Video Article

A Novel *In Vitro* Wound Healing Assay to Evaluate Cell Migration

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Abstract

The aim of this work is to show a novel method to evaluate the ability of some immunomodulatory molecules, such as antimicrobial peptides (AMPs), to stimulate cell migration. Importantly, cell migration is a rate-limiting event during the wound-healing process to re-establish the integrity and normal function of tissue layers after injury. The advantage of this method over the classical assay, which is based on a manually made scratch in a cell monolayer, is the usage of special silicone culture inserts providing two compartments to create a cell-free pseudo-wound field with a well-defined width (500 µm). In addition, due to an automated image analysis platform, it is possible to rapidly obtain quantitative data on the speed of wound closure and cell migration. More precisely, the effect of two frog-skin AMPs on the migration of bronchial epithelial cells will be shown. Furthermore, pretreatment of these cells with specific inhibitors will provide information on the molecular mechanisms underlying such events.

Video Link

The video component of this article can be found at https://www.jove.com/video/56825/

Introduction

It is largely known that wound healing in animals is a fundamental process to re-establish the integrity and normal function of tissue layers after injury[1]. Despite epithelial surfaces exposed to the external environment (*e.g.,* the skin, respiratory, and gastrointestinal tracts) form a protective barrier from physical and chemical insults, the formation of wounds can easily occur, especially after surgery or microbial infections[2]. As an example, colonization of lung tissue by the opportunistic bacterial pathogen *Pseudomonas aeruginosa*, especially in cystic fibrosis (CF) sufferers, leads to damage of the airways epithelium with consequent respiratory failure[3,4]. Wound healing is a complex host repair mechanism to restore the normal architecture of an injured tissue[5]. It is characterized by initial inflammation, followed by a regeneration period encompassing epithelialization, angiogenesis, and tissue remodeling with collagen production and cell differentiation[6,7,8]. To ensure epithelial integrity and to control microbial proliferation, all living organisms produce defense molecules, including antimicrobial peptides (AMPs)[9,10]. The wound healing process is very difficult to simulate *in vitro* due to the lack of cell debris and complex interactions among different cell types. However, the *in vitro* ability of a peptide to accelerate the closure of a pseudo-wound by stimulating migration of epithelial cells is indicative of its ability to heal a compromised epithelium. Indeed, cell migration is a rate-limiting event in wound healing, and studying factors that can affect cell migration will help to target therapies for improved wound healing.

Here, a highly reproducible experimental assay is provided based on special silicone culture inserts to evaluate cell migration *in vitro*. It is based on the creation of a 500 µm gap (pseudo-wound) on a confluent cell monolayer. The cells at the edge of the artificial "wounded" field will start migrating into the cell-free area, forming new cell-cell contacts. The culture insert represents a new tool for fast wound healing experiments. Two reservoirs separated by a 500 µm wall are provided, and they can be properly placed into a 3-cm dish plate or in the well of a 12-well plate. Filling each compartment of the insert with a cell suspension allows cells to grow in each designated area until confluence, while removal of the insert will engender a clean cell-free gap of approximately 500 µm (the same width as the separation wall). A proper cell culture medium supplemented with a test compound can then be added into the dish plate/well. Afterwards, the gap closure can be visualized at different time intervals under an inverted microscope, preferably one equipped with a video-camera for image acquisition. Finally, measurement of changes in the cell-covered area by the web-based automated image analysis program will allow the quantification of the speed of wound closure and cell migration. Overall, this method is a step forward with respect to the classical assay, where a scratch is manually made by incising confluent cell monolayers with a sterile needle or a pipette tip[11]. Indeed, the last procedure can destroy the plastic bottom of the dish plate/well and the surface coating, creating wrinkles. In addition, the "wounded" area does not have a well-defined width along the entire length of the gap, as this highly depends on the pressure applied by researchers to the needle/tip. Furthermore, the dislodged cells can form clumps of living and dead cells at the edges of the scratch; moreover, the spreading of living cells into the "wounded" area can interfere with the velocity of cell migration, generating non-reproducible results[12].

