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13. ABSTRACT (Maximum 200 Words)

Elucidating the degradive effectors responsible for focal proteolysis at the cell-matrix interface during metastasis is an ongoing challenge. We are dissecting the components of the proteolytic machinery required for breast cancer cells (metastatic MDA-MB231 and non-metastatic MCF-7) transendothelial migration and determining the stage of extravasation particularly reliant upon the metalloproteinase activity. Modulation of individual molecules demonstrates the functional cooperation of furin, cell surface adhesion molecules ($\alpha_v\beta_3$, CD44), and matrix metalloproteinases (MMP-2, MMP-9 and MT1-MMP) during the process of transendothelial migration. Confocal microscopy shows co-localization of molecules critical for MMP cell surface distribution, and disruption of these events and molecules reduces transendothelial migration of MDA-MB231 cells. MMP-2 and MT1-MMP localization matches the imprint of spatially restricted fluorogenic gelatin digestion. The digestion occurs in a stage-specific manner and becomes most evident during the migratory phase of tumor cell transendothelial migration. Of the above breast cancer cell lines, MDA-MB231 is able to undergo efficient transendothelial migration, and MT1-MMP emerges to be one of the key molecules involved for this event. The lack of several key components in MCF-7 cells impairs its ability to transmigrate. Our data are revealing how specific molecular interactions result in a cooperative proteolytic interface at the tumor cell surface within the breast tumor cell-endothelial cell microenvironment during transendothelial migration, which mimics cancer cell extravasation.

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Introduction:

Elucidating the degradative effectors responsible for focal proteolysis at the cell-matrix interface during metastasis is an ongoing challenge. We are dissecting the components of the proteolytic machinery required for breast cancer cell (metastatic MDA-MB231, and non-metastatic MCF-7) transendothelial migration (TEM). Specifically, we are determining the stage of extravasation that is particularly reliant upon metalloproteinase activity.

Body:

We have determined the MMP profile for the individual breast cancer cell lines MCF-7 and MDA-MB-231, and human lung microvascular endothelial cells by zymography. This analysis has also been performed on conditioned media taken from TEM cultures. Verification of key MMP (MT1-MMP) expression in these cells has been obtained at the protein level by western blotting. Both MMP-2 and MMP-9 activities are present by zymography. MT1-MMP is present only in MDA-MB-231 but not MCF-7 cells suggesting the possible involvement of trimolecular complex that is known to be critical for MMP-2 activation. These data are shown in the attached figure 1A-1C.

We have completed our investigation of inhibitory effects of biological and synthetic MMP-inhibitors, as well as a serine inhibitor, on transmigration of MDA-MB231cells. MMP inhibitors, the serine inhibitor, and TIMP-1 and TIMP-2 show a statistically significant reduction in TEM of breast cancer cells (figure 2A-2F). TIMP-1 and TIMP-2 did not exert an additive inhibitory effect.

Using antibodies specific to MMP-2, MMP-9 and MT1-MMP, we have localized these proteases on the cancer cells during the process of TEM (figure 3A-3C). With an antibody from Dr. W. Garten (Marburb University, Germany) we have also visualized furin in our TEM assay (figure 3D).

Further, we have observed co-localization of MMPs with cell surface molecules, specifically of MT1-MMP with TIMP-2 (figure 3A) during TEM. On the other hand we do not see MMP-2 co-localization of MMP-2 with $\alpha v\beta 3$ on breast cancer cells, which is clearly apparent in a melanoma cell line, WM 239 (figure 4). While both CD44 and MMP-9 can be visualized on MDA-MB231 breast cancer and WM 239 cells during TEM, these molecules do not co-localize (figure 3C).

With fluorogenic substrate, we have mapped proteolytic activity on individual cell as well as during the process of TEM (figure 5A-5B). We also have optimized conditions for measuring MMP-based fluorogenic substrate digestion in solution (figure 1B). As well, we have observed and qualitatively assessed proteolytic-matrix degradation in situ at the tumor cell surface at distinct stages of TEM, by confocal microscopy (figure 5C).

