A Novel 96-well Assay for Assessing Chemokinetic Modulators of Cell Migration and Invasion

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Abstract # 1178

Introduction

The failure of cells to migrate, or the movement of invasive cells into inappropriate locations, is intrinsic to many disease processes such as dysregulated wound healing and cancer. Here, we report the development and validation of a novel two-dimensional assay that can be used to investigate cell migration. This cell migration assay uses a 96-well plate populated with stoppers that exclude cells from the central migration zone of the well. An opaque mask fitted to the plate bottom restricts detection of signal to the central migration zone of each well. After cells are seeded and allowed to adhere, the polymeric stoppers are removed and cells are permitted to migrate (or invade) into the central migration zones defined by the mask. Next, cells are stained and the assay results are collected from a plate reader or inverted microscope.

The assay was used to evaluate the motility of several cell lines following their treatment with cell migration stimulators and inhibitors. FBS and EGF treatments stimulated the motility of SV-40 transformed human corneal epithelial cells (HCECs) in a dose-dependent manner. The addition of as little as 0.5% FBS and 0.1 ng/ml EGF markedly stimulated HCEC migration. Latrunculin A treatment inhibited the motility of NIH 3T3 fibroblast and HT-1080 fibrosarcoma cells in a dose-dependent manner. Complete inhibition of NIH 3T3 and HT-1080 cell migration was observed in the presence of at least 500 nM and 100 nM Latrunculin A, respectively. In addition, the Latrunculin A inhibition of HT-1080 cell migration was similar when the cells were examined in both the cell migration assay and a manual wound healing assay (WHA). The former assay, however, required less attended time (~5-fold) and provided more reproducible results than the WHA (% CVs of 13-18 vs 16-42). The cell migration assay, modified by coating the plates with collagen I, was used to assess the collagen-dependent migration of a T47D cell line, derived from human breast carcinoma, transfected with constitutively active R-Ras [R-Ras(38V)]. In this assay, R-Ras(38V) inhibited motility relative to vector control line which is consistent with the inhibitory effects of R-Ras(38V) on random cell migration and scratch wound healing. Preliminary studies using HT-1080 cells further suggest that this assay format may be used to assess the invasive abilities of cancer cells. This novel assay format provides a new tool for screening of test compounds for the chemokinetic modulation of cell migration and invasion.

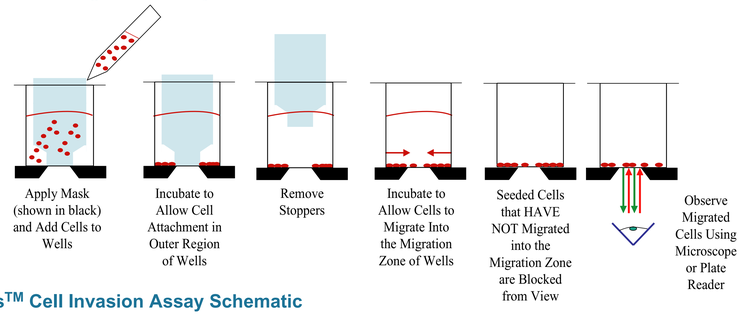
Materials and Methods

Reagents and Sources: Calcein AM (C3099) and EGF (E3476), Invitrogen; CellTracker™ Green (PA-3011), Cambrex BioSciences; Mitomycin C, MMC (47589), Calbiochem; Latrunculin A (T119), BIOMOL; FBS, Atlanta Biological; Growth Factor Reduced Matrigel™ (356231), BD BioSciences; and Growth Factor Reduced Cultrex™ BME (3433-005), Trevigen.

