Invadopodia: clearing the way for cancer cell invasion

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Abstract: The invasive nature of many cancer cells involves the formation of F-actin-based, lipid-raft-enriched membrane protrusions known as invadopodia or, more broadly, invadosomes. Invadopodia are specialized adhesive structures arising from ventral cell surface within cell-extracellular matrix (ECM) contacts and concentrate high proteolytic activities that allow cells to overcome the dense scaffold of local microenvironment, comprising a natural barrier to cell spreading. This degradative activity distinguishes invadopodia from other adhesive structures like focal adhesions, lamellipodia or filopodia, and is believed to drive cancer progression.

Keywords: Invadopodia; metalloproteases; serine protease; lipid raft; cancer invasion; metastasis

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For the first time invadopodia were spotted in cultured chicken embryo fibroblasts transformed by Rous sarcoma virus at the beginning of the 1980s and described as circular clusters (rosettes) of small patches with altered distribution of vinculin and alpha-actinin (1). Chen, in 1989, termed them invadopodia to emphasize their degradative abilities attributed to cancer cells (2). Alternatively, similar structures that could be observed in various normal human cells, such as macrophages, osteoclasts or dendritic cells, have been named podosomes (3).

Invadopodia structure and function

Invadopodia protrusions are rather stable structures that can last for several hours (4). They were established to have range from 0.05 to 0.1 μm in diameter and almost 2 μm in length (5). Structurally, they consist of two major components, an F-actin core enriched in actin-regulatory molecules, such as Arp2/3, N-WASP or WASP, and their upstream regulators Nck1, Cdc42 or WIP, next to cofilin, capping proteins, cortactin, and dynamin-2, and a protein ring surrounding the core. The ring is a structure formed of packed adhesion and scaffolding proteins, such as β1 integrins and CD44 receptor, as well as signaling molecules and membrane-associated proteases. Among them there are proteins such as focal adhesion kinase (FAK), src-associated proteins such as p130Cas and Tks5/FISH (tyrosine kinase substrate 5/five SH3 domains), and the small G proteins Arf1, and Arf6, serine proteinases like seprase or dipeptidyl peptidase IV, and various matrix metalloproteinases (MMPs) and ADAMs, as well as the urokinase-type plasminogen activator (uPA) receptor (uPAR) proteolytic system (6,7). Invadopodia are identified as dot-shaped areas of degraded fluorescently labeled extracellular matrix (ECM) proteins, that colocalize with invadopodia-associated protein components, *in vitro*. The identification of invadopodia in natural conditions is still very challenging due to technical limitations. Recently, membrane protrusions formed by cancer cells during intravascular migration have been shown in an embryonic zebrafish xenograft model, using high-resolution *in vivo* imaging (8-10). The ability of cancer cells

to form invadopodia correlates well with their *in vitro* and *in vivo* invasive potential (11).

Invadopodia formation

Invadopodia formation is a highly dynamic and complex process, which can be divided into three successive phases: initiation, stabilization, and maturation. The initiation phase includes the assembly of actin-based precursor complexes and cortactin-dependent actin polymerization that extends plasma membrane and drives elongation of cellular protrusions. A critical step in this phase is activation of the actin-related protein (Arp)2/3 complex that initiate actin nucleation and is necessary to start actin filament branching (12). During the stabilization phase, newly formed actin filaments are crosslinked into tightly packed bundles and anchored to plasma membrane by fimbrin, mDia2 and VASP, as well as myosinX and Tks5/FISH, to form a stable, three-dimensional functional structure (13,14). During the maturation phase, proteins with proteolytic activity are recruited to invadopodia, making them able *to* promote focal ECM degradation. The invadopodia formation process starts in response to different extracellular stimuli such as growth factors, cytokines and integrin-linked extracellular signals, but not only. The exposure to acidic pH, matrix rigidity, hypoxia, and reactive oxygen species (ROS) were also found to induce invadopodia formation (15-20). However, in all cases intracellular signal transducers are activated to initiate nucleation of F-actin.

Invadopodia-associated receptor tyrosine kinases

Transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), heparin binding EGF (HB-EGF) and tumor necrosis factor-alpha (TNF-alpha) have been found to stimulate invadopodia formation in a large number of cancer cell lines (21-24). All these factors are also known to be highly expressed in different malignancies and often correlated with a poor prognosis.