For specific manipulation of the individual complexes, our ongoing studies are utilizing: a) rCBD, b) HxCD, c) $\alpha v\beta 3$ blocking peptide, d) an antibody against furin and rfurin. As well cells have been transiently transfected with a CFP–tagged MT1-MMP expression construct.

Key research Accomplishments:

- Determination of MMP profile in isolated and TEM cultures at protein and activity levels (figure 1).
- Inhibition of breast cancer cell TEM by synthetic MMP inhibitors (figure 2).
- Cell surface and subcellular localization of the relevant MMPs and TIMPs (figure 3).
- Co-localization of cell surface proteins with specific MMPs during TEM (figures 3 and 4).
- Visualization and quantification of MMP-mediated substrate digestion at defined phases of TEM (figure 5).
- Generation of CFP-tagged MT1-MMP transfected cells (figure 6).

Reportable Outcomes:

A manuscript "*Dynamics of focal proteolysis during melanoma and breast tumor cell transendothelial migration*" by EB Voura, JL English, H-YE Yu, AT Ho, CM Overall, KLM Watson, and R Khokha has been submitted and revised for publication in the Journal of Biological Chemistry. We are awaiting the decision on its publication.

This data has been presented at the following International Conferences:

1. Annual US Army Era of Hope DoD Meeting, Orlando, Florida, USA, Sep'2002
2. AACR 94th Annual Meeting, Washington, USA. Education session, Jul'2003

Conclusions:

We have found that the two breast cancer cell lines, one metastatic (MDA-MB231) and the other non-metastatic (MCF-7), differ in their ability to undergo transendothelial migration. One basis for this differential transendothelial migration is that MCF-7 cells lack MT1-MMP as well as the $\beta 3$ integrin subunit, both of which have been shown to anchor other metalloproteinases (MMP-2) to the invasive front of the migrating cell. MT1-MMP could also act as a protease in its own right, independent of MMP-2 activation, for MDA-MB231 cells.

The above progress is in keeping with the milestones projected in the original application.

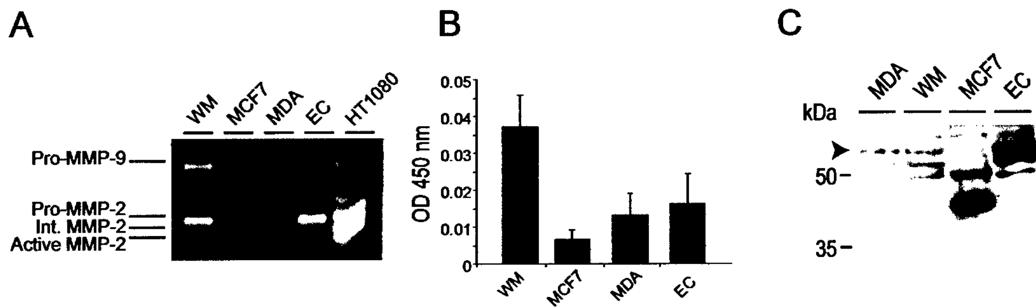


Figure 1

Expression of proteases by invasive cancer cells

(*A*) Zymogram of serum-free, conditioned media samples from WM239 (WM), MCF-7, MDA-MB231 (MDA) and EC. Highlighted are the various forms of the gelatinases seen on the gel. (*B*) MMP Gelatinase Activity Assay of serum-free conditioned media samples used for zymography. (*C*) Western blot of total cell lysates from MDA-MB231 (MDA), WM239 (WM), MCF-7 (MCF) and EC cultured in EC media stimulated with TNFα (10 ng/mL) show the expression of MT1-MMP using primary antibody Ab815 (1:1000).

