Video Article

A Modified *In vitro* Invasion Assay to Determine the Potential Role of Hormones, Cytokines and/or Growth Factors in Mediating Cancer Cell Invasion

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Abstract

Blood serum serves as a chemoattractant towards which cancer cells migrate and invade, facilitating their intravasation into microvessels. However, the actual molecules towards which the cells migrate remain elusive. This modified invasion assay has been developed to identify targets which drive cell migration and invasion. This technique compares the invasion index under three conditions to determine whether a specific hormone, growth factor, or cytokine plays a role in mediating the invasive potential of a cancer cell. These conditions include i) normal fetal bovine serum (FBS), ii) charcoal-stripped FBS (CS-FBS), which removes hormones, growth factors, and cytokines and iii) CS-FBS + molecule (denoted "X"). A significant change in cell invasion with CS-FBS as compared to FBS, indicates the involvement of hormones, cytokines or growth factors in mediating the change. Individual molecules can then be added back to CS-FBS to assay their ability to reverse or rescue the invasion phenotype. Furthermore, two or more factors can be combined to evaluate the additive or synergistic effects of multiple molecules in driving or inhibiting invasion. Overall, this method enables the investigator to determine whether hormones, cytokines, and/or growth factors play a role in cell invasion by serving as chemoattractants or inhibitors of invasion for a particular type of cancer cell or a specific mutant. By identifying specific chemoattractants and inhibitors, this modified invasion assay may help to elucidate signaling pathways that direct cancer cell invasion.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51480/>

Introduction

A novel invasion assay has been developed with the goal of identifying the involvement of specific hormones, cytokines and/or growth factors as chemoattractants in cancer cell invasion. The ability of tumor cells to invade through the extracellular matrix (ECM) is a hallmark of the metastatic phenotype¹⁻³. Invasion chambers have been extensively used as *in vitro* tools to study cancer cell invasion and migration and may provide knowledge as to the mechanisms of *in vivo* tumor invasion and metastasis. The chambers consist of cylindrical cell culture inserts nested within the wells of cell culture plates. The bottoms of the inserts are semi-permeable polycarbonate or polystyrene membranes of defined pore sizes.

In the standard invasion assay, cells are seeded onto the insert membrane with serum free media and placed into cell culture wells that are filled with serum or serum-like chemoattractants to set up a chemoattractive force. This force drives cells to move through the semi-permeable membrane (migration) or alternatively, through a coating of extracellular matrix (invasion), which can then be stained using conventional dyes to detect and quantify the number of cells. The cell number is then normalized according to the extent of migration and invasion in a noninvasive cell line. This method allows an investigator to evaluate the invasive potential of different cell types under a variety of conditions, including genetic manipulations.

Hormones, cytokines and growth factors are critical components of serum and are increasingly being shown to be associated with driving the invasive phenotype⁴. However, the nature, role, and specificity of these chemoattractive serum molecules in mediating invasion still remain elusive. The challenge remains to determine what specific factors in serum or serum-like chemoattractants are responsible for driving cancer cell invasion as well as what molecules may inhibit the invasion process. In this report, we describe a methodology to evaluate the potential involvement of hormones, cytokines and/or growth factors present in serum, as molecules driving the chemoattraction and invasion of cancer cells.

In this proposed modified protocol, charcoal stripping is used to remove hormones, cytokines and growth factors from 2% fetal bovine serum (FBS) to generate 2% charcoal stripped FBS (CS-FBS). Both 2% FBS and 2% CS-FBS are used as agents to set up the chemoattractive force