Most growth factors are associated with kinase activities (25,26). Proteins that catalyze the transfer phosphate groups to tyrosine residues of protein substrates comprise the largest class of growth factor receptors, known as receptor tyrosine kinases (RTKs), and were shown to be crucial for the invadopodia initiation and function (27). Upon binding of signals, RTKs recruit enzymatic effectors, including MAPK, PI3K, Src, PLCγ, JAK/STAT, to their intracellular domains, or act indirectly through adapter proteins, forming complexes capable of activating signaling pathways. Cell surface tyrosine kinase receptors like platelet-derived growth factor receptor alpha (PDGFRα), hepatocyte growth factor receptor (HGFR) or epidermal growth factor (EGFR), were recognized as mediators of changes in cellular actin distribution (28,29). Their activation resulted in formation of punctate clusters, consistent with invadopodia focal digestion of ECM (21,30). Eckert *et al.* have shown that this process was dependent on Twist1, one of key transcription factors regulating epithelial to mesenchymal transition (EMT). Twist1 directly induced PDGFRα expression and activation and Src-dependent phosphorylation of cortactin (31,32).

Invadopodia-associated non-receptor tyrosine kinases

Src is a member of the membrane-bound non-receptor tyrosine kinase family that is ubiquitously expressed in cells and identified as a key mediator of tumor malignancy (33). It is activated through the SH3- and SH2-mediated interactions with both various receptor tyrosine kinases (RTKs) and different adaptor proteins of CRK-associated substrate (CAS) family or focal adhesion kinase (FAK). The 80/85-kDa cortactin, an actin binding protein which is one of the major Src tyrosine kinase protein substrates, has been recognized as both a nucleation-promoting factor that activates Nck1-N-WASp-Arp2/3 complex and a stimulator of a matrix metalloproteinase (MMP) secretion in invadopodia (34-36). Using the crosslinking reagent, it was demonstrated that cortactin binds the Arp3 subunit of the Arp2/3 complex directly via the N-terminal acidic domain, making a bridge between the Arp2/3 complex and actin filaments (37). Cortactin is also known to bind and activate N-WASP and WASP via its C-terminal SH3 domain. All members of Wiskott-Aldrich syndrome protein (WASP) family and the WASP-family verprolin-homologous protein (WAVE), encoding by WASP, N-WASP, WAVE1/SCAR1, WAVE2, and WAVE3 genes, contain a conserved C-terminal verprolin homology, cofilin homology, and acidic region (VCA) domain that interacts with the Arp2/3 proteins, and thereby stimulate Arp2/3-mediated actin polymerization (38-40). The formation of the complex between ARP2/3 and N-WASP as well as binding their ligands such as CDC42,

WASP-interacting protein (WIP), and dynamin-2 is stimulated by cortactin phosphorylation (37,41,42). The phosphorylation of two tyrosine residues, Y421 and Y466, of the cortactin was shown as a critical point that regulates cortactin-Nck1 direct interactions and promotes free actin barbed ends generation during invadopodia formation process (34). The silencing of cortactin gene expression effectively inhibited ECM degradation at invadopodia sites same as the using MMP inhibitors, GM6001 or TIMP-2 (35). Moreover, the total inhibition of *in vitro* cell invasion was observed in cancer cells expressing the cortactin Y466F mutant, indicating that the phosphorylation of cortactin is essential also for matrix proteolysis and cancer cell migration (34). These data are consistent with other studies describing that the use of tyrosine kinase inhibitors resulted in the reduction of invadopodia formation as well as the ability to degrade ECM in cancer cells (22). Contrary to Src phosphorylation-dependent binding of Nck1, the ability of cortactin to bind N-WASP depends on phosphorylation of cortactin by ERK1/2 (38). Of the WAVE family proteins, WAVE3 but not WAVE1 and WAVE2, were found to be required for the invadopodia formation. However, similar to WAVE3, WAVE1 has been shown to play an indirect role in matrix degradation. It was observed that cells with the knockdown of WAVE1 had decreased secretion of MMP2, correlating with diminished invadopodia-related degradation activity (30). Instead, the silencing of WAVE3 resulted in the downregulation of MMP9 expression and activity due to inhibition of NFkB-p65 phosphorylation (24).

