migration: the switch from a path-generating to a path-finding migration mode that we have termed mesenchymal–amoeboid transition (MAT). Although proteases were used for constitutive migration of proteolytic cells of mesenchymal and epithelial origin, abrogation of pericellular proteolysis was followed by nonproteolytic movement. MAT thus represents a compensatory migration mode in vitro and in vivo to bypass physical matrix resistance by amoeboid shape change.

 Constitutive proteolytic migration of mesenchymal cells

Important concepts on cell migration are derived from the movement of fibroblasts and myoblasts (Lauffenburger and Horwitz, 1996). In 3D in vitro and in vivo tissues, fibroblasts develop elongated, spindle-shaped morphology mediated by cortical F-actin and occasional stress fibers (Welch et al., 1990). Fibroblast migration is dependent on \( \beta_1 \) integrin function (Doane and Birk, 1991) and is further coupled to the cleavage and remodeling of ECM components using MMPs and other proteases (Langholz et al., 1995). A similar type of mesenchymal behavior is developed by HT-1080 fibrosarcoma cells and MDA-MB-231 mammary carcinoma cells in 3D collagen matrices. These cells move as individual spindle-shaped cells that utilize \( \beta_1 \) integrin-mediated binding to collagen fibers for elongation and translocation. Similarly to fibroblasts, a range of ECM-degrading enzymes is expressed, coclustered with integrins at interactions to substrate, and used for the generation of tube-like matrix defects that correspond to known clearance tracks in other models (Nakahara et al., 1997; d’Ortho et al., 1998). Because the cell diameter of mesenchymal-like cells exceeds the mean pore size within the fiber network (Friedl et al., 1997), contact-dependent focal degradation of collagen barriers may facilitate migration by locally lowering the physical resistance of the ECM barrier (Murphy and Gavrilovic, 1999).

Figure 6. In vivo translocation and morphology of HT-1080/MT1 cells in the mouse dermis. Intravital microscopy of ECM structure (light brown) and labeled cells 3 h (A–D) and 8 h (B) after injection. (A) 3D reconstruction of injection site (asterisk), more distant cells passively scattered around the injection site, and matrix structure at low magnification (10X objective). Control cells (green) and cells pretreated with protease inhibitor cocktail (red). Asterisk, center of injection site. Black arrows, multicellular cord. (B) Position change of control cells (top) and cells pretreated with protease inhibitor cocktail (bottom) from the injection site depicted in A. False-color reconstruction was obtained for each fluorescence channel 3 h (green) and 8 h (red) after injection. Orthotopic superimposition was controlled by the position of coinjected fluorescent beads (circles) as well as the multicellular cord. White arrowheads, regions of cell translocation (right). (C) High resolution imaging of cell morphology and (D) elongation of control cells (green) and cells pretreated with protease inhibitor cocktail (red) at least 2 h after injection (40X objective). Elongation was calculated from 170 cells, six independent experiments; ***, \( P < 0.0001 \). Bars: (A and B [left]) 200 \( \mu \)m; (B [right] and C) 100 \( \mu \)m.
Constitutive nonproteolytic amoeboid migration strategies