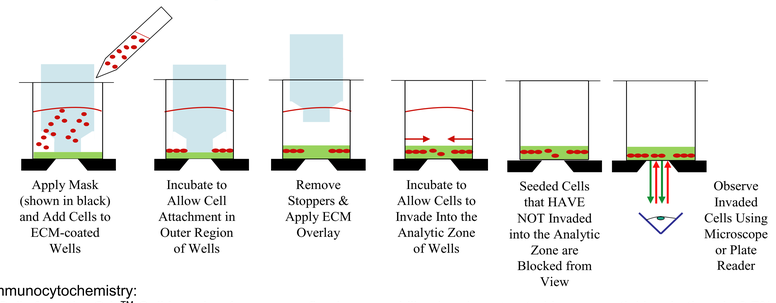
Antibodies and Sources: Polyclonal anti-MMP-9 (1:50 dilution, sc-10737), Santa Cruz Biotechnology; polyclonal anti-Cathepsin B (1:50 dilution, SA-361), BIOMOL and monoclonal anti-Cortactin (1:25 dilution, 5-180), Upstate; Alexa Fluor® 488 SFX kits containing highly cross-adsorbed conjugated goat anti-mouse antibodies (A31620) and goat anti-rabbit antibodies (A31628) with Image-iT™ FX signal enhancer and Alexa Fluor® 555 phalloidin (1:80 dilution, A34055), Invitrogen.

Cell Lines and Sources: NIH 3T3 fibroblast and HT-1080 fibrosarcoma cell lines, ATCC; SV40 transformed Human Corneal Epithelial Cells (HCEC) gifted from K. Araki Sasaki (Institute of Ophthalmology, Tane Memorial Eye Hospital, Kumamoto, Japan).

Oris™ Cell Migration Assay* Schematic



Oris™ Cell Invasion Assay Schematic



Immunocytochemistry:
- Cells from the Oris™ Cell Invasion Assay were fixed, permeabilized and pre-treated by sequential incubations in 3.7% formaldehyde, PBS, 0.5% Triton X-100, PBS, Image-iT™ FX signal enhancer (30 minutes), and PBS.
- Immunostaining was performed by incubating with primary antibodies (2 hours, 37°C) and Alexa Fluor® 488 conjugated secondary antibodies at 1:200 dilution (1 hour, 37°C) followed by an Alexa Fluor® 555 phalloidin counterstain (1 hour).
- Images were collected using a Nikon TE300 inverted microscope equipped with a Photometrics Coolsnap fx CCD camera, deconvolved using Slidebook™ v4.2 (Intelligent Imaging Innovations) and processed using Adobe® Photoshop® CS2 (Adobe Systems).

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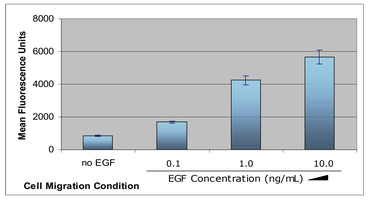
* Patent Pending

Cell Migration

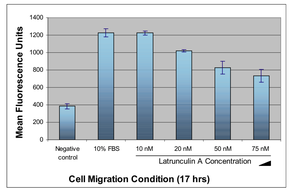
Stimulation of HCEC Cell Migration



MMC pre-treated HCECs were seeded to confluency, treated with EGF (0 - 10 ng) for 24 hours and stained with Calcein AM. Each column represents the mean fluorescence +/- SEM of 18 wells.

Results: EGF-stimulated migration of HCEC cells was demonstrated using The Oris™ Cell Migration Assay. The stimulatory effect of EGF was dose-dependent. Migration of cells in the presence of at least 0.1 ng/mL EGF was significantly higher than that observed in the absence of EGF.

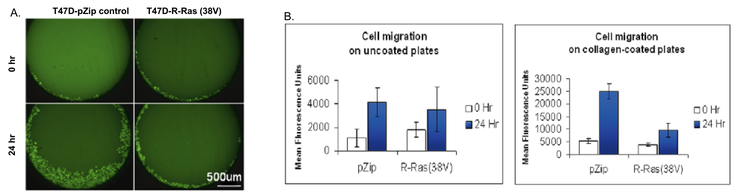
Inhibition of HT-1080 Cell Migration



HT-1080 fibrosarcoma cells (30,000 cells/well) were seeded, treated with Latrunculin A (0 - 75 nM), and labeled with Calcein AM. Each column represents the mean fluorescence +/- SEM of 5 wells. The negative control represents wells in which the stoppers remained in place until the staining occurred.