Src homology-3 (SH3) domain-rich tyrosine kinase substrate (Tks) adaptor proteins, Tks4 (SH3PXD2B) and Tks5/FISH, were identified as another Src substrates having an influence on both the number and activity of invadopodia (43,44). Both Tks4 and Tks5 were also identified as needful factors in cancer progression (45,46). These proteins, analogues of the NADPH oxidase (NOX) components like NOX organizer 1 (NOXO1) or p47phox, facilitate local generation of reactive oxygen species (ROS), which has been proved essential for invadopodia formation processes (47). The amino-terminal Phox homology (PX) domain that shows strong binding to phosphoinositides (PIs) such as phosphatidylinositol-3,4-bisphosphate [PI(3,4)P_2] and phosphatidylinositol-3-phosphate (PI3P/PtdIns3P) was found to be required for the invadopodia localization of both Tks4 and Tks5 (43,44).

Invadopodia lipid rafts

PIs, membrane-bound signaling phospholipids generated by the phosphoinositide 3-kinase (PI3K) family of lipid kinases, are involved in the stimulation of a wide range of secondary messenger molecules. On the other site, they also can bind various actin-binding proteins and function as a bridge connecting the cytoskeleton to the plasma membrane. They are known to colocalized with sphingolipid- and cholesterol-enriched microdomains, named lipid rafts, and provide an important element of lipid raft-related signal transduction processes (48,49). It is evident that lipid rafts, which are highly ordered and tightly packed regions of membrane, act as signaling platforms that regulate transmembrane crosstalk between cells and ECM. Raft domains laterally segregate proteins or form protein clusters by fusing of smaller domains into larger cholesterol-based protein assemblies and, therefore, they may reduce or promote interactions for signaling. Using different lipid raft-disrupting agents or blockers of glycosphingolipid synthesis have been shown to impair the formation and function of invadopodia as well as the invasive potential of cancer cells (50-53). Lipid rafts play also an important role in the delivery of newly synthetized proteins, including proteolytic enzymes, to the cell surface (54-56). The increased localization of membrane-anchored proteinases such as membrane type 1 matrix metalloproteinase (MT1-MMP) or ADAMs in lipid rafts, as well as upregulation of seprase, matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9), positively correlate with the ECM-degrading activity of invadopodia and enhanced invasion of cancer cells (53,57). As was found, the stimulation of cancer cells with TNF-alpha, the promigratory cytokine, resulted in the enhanced concentration of both the invadopodia-associated dipeptidyl peptidases and matrix metalloproteases in lipid rafts (58). While MMP2 and MMP9 are secretory proteins, small amounts of them have consistently been found to be associated with cell surface. Yu *et al.* have shown that CD44 which is the cell surface hyaluronan (HA) receptor serves as a docking molecule for a proteolytic form of the matrix MMP9 and thereby adds the proteolytic activity to the cell membrane in melanoma cells (59). Similarly, the αvβ3 integrin was demonstrated to associate with a proteolytically active form of MMP2 on the surface of melanoma cells to facilitate cell-mediated ECM degradation (60,61).

The direct association of αvβ3 or β1 integrins with MT1-MMP, resulting in the regulation of ECM-dependent proteolytic activity and MT1-MMP internalization at invadopodia (62). It is known that sorting and intracellular transport of MT1-MMP is critically important for invasion-promoting function in cancer cells and depends on many

different proteins, including vesicle regulators such as Rab GTPases (63).

In this context, it has been shown that the integrated co-trafficking of β3-integrin and MT1-MMP in Rab5a-dependent endo-exocytic cycles was necessary for HGF-stimulated formation of invadopodia and remodeling of the ECM (28). Consistently, integrin receptors which are heterodimeric transmembrane adhesion proteins and mediate interactions between the cytoskeleton and the ECM components such as fibronectin, type I collagen, and laminin, to regulate the cell shape, polarity, and movement of cells, were found as highly expressed factors in many cancers and were identified as crucial components of invadopodia (64,65). The cell surface receptor, CD44, is a ubiquitous transmembrane glycoprotein that is known to interact with various ligands including hyaluronic acid (HA), osteopontin (OPN) and different types of collagens as well as laminin and fibronectin. It was observed that interactions between CD44 and HA led to MT1-MMP-dependent invasiveness by the induction of the CD147-associated activation of EGFR-Ras-ERK signaling pathway (66). Matrix metalloproteinases (MMPs) have been shown to be key players in the invadopodia activity and positively correlated with tumor progression, metastasis, and poor overall prognosis.