to drive cancer cell invasion. Using 2% CS-FBS as a chemoattractive agent in comparison to normal 2% FBS affords several benefits including first, and most prominently, the ability to study the cancer invasion phenotype in the reduced/relative absence of hormones, cytokines and growth factors. It also enables the investigator to assess whether the collective removal of hormones, growth factors, and cytokines causes an increase or decrease in the invasive index. The assay is then designed to determine whether the addition of an individual component, designated as "X" can inhibit, decrease, increase, or restore the invasive index to its original value (see **Figures 2 & 3**). Although this methodology is specific for achieving the physiological levels of the component that is removed from the serum during charcoal stripping, it should also be noted that charcoal stripping can also affect signal transduction pathways. For example, it has been reported that charcoal stripping can decrease the alkaline phosphatase activity of osteoprogenitor cells and induce adipogenesis through reduction of MAPK activators⁵. Charcoal stripped media are commercially available and are based on the protocol outlined that combines charcoal with dextran and incubated with fetal bovine serum O/N⁶.

This assay was developed in response to a result reported by Zucker *et al.*⁷ The authors demonstrated that a mutant phenotype affecting gap junction communication demonstrated an increase in the invasion index when migrating toward media with normal 2% FBS. This increase was eliminated with the substitution of charcoal-stripped serum. From this result, the authors concluded that this mutant affected migration toward a lipophilic molecule (more specifically, a hormone, growth factor, or cytokine)⁷. It is well known that hormones, growth factors, and cytokines mediate signaling pathways involved in tumor promotion and invasion⁴. Thus, it is important for scientific investigators to determine what factor(s) are driving the tumor invasion of their particular cancer cells or mutants under study. The assay is designed to address the role of individual components at their physiological concentration as determined according to the difference between the concentration of the component "X" in normal FBS and the CS-FBS. Through the development of this assay, investigators can gain more insight into the specific invasive pathway governing their system.

Hormones, growth factors, and cytokines have often been classified as tumor promoters⁸. Some of these factors such as EGF are used as direct sources for chemoattractants in invasion chambers⁹. Thus, it seems likely that these represent the major components of the serum that direct tumor invasion. This protocol proposes a simple, yet significant, modification to the conventional *in vitro* invasion assay which allows an investigator to assess the involvement of hormones, growth factors, and cytokines in mediating the invasive potential of cancer cells. However, the assay is designed to provide the investigator with an answer as to whether the procedure will be effective for their study at an early step in the analysis, so as not to use precious time and resources if deemed unnecessary. This method uses CS-FBS and relies on the investigators discretion as to which molecules to pursue as lead candidates that potentially mediate cancer cell invasion. The results from these analyses should prove to be useful in identifying which serum components serve as chemoattractants or inhibitors for the particular cell line or mutant being studied. In addition, this approach may help the investigator identify key signaling pathways either promoting or inhibiting cancer cell invasion; thus directing future drug design.

Protocol

1. Prepare the Different Media and Additional Components

1. Prior to experiment, prepare media consisting of DMEM or other specified media with the addition of either normal FBS, charcoal stripped FBS, or charcoal stripped FBS plus the component to be tested. Note that multiple components can be tested in each experiment.
2. Weigh out and dilute the hormones, growth factors, or cytokines appropriately to be dissolved in the charcoal stripped serum at the physiological concentration.

2. Prepare the Collagen Matrix on Ice

1. Prepare 2 ml collagen I matrix at 2.2 mg/ml by adding the following sterile filtered components on ice: 200 μ l 10x PBS (pH 7.4), 5.4 μ l 1 N NaOH, 600 μ l of double distilled H₂O, and 1.2 ml collagen I (at 3.63 mg/ml).
2. Keep collagen I solution on ice until ready to plate.

3. Prepare Migration/Invasion Plates for Assay

1. For each cell line to be tested, use one 24-well chamber plate in which 12-wells contain inserts. Use the additional 12 wells that do not contain inserts for adding the chemoattractant media and transferring the inserts for the experimental set up.
NOTE: A collagen matrix on plates with a polyethylene terephthalate (PET) membrane and 8 μ m pore size is optimal for the cell lines use here. However, a matrigel matrix in precoated plates can also be substituted with the pore size decreased according to the cell line being investigated.
2. Clearly label the plate, using 3 wells per condition being analyzed (FBS migration, CS-FBS migration, FBS invasion, and CS-FBS invasion as well as FBS-migration for a control, noninvasive cell line). Assay migration by movement through pores in a PET membrane, and assay invasion by movement through a collagen or matrigel matrix and then through pores in the membrane. Use different color markers for each cell condition to aid in the plating process.