In addition, thanks to a scratch image analysis platform, users can rapidly receive (within minutes) quantitative data on the migratory behavior of the selected cells without the necessity of acquiring additional software. This platform is capable of analyzing phase contrast microscopy images of low (~5X), medium (~10X), and high (~20X) magnification. After uploading a zip file of images (in *.jpg, *.jpeg, *.jp2, *.png, *.gif, *.tiff format)

the analysis is automatically conducted to generate a summary file that shows the percentage of both cell-covered areas and scratch areas, as well as the speed of cell migration, at distinct time intervals.

In this work, by using a frog-skin AMP-derivative, *i.e.* Esc(1-21) and its diastereomer Esc(1-21)-1c[13], and a bronchial cell line expressing the functional CF transmembrane conductance regulator (CFTR)[14,15], an example of peptide-induced cell migration in comparison with untreated (control) samples is provided. Note that the airway epithelium and CFTR play a crucial role in maintaining lung function and wound repair[16]. Furthermore, by means of selective inhibitors (*e.g.,* AG1478)[17] of the epidermal growth factor receptor (EGFR), evidence that migration of bronchial cells induced by the aforementioned peptides involves activation of EGFR[12,18] is reported.

In summary, the goal of this procedure is to show how the usage of such silicone culture inserts represents a fast and easily accessible assay to accurately determine migration of adherent cells (*e.g.,* bronchial epithelial cells) and the molecular mechanisms controlling such events.

Protocol

1. Cell Preparation

1. Seed 2.5x10^6 cells in 10 mL of Minimum Essential Medium (MEM) supplemented with 2 mM glutamine (MEMg), plus 10% fetal bovine serum (FBS), antibiotics (0.1 mg/mL of penicillin and streptomycin), and puromycin (0.5 µg/mL for selection and maintenance of the cell line) in a T75 flask. Incubate the flask at 37 °C and 5% CO_2 for 2 days. Before starting the experiment, check the confluence of cells under an inverted microscope.
   NOTE: The cells used for the experiment are immortalized human bronchial epithelial cells transduced with a lentiviral vector conferring resistance to puromycin. They stably express a functional CFTR[14,15].
2. Once the cells' confluence has reached 90-100%, aspirate the medium from the flask and discard it into a waste bottle under a biological safety cabinet class II. Wash the cells with 6 mL of phosphate buffered saline without calcium and magnesium (CMF-PBS). Gently rock the flask manually and discard CMF-PBS.
   NOTE: Be careful not to touch the cell monolayer with the pipette.
3. Add 10 mL of CMF-PBS and incubate the flask at 37 °C and 5% CO_2 for 10 min.
4. Aspirate CMF-PBS and discard it. Then add 2 mL of trypsin/EDTA to the flask.
5. Gently rock the flask, allowing the solution to completely coat the cells, and incubate the flask at 37 °C and 5% CO_2 for 10 min until the cells are visibly detached under a microscope.
   NOTE: At the end of the incubation time, the cells should appear rounded and not attached to the plastic surface. If the cells are not well detached, manual agitation may be necessary.
6. Add 10 mL of MEMg plus 10% FBS to inactivate trypsin and collect the cells by washing the bottom of the flask. Transfer the volume into a conical 50 mL tube.
7. Centrifuge the tube for 5 min at 80 x *g*.
8. Aspirate the supernatant and re-suspend the cells in 6 mL of MEMg plus 10% FBS. Pipette repeatedly to break up any clumps.
9. Take out 10 µL of cell suspension with a micropipette and inject the volume under the cover glass previously put over a Burker or Neubauer chamber.
10. Count the cell number.

2. Cell Seeding in the Culture Inserts

1. In each well of a 12-well plate, transfer the culture insert with sterile tweezers (**Figure 1**). Use tweezers to press along the inserts edges in order to fix them to the surface of the plate.
   NOTE: Inserts have a sticky underside that allows adhesion.