Invadopodia-associated proteolytic enzymes

Membrane-type metalloproteinases

MT1-MMP, known as MMP14, is an integral type I transmembrane protein of the MMP family synthesized as a ~64-kDa proenzyme. MT1-MMP is cleaved into the active ~57-kDa form by furin-like proprotein convertases during trafficking from the trans-Golgi network to the cell surface. It was found to localize predominantly in invadopodia. MT1-MMP shows a broad substrate specificity and plays diverse cellular functions owing to their degradation ability. In addition to the catalytic domain, the extracellular region contains a hemopexin-like (PEX) domain which is involved in homodimerization of MT1-MMP, a crucial process for its collagenolytic activity as well as for the activation of proMMP-2 on the cell surface. The PEX domain participates also in heterodimerization/oligomerization of MT1-MMP with the cell surface adhesion molecules. The transmembrane-cytoplasmic fragment, including a short cytoplasmic tail composed of 20 amino acid residues, was shown to be required for invadopodia localization and crucial for cellular invasion (67). It is known that the accumulation of MT1-MMP at invadopodia, which is required for initiation and maintenance of matrix degradation, is supported by the formation of actin and cortactin aggregates. MT1-MMP depletion did not affect the actin-cortactin interactions, but results in proteolytic non-active invadopodia (6). MT1-MMP degrades multiple ECM components, including collagen types I, II, and III, fibronectin, laminin-1, vitronectin, aggrecan, gelatin, α2-macroglobulin, αl proteinase inhibitor (α1Pi) and proteoglycans (68). Cleavage of ECM proteins by MT1-MMP may additionally release various bioactive matrix fragments named matrikines, acting as extracellular modulators. It was demonstrated that laminin-111 derived peptides, AG73 and C16, significantly stimulated invadopodia-associated activities through β1 integrin-dependent Rac1-Erk1/2 signaling pathway (69). MT1-MMP was found to be involved in the activation of MMP2 and possibly MMP8 and MMP13 (70-73). In addition, MT1-MMP may proteolytically modify protein receptors such as CD44, αV integrin, syndecan-1 and low density lipoprotein receptor related protein (LRP) or cytokines, including TNF-alpha and IL-8, by which it affects cell motility and has a direct effect on the immune system (6,68,74,75). The net concentration of active MT1-MMP in cell membrane is generally low, and its upregulation correlates with cancer progression (68,76). From the cell surface, MT1-MMP is rapidly internalized by a combination of clathrin- and caveolin-dependent as well as dynamin-mediated endocytosis (71). MT1-MMP by cleaving of ECM components can be directly or indirectly involved in the regulation cell-cell and cell-matrix interactions. It can release, activate or inactivate different ECM signaling molecules. It can also trigger intracellular signaling pathways by shedding of cell surface receptors (68). It was shown that the silencing of MT1-MMP gene expression resulted in reduced activation of phorbol-12-myristate-13-acetate (PMA)-induced transcription factors such as ELK1, EGR1, ETS1, and ETS2, caused by dysregulation of the phosphorylation of NF-κB/p105. Similarly, mutations within the cytoplasmic domain of MT1-MMP altered RhoA/ROK expression or RhoA-dependent shedding of CD44, and changed the phosphorylation status of Erk1/2, STAT3, and Akt in various type of cancer cells (77). In addition to proteolytic functions, MT1-MMP was described to control cell migration also through non-proteolytic mechanisms. The TIMP2-dependent activation of Erk1/2 was found to be

mediated by the mechanism that requires the cytoplasmic tail but not the proteolytic activity of MT1-MMP (78). It was suggested that this functional diversity of MT1-MMP might be tightly dependent on its partitioning into raft and non-raft membrane domains (56).