4. Dispense the Invasion Matrix

1. Carefully pipette 75 μ l of the collagen matrix solution into the inserts to be used for invasion assays. Use caution to avoid bubbles. Disperse bubbles by applying an inverted pipette tip to the surface.
2. Transfer the plate with the collagen-coated inserts to a 37 °C and 5% CO₂ incubator for 30 min to enable the gel to solidify.

5. Plate the Cells onto the Membrane or Invasion Matrix

1. Meanwhile, trypsinize cells and add media with 10% FBS. Spin cells at 200 x g for 5 min on a table top centrifuge and rinse 3x in serum free media.
2. Resuspend in serum free media. Count cells with a hemocytometer or automated slide counter. Add serum free media to a final concentration of 5×10^4 cells/ml.
3. When the collagen matrix has solidified (after 30 min), add 700 μ l of media with either 2% defined FBS or charcoal-stripped FBS to each well. Of the 12 inserts per plate:
 - 3 inserts have collagen and wells with media + 2% FBS
 - 3 inserts have collagen and wells with media + 2% CS-FBS
 - 3 inserts have no collagen and wells with media + 2% FBS
 - 3 inserts have no collagen and wells with media + 2% CS-FBS
1. Use additional plates depending on the number of factors being tested and with a no collagen control corresponding to each condition.
4. Add cell suspension to the inserts at 5×10^4 cells/ml, plating 500 μ l cells per insert in all migration and invasion inserts.
5. Incubate the cells for 22 hr at 37 °C.

6. Quantify the Number of Migrating and Invading Cells

1. Set up staining of wells using methanol fixative, eosin, and hemotoxylin, in separate wells.
2. Use cotton swabs to remove cells and matrix from each well. Repeat with second swab application for each well.
3. With forceps, dip each insert 5 times for 1 sec into each of the 3 solutions in succession.
4. Allow inserts to dry O/N.
5. Either i) remove filters with a scalpel, cutting carefully around the edges and mount on a slide with coverslip and immersion oil, or ii) allow the inserts to dry O/N inverted and use the inserts directly for microscopy.
6. The next day, view slides or inserts under a microscope with a 20X objective and take 5 images from different regions of the filter. To improve consistency, take 4 outer fields and one center.
7. Count cells for all conditions using the ImageJ software and apply to the formulas below.
8. Determine the percent invasion as follows:
 Mean # of cells invading through collagen I insert = a
 Mean # of cells migrating through control insert = b
 $\% \text{ Invasion} = (a / b) * 100$
9. Determine the Invasion Index in 2% FBS as follows:
 % invasion of cells being assayed (in 2% FBS) = c
 % invasion of control noninvasive cells in (2% FBS) = d
 $\text{Invasion Index (FBS)} = (c / d)$
10. Determine the Invasion Index in 2% CS-FBS as follows:
 % invasion of cells being assayed (in 2% CS-FBS) = e
 % invasion of control noninvasive cells in (2% CS-FBS) = f
 $\text{Invasion Index (CS-FBS)} = (e / f)$

7. Repeat Experimental Protocol Comparing Charcoal-stripped FBS to Charcoal-stripped FBS + X_n with Multiple Factors Combined

1. Repeat the procedure multiple times as needed using different components for "X" or a combination of components.
2. Apply the calculations to determine the contribution of each factor "X" to the migration and invasion effects.

