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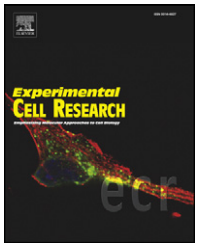

Research Article

Competitive enhancement of HGF-induced epithelial scattering by accessory growth factors

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ABSTRACT

HGF signaling induces epithelial cells to disassemble cadherin-based adhesion and increase cell motility and invasion, a process termed epithelial–mesenchymal transition (EMT). EMT plays a major role in cancer metastasis, allowing individual cells to detach from the primary tumor, invade local tissue, and colonize distant tissues with new tumors. While invasion of vascular and lymphatic networks is the predominant route of metastasis, nerves also can act as networks for dissemination of cancer cell to distant sites in a process termed perineual invasion (PNI). Signaling between nerves and invasive cancer cells remains poorly understood, as does cellular decision making that selects the specific route of invasion. Here we examine how HGF signaling contributes to PNI using reductionist culture model systems. We find that TGFβ, produced by PC12 cells, enhances scattering in response to HGF stimulation, increasing both cell–cell junction disassembly and cell migration. Further, gradients of TGFβ induce migratory mesenchymal cells to undergo chemotaxis towards the source of TGFβ. Interestingly, VEGF suppresses TGFβ-induced enhancement of scattering. These results have broad implications for how combinatorial growth factor signaling contributes to cancer metastasis, suggesting that VEGF and TGFβ might modulate HGF signaling to influence route selection during cancer progression.

Introduction

A central feature of malignant cells is their ability to detach from the primary tumor, invade through local tissues, migrate to distant sites, and colonize distant tissues with secondary tumors. The traditional view is that cancer cells first infiltrate vascular and lymphatic networks en route to metastatic locations. Additional routes of cancer invasion exist, although these remain much more poorly understood than vascular and lymphatic invasion, or VLI. One important example of an alternative route for metastatic cells occurs when detached tumor cells invade and colonize nerve bundles, a process termed perineural invasion, or PNI [1]. In certain cancer types, such as prostate [2] and pancreatic cancers [3], PNI is thought to be the primary route for establishment of metastatic disease.

Cellular signaling between cancer cells and nerves is required for PNI. Although it is proposed that nerves produce factors that allow cancer cells to home their migration towards nerves, resulting in nerve colonization [4], signaling systems required for PNI remain undefined. Further, although specific tumor types are associated with either VLI or PNI, how tumor cells select VLI or PNI as a primary route of invasion is also unknown. Signal transduction pathways that are activated in invasive cells and that drive

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invasive behavior are likely candidates. Hepatocyte growth factor (HGF) signaling plays an important role in cancer progression [5]. HGF binds and activates the c-met receptor tyrosine kinase [6], whose overexpression or mutation is tightly associated with cancer progression, particularly cancer metastasis [5]. Consistent with a role in cancer invasion, HGF signaling induces epithelial–mesenchymal transition (EMT) [7,8]. However, it is unclear whether HGF signaling can specify a particular route of invasion.

Cancer cell lines can be grown with primary cultures of dorsal root ganglion, resulting in enhanced cell migration and colonization of nerve tissues [9]. Such models are likely to prove powerful in the dissection of signal transduction pathways that drive or permit PNI. However, perturbations in cellular signaling pathways that are inherent to cancer cells confound dissecting signaling that drives PNI. In this report, we employ a reductionist co-culture model system to examine how HGF signaling impacts cellular behaviors associated with PNI. Instead of a cancer cell line as a model for invasive cells, we use MDCK cells, a cell line derived from normal canine kidney tissue and that expresses c-met [7]. MDCK cells are co-cultured with PC12 cells, a rat adrenal pheochromocytoma cell line that differentiates into nerves when stimulated with growth factors. We find that PC12 cells exacerbate HGF-induced scattering, an effect that can be reproduced with conditioned medium or with purified transforming growth factor β (TGFβ). In addition to increasing MDCK cell scattering in response to HGF, TGFβ acts as a chemoattractant. The effects of TGFβ occur when cells adhere to collagen; on plastic it is vascular endothelial growth factor (VEGF) that enhances scattering. Interestingly, VEGF and TGFβ oppose each other in driving increased scattering. On collagen, VEGF blocks TGFβ increases in HGF-induced scattering, while on plastic TGFβ blocks VEGF increases in HGF-induced scattering. We propose a simple working model whereby signaling by additional growth factors (TGFβ or VEGF) predisposes metastatic cells to select a specific route of invasion.