In some cell types, migration results from more dynamic, transient and less defined cell–substrate contacts associated with amoeboid shape (Friedl and Bröcker, 2000; Friedl et al., 2001; and references therein). The concept of amoeboid movement is most clearly established from the single cell state of the lower eukaryote Dicystostelium discoideum. Dicystostelium is an ellipsoid cell that translocates via rapidly alternating cycles of morphological expansion and contraction, relatively low-affinity integrin-independent interactions with the substrate, and extraordinary deformability (Yumura et al., 1984; Devreotes and Zigmond, 1988; Killich et al., 1993; Friedl et al., 2001). In higher eukaryotic cells, such as leukocytes and certain types of tumor cells, hallmarks of amoeboid motion are retained, including rapid low affinity gliding coupled to shape change, squeezing through pre-formed matrix gaps, and propulsive elongation along paths of least resistance (Lewis, 1934; Schor et al., 1983; Friedl et al., 1998, 2001). In collagen matrices, T cell migration is independent of structural matrix remodeling (Schor et al., 1984; Devreotes and Zigmond, 1988; Killich et al., 1993; Friedl et al., 2001). In collagen matrices, T cell migration is independent of structural matrix remodeling (Schor et al., 1984) and is not sensitive to protease inhibitor treatment (unpublished data) or the inhibition of β1 integrin function, the latter both in vitro and in vivo (Friedl et al., 1998; Brakebusch et al., 2002). Thus, amoeboid migration appears to comprise morphodynamic supramolecular mechanisms to bypass tissue barriers independent of ECM degradation.

Mesenchymal–amoeboid transition: induced amoeboid migration

In HT-1080 fibrosarcoma and MDA-MB-231 mammary carcinoma cells, the mechanism of sustained migration after abrogation of pericellular proteolysis was a striking change in cell morphology and migration “style” reminiscent of transition toward amoeboid movement. To provide maximum inhibition efficiency, five pharmacological inhibitors were used to simultaneously target different protease classes known to contribute to ECM degradation and remodeling, including MMPs, serine proteases, and cathepsins, all of which are expressed by HT-1080 and MDA-MB-231 cells. We reasoned that more specific approaches, such as genetic ablation of individual proteases, might be hampered by proteolytic redundancy and functional compensation.

The effectiveness of the protease inhibition strategy was shown by a combined biochemical and structural approach: (a) near-total inhibition of FITC release using FITC-labeled fibrillar collagen; (b) abrogation of macroscopic structural degradation of the collagen lattice by HT-1080 cells; and (c) the lack of newly generated tube-like matrix defects in the wake of the cells visualized by 4D confocal backscatter microscopy.

Inhibition of constitutive proteolysis, instead of causing the cells to become “trapped” within the fibrillar network, induced a program of morphodynamic and molecular changes that allowed the maintenance of the migratory action (Fig. 7). These changes included a reduced elongation yet increased morphodynamic flexibility, the loss of focal β1 integrin and MT1-MMP clustering at interactions with matrix fibers, and a more diffuse cortical actin distribution, including small actin-rich nucleation foci at fiber binding sites. These features are coupled to the forward flow (streaming) of cytoplasm through cell regions of narrow space confined by outside constriction and, together, result in a phenotype mimicking amoeboid moving cells (Yumura et al., 1984; Devreotes and Zigmond, 1988; Killich et al., 1993; Friedl et al., 2001). Because this induced amoeboid behavior generated significant migratory activity, yet the transmigrated collagen architecture remained intact by means of biochemical and structural analysis, our data show an alternative inhibitor-induced nonproteolytic migration type (Fig. 7).

Induced protease-independent movement was observed for two different collagen-based tissue models, 3D collagen lattices in vitro and the mouse dermis in vivo. As reconstructed by intravital microscopy, the loose connective tissue of the mouse dermis exhibits strong structural similarity to 3D collagen matrices. Both models provide 3D fibrillar collagenous strands bordering matrix gaps in the range of 1–15 μm (unpublished data). Migratory alignment and shape change of tumor cells (cell diameter in the range of 15–25 μm [Friedl et al., 1997]; 10–30-fold larger in volume than amoeba or leukocytes) along preformed fiber strands may hence be sustained by constriction of the cell body and propulsion of the cytoplasm through narrow space as a nonproteolytic, physical strategy to bypass structural ECM barriers. Although the spacing of such loose connective tissue supports protease-independent cell movement, future studies will be necessary to determine under which conditions transmigration of more densely interconnected ECM, such as basement membranes, can occur by nonproteolytic means.