Results: Latrunculin A inhibition of HT-1080 cell migration was demonstrated using the Oris™ Cell Migration Assay. A dose response to Latrunculin A was observed after 17 hours of incubation. In data not shown, complete inhibition was detected in the presence of 100 nM Latrunculin A at 6 hours of incubation.

T47D Cell Migration on 2D Collagen-Coated Surfaces



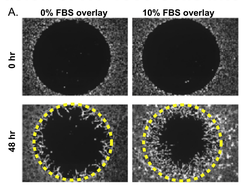
T47D cell lines were tested (100,000 cell /well) for the ability to migrate on uncoated and collagen-coated plates. The positive control consisted of pZip cells, a T47D cell line derived from a human breast carcinoma and transfected with a null vector. The negative control consisted of R-Ras (38V) cells, a T47D cell line transfected with a constitutively active R-Ras-expressing vector. In other assays, R-Ras (38V) inhibits random cell migration and wound closure. In the Oris assay cells were incubated for 24 hours to permit migration then labeled with 5 uM Calcein AM. Plate reader results are depicted graphically where each column represents the mean +/- S.D. of 18 wells.

Results: The collagen-dependent T47D cells (pZip and 38V) were able to adhere to the uncoated and the collagen-coated plates; however, the morphology of the cells was improved by plating onto the collagen-coated plates (A). Similarly, the coefficient of variance (CV) was dramatically lower when pZip and R-Ras (38V) cells were seeded on the collagen-coated plates (C) compared to that observed when the cells were seeded on the uncoated plates (B). The inhibitory effect of R-Ras (38V) expression could not be validated when the cell migration assay was performed using uncoated tissue culture plates. However, when the assay was performed by seeding the R-Ras (38V) cells onto collagen-coated plates, the cells were inhibited in their motility; thus validating previously published findings (Reference 1).

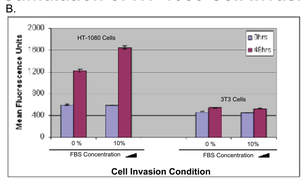
Cell Invasion

The Oris™ Cell Migration Assay is the only 2-D, membrane-free closure assay that enables researchers to study the migration of cells on layers of ECM proteins such as type 1 collagen. We now report results from a novel 3-D cell invasion assay that has been developed by modifying the Oris™ migration assay format. Preliminary data, presented here, depicts marked differences in the abilities of invasive HT-1080 cells and non-invasive NIH 3T3 cells to move through a 3-dimensional ECM. We confirm invasion status of the HT-1080 population by identifying key hallmarks including the presence of invadopodia and the expression of cellular proteases.

Invasion of HT-1080 Cells into ECM



Stimulation of HT-1080 Cell Invasion

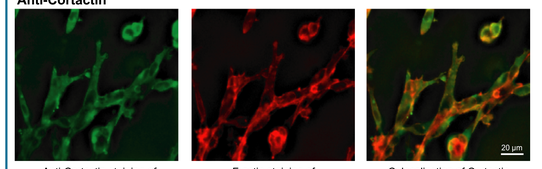


HT-1080 cells, serum starved for 18 hours, were seeded (50,000 cells/well) on an ECM coated plate (4 mg/ml). Stoppers were removed and ECM (+/- 10% FBS) was overlayed on the cells. Following a 48 hour incubation the cells were labeled with Calcein AM and imaged by using a Zeiss Axiovert microscope (5X magnification). Fluorescence in the analytic zone was quantified by using a plate reader. Each column represents the mean +/- SEM of at least 22 wells. A non-invasive cell line, NIH 3T3, served as the negative control.