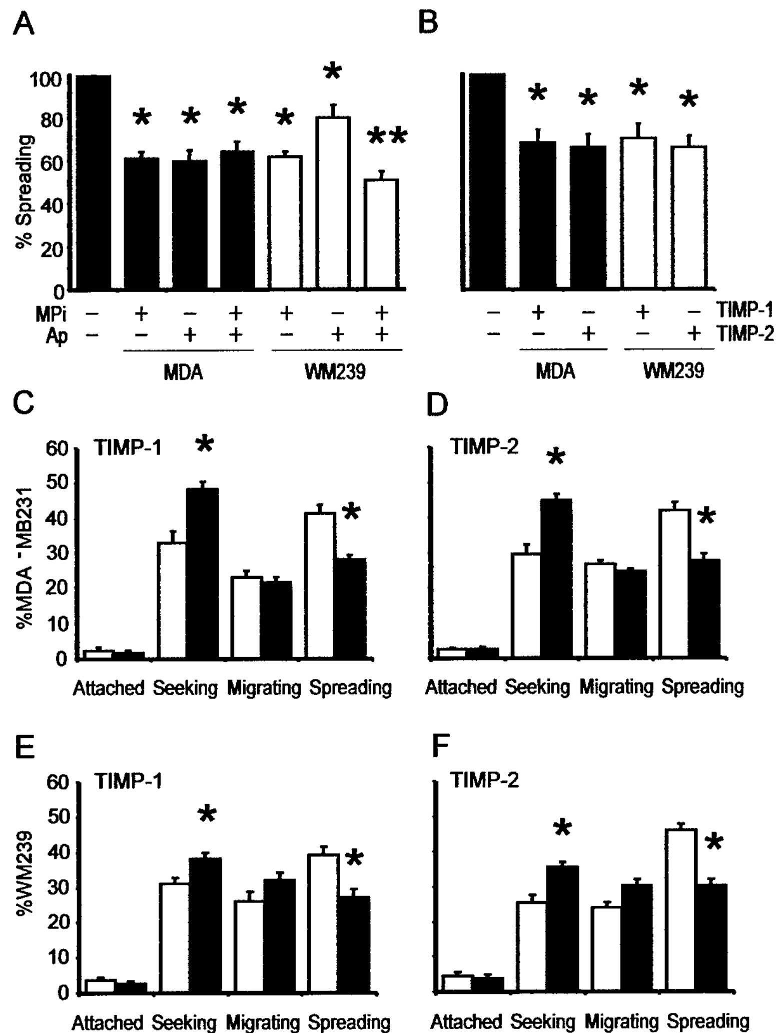
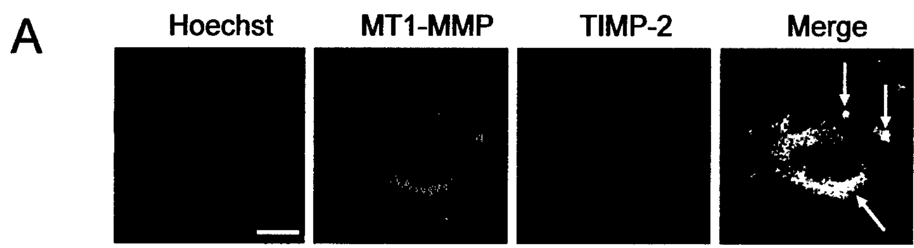
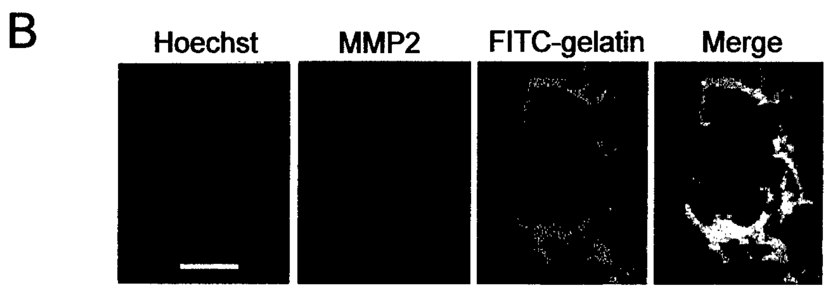


Figure 2

Modifying agents demonstrate the importance of proteolysis in the migratory process (*A*) Percent spreading MDA-MB231 (gray bars) and WM239 (white bars) relative to no inhibitor control (black bar). Proteinase inhibitors MPi (20 μM) and aprotinin (100 U) were used. * Indicates significant P value compared to controls and ** indicates significant P value relative to both controls and MPi or aprotinin alone. (*B*) The reduction in spreading cells with recombinant TIMP-1 or TIMP-2 (each at 2μg/mL). (*C* and *D*) Percent cells at each stage of extravasation upon treatment with the indicated TIMP (black bar) compared to untreated controls (white bars) for MDA-MB231 and (*E* and *F*) WM239. Error bars=mean±S.E.M.