The finding that blocking proteases induces transition from a mesenchymal phenotype to amoeboid movement sheds light on how disseminating tumor cells may react in response to protease inhibitor–based therapy. In established tumor disease, tumor cells have already reached interstitial tissue compartments (Stetler-Stevenson et al., 1993) that are likely to support shape-driven migration independent of pericellular proteolysis. Therefore, protease-independent tumor cell dissemination may represent a candidate escape
Molecular implications of MAT

The putative mechanistic link between loss of pericellular proteolysis and MAT can be best explained by changes in cell–matrix interaction strength. Although different experimental systems are difficult to directly relate, exaggerated mesenchymal-like phenotypes can be induced by strengthening cell–ECM contacts or reducing their turnover: by stabilizing interactions toward collagen fibers after arresting β1 integrins in a high-affinity ligand-bound state (Maaser et al., 1999) or by stabilizing stress fibers leading to spread-out, elongated morphology through activation of the small GTPase Rho (Nobes and Hall, 1999). Conversely, reduced axis length and amoeba-like morphodynamics may emerge from weakening substrate-binding strength: by reducing integrin adhesive function (Dufour et al., 1988) or by inhibiting Rho-kinase (Nobes and Hall, 1999). In addition to integrins, several of the proteases blocked in this study are known to regulate cellular morphodynamics by other mechanisms, including a mesenchymal phenotype (Locher et al., 1997). MMP-3 overexpression in epithelial cells leads to the cleavage of E-cadherin and induces epithelial–mesenchymal transition, i.e., single cell detachment and mesenchymal invasion (Locher et al., 1997). Proteases further control cell adhesion, cytoskeletal organization, and the release of soluble factors (Sternlicht and Werb, 2001 and references therein). These functions comprise cleavage of adhesion receptors (e.g., L-selectin); cleavage and activation of cytokines (e.g., IL-1 and TNF-α) or growth factors (e.g., EGF and bFGF); and the regulation of focal contact assembly and turnover by cleavage of talin or integrin cytoplasmic domains through, for example, calpain. The identification of pathways involved in MAT will thus require further studies on different cytoskeletal and signaling checkpoints that control cell shape and cell–matrix interactions.

MAT: comparison to other morphodynamic processes

Plasticity and adaptation in cell behavior are well established in other morphodynamic processes governed by cell–cell and cell–substrate interactions. In epithelial–mesenchymal transition, a usually nonmigrating epithelial collective abandoning cell–cell cohesion after cadherin-mediated cell–cell contacts are weakened, allowing detachment of single cells and conversion toward mesenchymal morphology and migration (Birchmeier and Birchmeier, 1995). In another example, differentiating muscle precursor cells quit cell movement and fuse via N- and E-cadherin to form a multicellular contractile syncytium (Redfield et al., 1997). In conclusion, MAT may represent a novel transient morphodynamic alteration and adaptation pathway in molecular cell dynamics. Stringent, adhesive, and proteolytic cell–ECM contacts, as developed by mesenchymally moving cells, can be converted toward less well characterized, more diffuse, and less proteolytic interactions with extracellular scaffolds, reminiscent of amoeboid single cell movement. Residual amoeboid movement may hence represent a robust “salvage” pathway of cell movement in loose connective tissue after the abrogation of pericellular proteolysis.

Materials and methods

Cell migration in 3D collagen matrices: reconstruction by time-lapse video microscopy and confocal microscopy