Results: Serum starved HT-1080 cells form invadopodia-like projections within the 3-dimensional overlay of ECM. This invasive movement is potentiated by 25 - 30% when 10% FBS is present in the ECM overlay. In contrast, NIH 3T3 cells do not invade into the 3D ECM overlay, even in the presence of FBS.

Characterization of Invadopodia

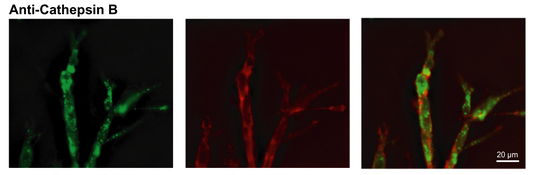
Anti-Cortactin



Anti-Cortactin staining of HT-1080 cells | F-actin staining of HT-1080 cells | Colocalization of Cortactin and F-actin

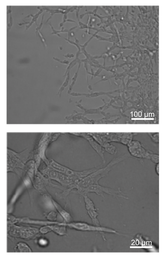
F-actin is present in all eukaryotic cells, it is a major component of the cytoskeleton and functions to define and maintain the cell shape. Cortactin is a cellular protein that is present in and promotes the formation of lamellipodia and invadopodia. These structures propel cells over surfaces as they move toward a target. Results depicted above demonstrate distinct areas of Cortactin and F-actin colocalization characteristic of invadopodia (References 2 & 3).

Anti-Cathepsin B



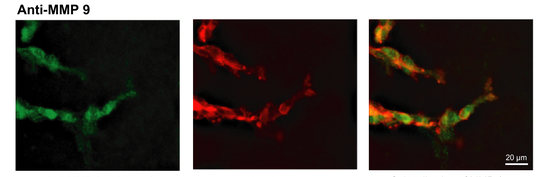
Anti-Cathepsin B staining of HT-1080 cells | F-actin staining of HT-1080 cells | Colocalization of Cathepsin B and F-actin

Cathepsin B is a lysosomal protease that facilitates cell invasion by degrading the surrounding extracellular matrix. Results depicted above demonstrate distinct areas of lysosomal and peripheral cellular expression of Cathepsin B characteristic of protease expression observed in cancer cell invasion (Reference 4).



Brightfield microscopic images of invadopodial projections formed within the ECM overlay using Oris™ Cell Invasion Assay.

Anti-MMP 9



Anti-MMP-9 staining of HT-1080 cells | F-actin staining of HT-1080 cells | Colocalization of MMP-9 and F-actin

MMP-9 is a protease that is involved in matrix degradation in cancer cell invasion. Results depicted above demonstrate evidence of MMP-9 expression and secretion into surrounding areas of ECM, which are established characteristics of proteases observed at invadopodia sites (References 2 & 3).

Conclusions

The Oris™ Cell Migration Assay enables the study of cell migration in the presence of chemokinetic modulators in a convenient, quantifiable 96-well plate format. Data presented here demonstrate the added benefit of observing the morphology of the adherent cells as they migrate on an ECM.

The Oris™ Cell Invasion Assay enables the study of cell invasion through a 3D ECM in a convenient, quantifiable 96-well plate format. Data presented here demonstrate that the cells moving within the 3D ECM exhibit hallmark features of invadopodia, as evidenced by immunocytochemistry.

1 Araki-Sasaki K et al; Invest Ophthalmol Vis Sci. 1995. "An SV40-immortalized human corneal epithelial cell line and its characterization"; 36 (3) : 614 - 21.
2 Weaver, AM; Clin Exp Metastasis 2006. "Invadopodia: specialized cell structures for cancer invasion." 23:97-105.
3 Furmaniak-Kazmierczak et al; Circulation Research 2007. "Formation of extracellular matrix-digesting invadopodia by primary aortic smooth muscle cells." 100:1328-1336.
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