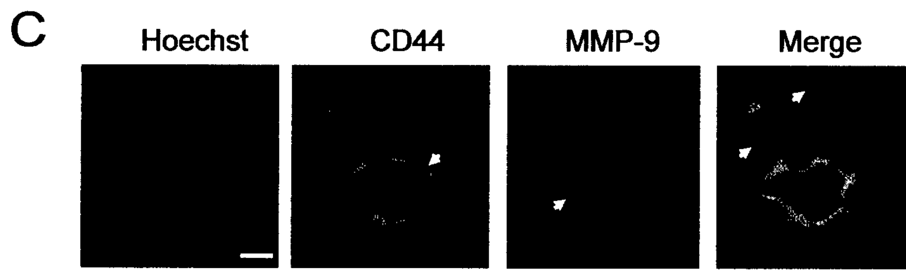


Figure 3

Figure 3

(*A*) Identification of proteins co-localized while undergoing the process of transendothelial migration. Blue cell tracker and Hoechst labeled tumor cells (bright blue cells and nuclei) incubated on EC for 3 hours. Both tumor and EC cells were stained for actin (pale blue). Upper panel shows 1 μm sections of a non-permealized seeking MDA-MB231, the lower panel shows a non-permealized migrating WM239 labeled with MT1-MMP (green) and TIMP-2 (red). Arrows in merged image indicate co-localization (yellow). Bars=10 μm. (*B*) 1μm confocal section of a Hoechst and blue cell tracker (blue) tagged WM239 cell with surface MMP-2 (red) corresponding matrix digestion (green), and the merged image. (C) Blue cell tracker and Hoechst labeled tumor cells (bright blue cells and nuclei) incubated on EC for 3 hours. Both tumor and EC cells were stained for actin (pale blue). 1 μm sections through a seeking MDA-MB231 cell (upper panel) and a seeking WM239 cell (lower panel) labeled for CD44 (green) and MMP-9 (red). Arrowhead and arrow point to CD44 in heterotypic tumor cell-endothelial cell contacts or on the cell surface respectively. Arrowheads also indicate little cell surface, or abundant MMP-9 staining of blebs merged. Bars=10 μm. (D) Lamellipodia from MDA-MB231 (upper panels) and WM239 (lower panels) express furin (green) in 1 μm confocal sections when cultured alone on Matrigel. Furin localized to leading lamellipodial edges (arrowhead) with actin (red). Seeking Hoechst and blue cell tracker labeled (blue) MDA-MB231 (upper panel) and WM239 (lower panel) in 1 μm confocal sections. These images, taken on non-permealized transendothelial migration samples, show furin (red) at the cell surface and on blebs. Bars=10 μm.

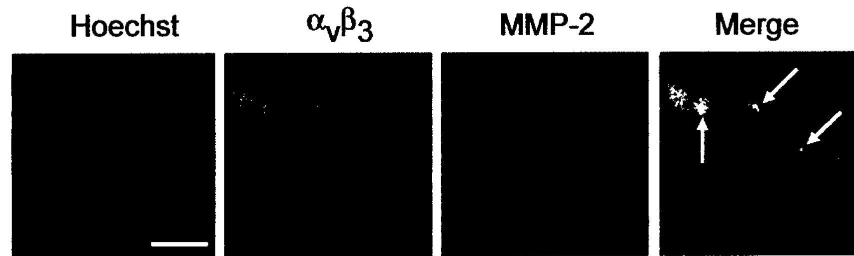


Figure 4

1 μm confocal sections of a blue cell tracker and Hoechst labeled WM 239 cell (bright blue cells and nuclei) incubated on EC for 3 hours and undergoing the spreading stage of transendothelial migration. Experiments were subjected to labeling of actin in both tumor and EC cells (pale blue). Labeling of $\alpha_v\beta_3$ (green) and MMP-2 (red). Arrows indicate spectral co-localization (yellow) in merged image. Bars=10 μm.