Subconfluent HT-1080 fibrosarcoma cells stably transfected with MT1-MMP (Denyagina et al., 1997) or MDA-MB-231 breast carcinoma cells (Ishibashi et al., 1999; Bachmeier et al., 2001) were detected by EDTA (2 mM), washed, incorporated into 3-D collagen lattices (1.67 mg/ml; native dermal bovine type I collagen; Vitrogen; Cohesion Inc.), and monitored by time-lapse video microscopy (Friedel et al., 1997; Gunzer et al., 2000). The collagen was resistant to trypsin and sensitive to degradation by MT1-MMP (not depicted), confirming its native state. HT-1080/MT1 cells served as model for high constitutive collagenase expression and activity, allowing a robust visualization of surface MT1-MMP and fiber breakdown in the process of migration. In control experiments, HT1080/neo cells as well as HT-1080 wild-type cells were used for collagen degradation and migration experiments yielding similar results, including MAT (not depicted). For inhibition studies, blocking anti-β1 integrin mAb 4B4 (10 μg/ml; Coulter) or protease inhibitor cocktail (Table I) was added to the lattice before polymerization as well as to the supernatant. Protease inhibitor cocktail consisted of BB-2516 (marimastat; provided by British Biotech Inc.), leupeptin (Molecular Probes), pepstatin A, E-64, and aprotinin (all from Sigma-Aldrich). Protease inhibitor cocktail did not affect cell viability in collagen after 48 h, as assessed after cell release from the collagen lattice by collagen digestion, propidium iodide staining, and flow cytometry, or upon liquid culture after 72 h of exposure.

For 3D time-lapse confocal microscopy (Leica-SP2 system), cells within the lattice were labeled by calcine-AM (1 μM), scanned at 2.5-min time intervals for simultaneous fluorescence and backscatter signal, and reconstructed (Friedel et al., 1997). MT1-MMP and β1 integrins were detected by rabbit AB815 (Chemicon), secondary rhodamine-conjugated goat anti-rabbit Fab’/fragments (Jackson ImmunoResearch Laboratories), and a cocktail of FITC-labeled mAb 4B4 and K20 (Coulter), respectively.

Computer-assisted cell tracking and reconstruction of cell morphology

Locomotor parameters were obtained by computer-assisted cell tracking and reconstruction of the x and y coordinates of the cell paths (Maaser et al., 1999). The population speed was calculated from each 15-min tracking interval for all cells divided by the number of cells. Single cell speed represents the total length of the path divided by time. The proportion of mesenchymal to amoeboid migration was assessed 9 h (HT1080/MT1) or 18 h (MDA-MB-231) after addition of protease inhibitor cocktail by visual blinded analysis of a 60-min time interval by two independent observers. Cells of indeterminate morphology representing either transition states of partially elongated, dividing, or nonmigrating cells precluded clear categorization and were excluded from analysis (15–40% of total population). In control cells, indeterminate morphology was seen in 20% of the cells, mainly representing dividing cells and periods of retraction of otherwise elongated mobile cells (see Video 3). In the presence of inhibitor cocktail, the indeterminate fraction was increased to 40%, containing an additional subset of partially retracted cells (elongation index >2) that lacked the criteria of full amoeboid as well as mesenchymal morphodynamics (Video 3). Elongation was calculated from still frames as elongation index for each cell (axis length divided by width) and displayed as box-plot ranging from the 25th to 75th percentile including the median and whiskers from the 5th to 95th percentile. Unless indicated otherwise, statistical analysis was performed using the unpaired two-tailed Mann-Whitney U test.