Representative Results

The invasion index is calculated for each condition according to normalization to a noninvasive cell line. For our experiments, we use the 1205Lu melanoma cell line and established variant stable cell lines as our invasive lines as well as the premalignant noninvasive variant, WM793 from which the 1205Lu cells were derived¹⁰ which serves as a logical control. We also utilize collagen I as the invasion matrix because that is the primary component of the dermis. This is in accordance with a previous study whereby the optimal invasion matrix varies based on the cell line and the extent of concordance with *in vivo* results¹¹. This invasion assay is outlined schematically according to the possible results the investigator might obtain. Initially, the invasion index for 2% FBS should be significantly higher or lower than the invasion index for CS-FBS in order to pursue this assay (**Figures 1 & 2**). If a significant increase or decrease in the invasion index is apparent with charcoal-stripped FBS, this assay is not useful for the investigator (**Figures 2 & 3**). If this increase is eliminated with charcoal-stripped FBS, the investigator already has the knowledge that the enhanced invasion is directed toward a hormone, growth factor, or cytokine (**Figures 2 & 3**). Then, the investigator must utilize information about the specific tumor type and mutation to determine which candidate(s) present plausible mechanisms as chemoattractants. The investigator may begin by trying one or several components individually at the physiological concentration by adding the component at the concentration difference between 2% FBS and 2% CS-FBS (**Table 1**). If a component added to charcoal-stripped serum significantly increases or decreases the invasion potential as compared to the charcoal-stripped serum alone, that candidate, "X", may serve as a partial or complete rescuer depending on the extent of increase (**Figures 2 & 3**). Two or more components can be combined to achieve an additive or synergistic increase, (denoted as $X_1 + X_2 + X_3 \dots$) (**Figure 3**). A factor which decreases the extent of invasion would be classified as an inhibitory factor (**Figures 2 - 4**). In our experiments, we showed that the use of a mutant melanoma cell line (1205Lu T154A) migrating towards a chemoattractant with its hormones, growth factors, and cytokines removed caused a decrease in tumor invasiveness. Three hormones

were tested to identify a possible "X". These include estrogen (estradiol), progesterone, and thyroid hormone (T4). Two of these hormones had no effect on the invasion index (progesterone and thyroid hormone), whereas estrogen was identified as an inhibitory factor (**Figure 4**). According to the potential outcomes listed in **Figure 3**, the experimental results in this example demonstrate a decrease with Condition 2 and an inhibition with Condition 3. It is likely that estrogen will be used further in this assay, since it may be important in the identification of pathways that sequester certain signaling factors for future drug design strategies. Additional experiments will be directed at growth factors and cytokines to identify other components that may serve as "phenotype rescue" pro-invasion chemoattractants. Identification of both pro-invasive and inhibitory molecules may collectively elucidate the invasive potential for the cell lines and mutant variants under investigation.

EXPERIMENTAL SETUP

Figure 1. Experimental setup and design. Model of the set up for the experimental process showing cells plated either onto the PET membrane directly or the collagen layer in serum free media. The cells are either migrating or invading through the collagen matrix toward a chemoattractant which is modified according to the protocol and described as Condition 1, 2, and 3. The migrated or invaded cells are then fixed and stained. [Please click here to view a larger version of this figure.](#)

Figure 2. Potential outcomes from one standard invasion assay membrane result. This model demonstrates the potential results that the investigator may encounter for the invasion assay and how to interpret these results. Each circle is a representative result for a stained filter using the same 3 conditions that are outlined in **Figure 1**. Each dot represents a cell that has passed through the invasion matrix and stained on the bottom of the membrane. The cells would be appropriately quantified and analyzed accordingly. [Please click here to view a larger version of this figure.](#)

PHENOTYPE EVALUATION

Compare the Ratio of:
$$\frac{\text{Invasion Index (CS-FBS)}}{\text{Invasion Index (FBS)}}$$

Possible outcomes:

If the Ratio of the Invasion Indexes of:

$(\text{CS-FBS}) / (\text{FBS}) = 1 (\pm \text{s.d.})$, *Do not pursue assay*

$(\text{CS-FBS}) / (\text{FBS}) < 1 (\pm \text{s.d.})$ = *Decrease*

$(\text{CS-FBS}) / (\text{FBS}) > 1 (\pm \text{s.d.})$ = *Increase*

For individual hormones, growth factors, cytokines, X

If the Ratio of the Invasion Indexes of:

$(\text{CS-FBS} + \text{X}) / (\text{FBS}) = 1 (\pm \text{s.d.})$ = *Restore*

$(\text{CS-FBS} + \text{X}) / (\text{FBS}) > (\text{CS-FBS}) / (\text{FBS}) < 1 (\pm \text{s.d.})$ = *Decrease*

$(\text{CS-FBS} + \text{X}) / (\text{FBS}) < (\text{CS-FBS}) / (\text{FBS}) > 1 (\pm \text{s.d.})$ = *Increase*

$(\text{CS-FBS} + \text{X}) / (\text{FBS}) = (\text{CS-FBS}) / (\text{FBS})$ = *No effect*

$(\text{CS-FBS} + \text{X}) / (\text{FBS}) < (\text{CS-FBS}) / (\text{FBS})$ = *Inhibition*

Repeat with different "X"

For multiple hormones, growth factors, cytokines, $X_1 + X_2 + X_3 \dots$

If the Ratio of the Invasion Indexes of:

$(\text{CS-FBS} + X_1 + X_2 + X_3 \dots) / (\text{FBS}) = 1 (\pm \text{s.d.})$ = *Restore*

Figure 3. Data interpretation for invasion with CS-FBS $\pm$ X. This outline describes how the calculations of the Invasion Index determine the experimental conclusions. CS-FBS is the charcoal-stripped serum and X refers to the individual components added to the CS-FBS media described in the text. The authors set the standard deviation (s.d.) as the accepted variability due to experimental error in our conditions. However, depending on the conditions under observation and the number of replicates, this factor can be adjusted to be within the range of statistical significance for the investigator. The experiment can be repeated with multiple "X" molecules, (denoted as $X_1 + X_2 + X_3 \dots$). [Please click here to view a larger version of this figure.](#)

Invasion Index for 1205Lu T154A Cells

Figure 4. Invasion assay results. This graph depicts the experimental results obtained when using a mutant variant cell line³ established from 1205Lu metastatic melanoma cells. The concentrations of the added hormones are as follows: estrogen (0.566 pg/ml), progesterone (0.001 ng/ml), thyroid hormone (0.283 µg/dl). The control, noninvasive cell line used for these experiments was WM793B. This experiment was repeated 3 times with similar results, and a representative graph is shown.

		Units	Defined FBS	CS FBS	Difference	[2% FBS]- [2% CS-FBS]	Source	Catalog Number
Hormones	Estrogen (Estradiol)	pg/ml	28.30	8.76	19.540	0.566	Life Technologies FBS cat#16000 c.s FBS cat#12676	Sigma E2257
	Insulin	uIU/ml	8.61	undetected	8.610	0.172		Sigma I3536
	Progesterone	ng/ml	0.05	undetected	0.050	0.001		Sigma P8783
	Testosterone	ng/ml	0.10	undetected	0.100	0.002		Sigma T1500
	Thyroid Hormone (T4)	ug/dl	14.14	undetected	14.140	0.283		Sigma T1775
Growth Factors	IGF	ng/ml	111.00	49.30	61.70	1.23	Thermo Scientific Hyclone FBS cat#SH30070 c.s FBS cat# SH30068	Sigma I3769
	TGF-β	ng/ml	12.60	7.30	5.30	0.11		Sigma SRP3170
	FGF-2	pg/ml	37.30	32.70	4.60	0.09		Sigma SRP4037
		Units	Human Sera				Source	Catalog Number
Cytokines	G-CSF	pg/ml	14.7 ± 13.2	N/A			PMID: 21774806	Sigma SRP3331
	GM-CSF	pg/ml	40.9 ± 108.6					Sigma SRP3201
	MCP 1	pg/ml	213.5 ± 100.7					Sigma SRP3109
	TGF-α	pg/ml	3.2 ± 4.0					Sigma T7924

Table 1. Examples of concentration ranges of select hormones, growth factors and cytokines in bovine and human sera. The concentration difference between 2% FBS and 2% CS-FBS represents the amount added to achieve physiological concentrations.