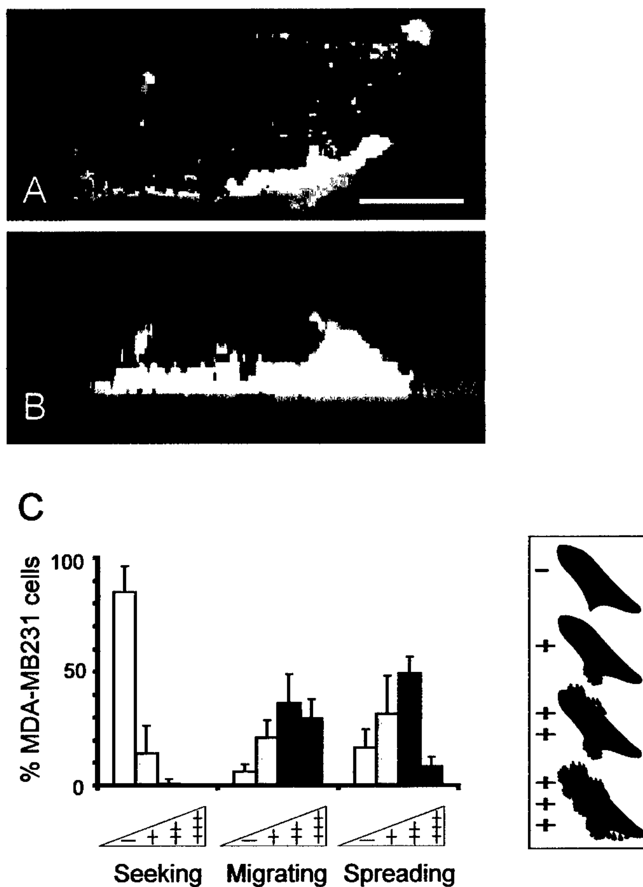


Figure 5

Matrix degradation is evident during tumor cell transendothelial migration. 3 dimensional optical rotation of Dil labeled MDA-MB231 (red) from the top (*A*) and side (*B*) as it digested and internalized the matrix (green).

"-" = no digestion around cell contour, "+" = single patch of digestion, "++" = patchy digestion and "+++" = digestion around the entire cell contour. The cartoon beside represents the scoring scheme. Error bars=mean±S.E.M.. .

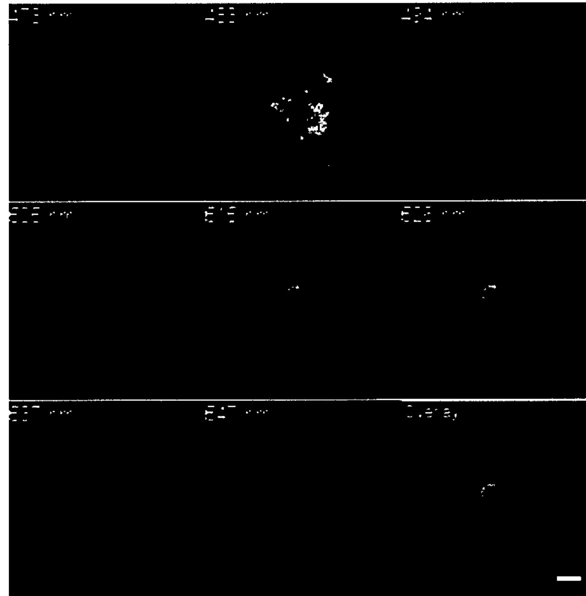


Figure 6

A wavelength scan (Zeiss LSM-META) at the indicated 10 nm intervals through a CFP-MT1-MMP expressing cell (blue) digesting FITC gelatin (green).
The 483 and 515 images were used in the overlay image.