Materials and methods

Cell culture

MDCK cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). PC12 and RT4-D6P2T cells were maintained in RPMI medium containing supplemented with 10% fetal bovine serum and, for PC12 cells, 10% horse serum. Medium used for these cell lines in co-culture type experiments was 4 parts DMEM and 1 part RPMI, supplemented with 10% FBS and, for PC12 cells, 5% horse serum. MRC-5 cells, a human fetal lung fibroblast cell line, were maintained in DMEM supplemented with 10% FBS and were grown to generate conditioned medium containing HGF. Each lot of conditioned medium was assayed by titration for activity in inducing scattering of MDCK cells and diluted appropriately. Twenty percent conditioned medium was used to induce scattering in all experiments. Purified growth factors used were NGF (human protein used at 0.1 μg/ml final concentration), TGFβ2 (human protein, used at 0.1 ng/ml final concentration), or VEGF (mouse protein used at 10 ng/ml).

Cell-based assays

In co-culture experiments, a cloning ring was placed in the center of a 6-well plate. 100,000 PC12 cells were seeded inside the cloning ring, while 24 h later, 200,000 MDCK cells were seeded outside of the cloning ring. After culturing for an additional 24 h, the cloning ring was removed and the appropriate growth factors added at the desired concentration. Invasion assays were performed by coating transwell filters with collagen, then seeding 75,000 MDCK cells onto the upper surface of the transwell filter system. After 24 h, cells were stimulated with growth factor and cultured an additional 24 h. Cells were washed with ice-cold PBS, fixed with 4% (w/v) paraformaldehyde on ice, and stained with crystal violet. The upper surface of the transwell filter was swabbed of cells, leaving only cells that had invaded through the filter. Results were quantified by imaging the filters with light transmitted through the filter. For immunofluorescence imaging of MDCK cells responding to growth factors, 10,000 MDCK cells were seeded onto collagen-coated coverslips and cultured for 24 h. Cells were stimulated with the appropriate growth factors and cultured for an additional time period. Cells were then washed with ice-cold PBS, fixed, with 4% (w/v) paraformaldehyde on ice, and processed for fluorescence imaging using cadherin (BD Biosciences), osteonectin (Leica), or phosphoSMAD2/3 (Santa Cruz) primary antibodies and Alexa dye-conjugated secondary antibodies. To visualize scattering of MDCK cells, 10,000 cells were seeded on collagen-coated DeltaT (Bioptechs) imaging dishes in DMEM +10% FBS and cultured overnight. Prior to imaging, media was changed to HEPES buffered DMEM +10% FBS. A heated microscope stage using Delta T4 temperature regulation system (Bioptechs) maintained temperature at 37 °C. Multiple positions were imaged for each experiment. The appropriate growth factors were added immediately prior to initiating imaging.

Dunn chamber assay

To image chemotaxis of MDCK cells in a gradient, 50,000 cells were seeded onto a collagen-coated coverslip and cultured for 24 h. The coverslip was affixed to the Dunn chamber, containing HEPES-buffered DMEM + 10% FBS. Both chambers contained HGF and the outer chamber contained 0.4 ng/ml TGFβ. Multiple positions on the coverglass were selected for imaging for each experiment, always within the bridge region between the chambers of the Dunn chamber system. Phase contrast images of cell scattering were taken at 16× magnification using a 10× (0.30 aperture) objective and 1.6× slider. SlideBook software was used to track specific cells and their location in the resulting image series.

Results

PC12 cells enhance HGF-induced detachment of MDCK cells

To test whether HGF signaling function in PNI, we used a reductionist tissue culture system that recapitulates elements of PNI. MDCK cells are co-cultured with PC12 cells and then stimulated with HGF. The two cell types are separated by a barrier, removal of which initiates the experiment and allows cell communication. MDCK cells are an epithelial cell line derived from normal canine kidney that has become a well-established model for epithelial cell biology. In the absence of HGF stimulation, MDCK cells form an adherent, non-migratory epithelial tissue that closely recapitulates morphology and function observed in the tissue of origin. MDCK cells express the c-met receptor tyrosine