Discussion

Tumor metastasis is a multistep process. The cells must break through the basement membrane, intravasate into either the lymphatic system or blood microvessels, in which they are transported to distant sites. The tumor cells then extravasate and colonize into a macrometastasis¹². Progression through the epithelial to mesenchymal transition (EMT), tumor invasion, and metastasis has been enhanced by steroid hormones^{13,14}, growth factors¹⁵⁻¹⁸, and cytokines¹⁹⁻²¹. These molecules are so critical to addressing the signaling pathway of tumor progression, that the development of an assay to address their role in tumor invasive potential is an important step toward deciphering the intricate signaling pathways.

This assay of using charcoal-stripped serum has an advantage over existing methods because it aims to identify the driving force to which the tumor cells are migrating. A previous assay has been published using normal primary breast fibroblasts in invasion chambers. In this study, the authors used charcoal-stripped media and added back estrogen in matrigel chambers which did not cause invasion as expected since the cells were not tumorigenic. However, the presence of macrophage-conditioned media in the presence or absence of estrogen increased invasion significantly and to a similar extent²². While this assay was similar that it used invasion assays with charcoal-stripped serum in the absence or presence of estrogen, it was not a tumor assay and was directed at the study of paracrine interactions between macrophages and fibroblasts²². Another assay used charcoal-stripped media to show that the addition of low levels of estrogen increased the proliferation of breast cancer cells but caused resistance to kinase inhibitors²³. Our study is unique in that it is an invasion assay applicable to various types of cancer cells designed to study the effect of individual hormones, growth factors, and cytokines either alone or in combination. Furthermore, subject to the available literature, we have determined the concentrations of hormone necessary to add to the charcoal-stripped serum to achieve physiologically relevant levels (Table 1). The addition of the individual component, "X" at levels normally present in serum is a critical parameter of the assay. While this assay may seem to be limited by the published levels of hormones, growth factors, and cytokines in serum, the investigator can use ELISA assays to determine the levels of additional factors.

While the protocol is specific for using collagen I, matrigel could also be substituted if the investigator was consistently using that matrix. The reason we prefer to use collagen I is because it is a single component which is defined and is the primary constituent of the dermis. In addition, we use 75 µl of 100 µg/ml collagen to make the invasion matrix. This can also be modified to achieve the desired results. It is best to optimize the concentration so that a reasonable number of cells can be counted on the filters. If the cells are highly invasive, it may be better to use 100 µl of collagen I for the matrix or to increase the collagen concentration. While this technique may help the investigator obtain important information, it is only useful if the tumors cells are invading towards a chemoattractant which is removed by the charcoal-stripping of the serum. If there is no difference between the results for control and charcoal-stripped serum, then the assay would not be worth pursuing. The assay also addresses the results for individual chemoattractants as well as suppressors of chemotaxis. Furthermore, in the comparison of possible results (Figure 3), we used the standard deviation as the level of accepted experimental error for each experiment. However, the actual values can be determined by the investigator based on the standard deviation or standard error and a significant *p* value.

Furthermore, the information gleaned from this assay could prove useful in the design of pharmacological inhibitors to certain signaling pathways. For example, if one or more hormones, growth factors, and/or cytokines is found to be implicated in the invasion mechanism for a particular tumor cell mutation, than an existing pharmacological agent can be used or a new drug designed to inhibit that pathway. The assay is also an effective tool for studying the particular tumor mutation and whether altering the amino acid residue or its position maintains the same affinity to the chemoattractant that was identified by this assay. In this way, our assay offers a novel approach to provide clues as to the invasion mechanism of cancer cells and possibly methods to prevent tumor invasion.

Disclosures

The authors have nothing to disclose.

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