RT-PCR

Primers and RT-PCR conditions were used as previously described (Hofmann et al., 1999; Konttinen et al., 1999; McCulloch et al., 2000). Additional primers were cathepsin B, 5′-CCGCTGAGAGTTCTGATGCAC-3′ (direct) and 5′-CTATGGAGTACTGAGGCA-3′ (reverse); cathepsin D, 5′-CCACAGCCCAATACCCCAATCACCACCCCTCCAG-3′ (direct) and 5′-CAGTCGGTGAGGCAAGGCTTAACACG-3′ (reverse); cathepsin K, 5′-TCC- ATGCCATAACCTGGGTCCTT-3′ (direct) and 5′-CCACAGGATCCTTCTTCACACGG-3′ (reverse); cathepsin L, 5′-CCGCGAGTTGAACATGGCCTT-3′ (direct) and 5′-CCGCGGTTCCATCATGCTGAA-3′ (reverse); MT1-MMP, 5′-CCCTATGGAGCTATTTTCAGAGG-3′ (direct) and 5′-CCATCATCTCCAGTGAAA-3′ (reverse); u-PA, 5′-GTTACCCCGCGCTGT-3′ (direct) and 5′-
CCACTGGCGTACCTGCCA-3' (reverse); u-PA, 5'-AAATTGCTGTGCTGCTGCTG-3' (direct) and 5'-CCCTGCTGCTGCTGCTGCTG-3' (reverse); and PAI-1, 5'-GAAAGGTATGAGATGCAGACC-3' (direct) and 5'-ACTATGAGCTGATGGTACCGG-3' (reverse). Uniformity of region was confirmed using the NCBI blast program; specificity was determined from the length of each PCR product. Total RNA was isolated (QiAGEN RNAEasy kit), transcribed to cDNA by AMV reverse transcriptase, and amplified by PCR (30 cycles). Absence of cDNA contamination was confirmed by setting total cell lysates to PCR in the absence of reverse transcriptase.

Collagen degradation assays
HT-1080/MT1 or MDA-MB-231 cell suspension was copolymerized with nonlabeled rat tail collagen (Becton Dickinson) containing 2% FITC-labeled collagen monomers (Molecular Probes). After migration for 40 h, solid-phase collagen containing the cells was pelleted, while FITC released into the supernatant was analyzed by spectrofluorometry. 100% values were obtained by complete collagenase digestion of cell-free collagen lattices. Background fluorescence was calculated by pelleting nondigested cell-free lattices. No autofluorescence was detected for either cells or inhibitor. FITC-collagen was sensitive to degradation by recombinant MT1-MMP, MMP-2, and trypsin. While FITC detected both classical collagenases as well as trypsin-like activity, FITC release induced by HT-1080/MT1 cells was reduced by 80% by the MMP inhibitor BB-2516 (Fig. 2 C), indicating MMPs as primary collagenases in these cells. To exclude that FITC release merely reflected telopeptidase activity and incomplete fiber degradation, 500,000 HT-1080/MT1 cells in a drop of medium were plated onto native nonlabeled 3D collagen (100 μm in depth). Fiber breakdown was detected after 40 h by Coomassie blue staining and confocal backscatter microscopy.

Intravital multiphoton microscopy
Reconstruction of HT1080/MT1 cell positioning and ECM structure were monitored by multiphoton microscopy (Maiti et al., 1997) of the mouse dermis in adult C57BL/6 mice, using a novel modification of a bone marrow intravital video microscopy model (Mazo et al., 1998). The epidermis and upper dermis, including hair follicles of the frontoparietal scalp, were monitored by multiphoton microscopy (Maiti et al., 1997) of the mouse dermis at 800 nm. Multiphoton excitation light of 800 nm was introduced into the imaging system controlled by the Lasersharp software (all from Bio-Rad Laboratories). Multiphoton excitation light of 800 nm was introduced into the imaging system controlled by the Lasersharp software (all from Bio-Rad Laboratories). Multiphoton excitation light of 800 nm was introduced into the imaging system controlled by the Lasersharp software (all from Bio-Rad Laboratories). Multiphoton excitation light of 800 nm was introduced into the imaging system controlled by the Lasersharp software (all from Bio-Rad Laboratories). Multiphoton excitation light of 800 nm was introduced into the imaging system controlled by the Lasersharp software (all from Bio-Rad Laboratories). Multiphoton excitation light of 800 nm was introduced into the imaging system controlled by the Lasersharp software (all from Bio-Rad Laboratories). Multiphoton excitation light of 800 nm was introduced into the imaging system controlled by the Lasersharp software (all from Bio-Rad Laboratories).

Online supplemental material
Quicktime movies of the image series from which figures were made are available at http://www.jcb.org/cgi/content/full/jcb.200209006/DC1.

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