Gelatinases

The focal degradation of ECM was found to be correlated also with the localization of MMP2 and MMP-9 at invadopodia. As was shown, the selective reduction of MMP2 and/or MMP9 activity with specific inhibitors or by siRNA-mediated gene silencing, significantly inhibited invadopodia-related ECM degradation and cell invasion (79,80). Gupta *et al.* have shown that downregulation of MMP9 resulted in more adhesive and less invasive phenotype in prostate cancer cells and was correlated with the expression of highly glycosylated variant 6 of CD44 (CD44v6) (81). MMP2 and MMP9 are well known gelatinases which through fibronectin type-II repeats inserted in their catalytic domains bind and process both native and denatured (gelatins) collagens. Their secretion is based on transport from the trans-Golgi Network (TGN) (82). Recent studies have shown that Rab40b GTPase-dependent transport and sorting into VAMP4-containing secretory vesicles of MMP2 and MMP9 is required for invadopodia formation and invadopodia-related ECM degradation, observed during breast cancer metastasis, and is tightly associated with the Tks5 adaptor protein (83,84). MMP2 and MMP9 which cleave major components of the basement membrane (BM) were described to play a key role in the early steps of cancer cell invasion. Both proteins, like most of other members of a family of zinc-dependent endopeptidases, are secreted as inactive pro-enzymes that can be activated through an autocatalytic process or through cleavage by other metalloproteases, like MT1-MMP or seprase.

Serine proteases

Seprase, also known as fibroblast activation protein-α (FAP-α) or F19 cell surface antigen, is a 170-kDa membrane glycoprotein with gelatinase activity. Seprase together with Dipeptidyl peptidase IV (DPP4/CD26) belong to the most recognized serine protease which act in concert to degrade components of ECM and are known to localize at invadopodia (85,86). It forms transmembrane homo- or hetero-dimeric glycoprotein complexes with a post-proline dipeptidyl aminopeptidase activity. Seprase shares 52% amino acid sequence identity with CD26, but it differs in its cellular and substrate specificity. While, CD26 is a ubiquitously expressed cell surface protein which releases X-proline dipeptides from the N-terminus of different peptides, seprase is undetectable in normal cells. Instead, it is selectively expressed by myofibroblast-like cells and by several types of highly invasive cancer cells and cleaves larger proteins. Seprase was found to form a complex with $\alpha_3\beta_1$ but not $\alpha_6\beta_1$ integrin at sites of invadopodia matrix degradation, in response to type I collagen (61). Using confocal microscopy, it was found that the membrane concentration of seprase was several fold higher in invadopodia than in other adhesive structures, and its level correlated with MMP2 expression, and matrix degrading activity in malignant melanoma cells (85). Both seprase and MMP2 were shown to concentrate in lipid rafts in breast cancer cells upon TNF-alpha treatment (58). It was also observed that seprase could form supramolecular complexes with urokinase plasminogen activator receptor (uPAR). These interactions were dependent on vitronectin and β_1 integrin and tightly associated with membrane domains of invading melanoma cells (87).

Disintegrin and metalloproteinases

The other class of enzymes identified as important functional components of invadopodia is the family of a disintegrin and metalloproteinases (ADAMs). ADAMs, also known as sheddases, comprise a group of membrane-anchored, multidomain proteases that may regulate cell behavior by proteolytic processing cellular and ECM components (88). Aside from the extracellular metalloprotease domain, they contain propeptide, disintegrin-like, cysteine-rich, and epidermal growth factor (EGF)-like transmembrane and cytoplasmic domains. Different ADAMs were found to localize at invadopodia, including ADAM12 and ADAM19 (89). The disintegrin domain of ADAM12 was indicated to modulate integrin-dependent cell adhesion at invadopodia (90). As was shown, knockdown of ADAM12 in breast cancer cells significantly impaired invadopodia formation and function (91). Both ADAM12 and ADAM19 were found to interact with the adaptor protein Tks5 which is a key component of invadopodia (89). Moreover, hypoxia-induced secretion and activity of ADAM12 had an impact on the formation of invadopodia via ectodomain shedding of the heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) (92).

Summary

The broad interest in invadopodia is related to their significant role in cancer invasion and metastasis. Although invadopodia are very complex and dynamic structures, it is expected that their key regulators would be good targets for anticancer therapy. Plasma membrane organization and the clustering of lipid rafts into active signaling platforms which mediate signal transduction from and into cells could serve as good target for the future research on specific drugs for the treatment of invadopodia-promoted diseases.

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