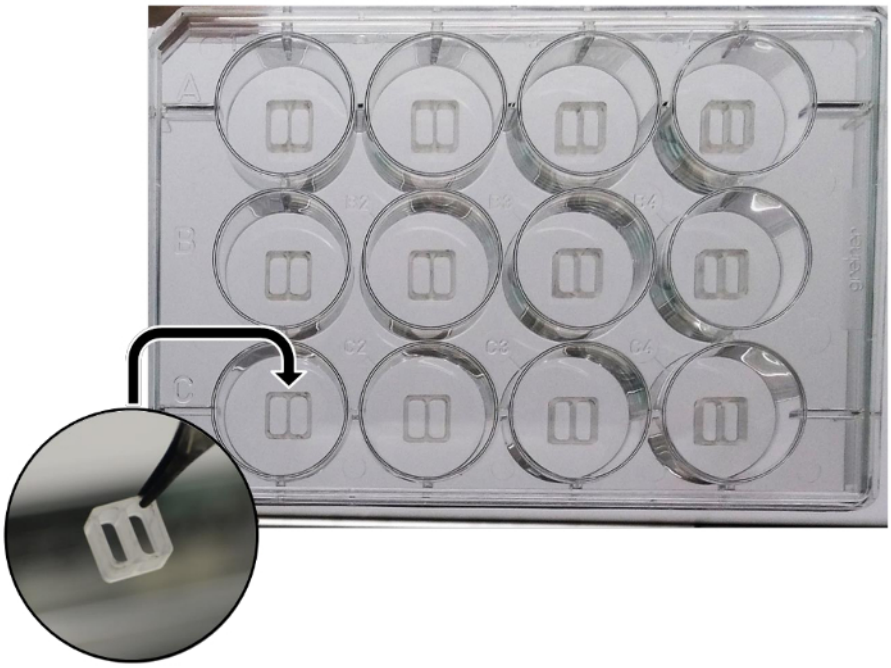

Figure 1: Schematic representation of the silicone culture inserts, properly put into wells of a 12-well plate. Please click here to view a larger version of this figure.

2. Properly dilute the cell suspension in MEMg plus 10% FBS. Fill each compartment of the insert with 70 µL of cell suspension (about $3.5x10^4$ cells/chamber).
   NOTE: The density of cells applied in each compartment depends on the type of cells. It is recommended to use a cell density that leads to complete confluence within 24 h.
3. Under the microscope, check that cells are not leaking from the insert compartments and incubate the 12-well plate for 24 h at 37 °C and 5% CO_2.

3. Pseudo-Wound Healing Assay

1. After incubation, visualize the cells under the inverted microscope to verify that confluent cell monolayers have been formed.
2. Re-suspend the test compounds (*e.g.,* AMPs) in 1 mL of MEMg.
   NOTE: Prepare fresh AMPs dilutions starting from the stock solution stored at -20 °C.
3. Gently remove the inserts by sterile tweezers. Be careful not to break the cell monolayers. Transfer the inserts onto absorbent paper.
   NOTE: To re-use the same inserts, sterilize them in 70% ethanol for at least 3 h. It is recommended to throw them away afterwards.
4. To remove non-adherent cells, add 1 mL of MEMg per well using a micropipette. Close the plate and gently rock it.
   NOTE: Do not add medium directly on top of cell monolayers to avoid their disruption.
5. Aspirate the medium and replace it with 1 mL of MEMg per well. Close the plate and visualize the cell-free gaps (created by the inserts) under the inverted microscope at 4X magnification, equipped with a video-camera. Acquire images at time zero (T0) and save them in a .jpg format.
6. Remove the medium from the wells, wash them with 1 mL of PBS, and discard it.
7. Add the test compounds (prepared at point 3.2) to the wells. For untreated control samples, add 1 mL of MEMg. Incubate the plate at 37 °C and 5% CO_2.
8. After 15, 20, and 24 h treatment, observe the cell migration under the microscope at 4X magnification and acquire images.
   NOTE: During this step, try to capture images in the same areas as for T0. The choice of time intervals at which images are captured depends on the cell migration speed.
9. To study the effect of some selective inhibitors, *i.e.* AG1478, on cell migration, aspirate the medium from each insert compartment before removing inserts.
   1. Wash each compartment with fresh MEMg and fill it with 70 µL of MEMg supplemented with AG1478.
   2. After 30 min of incubation at 37 °C and 5% CO_2, proceed as described from point 3.3.
      NOTE: During the washing step and pretreatment of cell monolayers with the specific inhibitors, be careful not to remove the inserts.

4. Image Analysis

1. Upon completion of the experiment, select images of the most representative samples of the various experimental groups, and create a zip file containing single images at T0, T15, T20, and T24 h.
   NOTE: Single images are taken at the selected time intervals for all samples. Run triplicates for each experiment, which is repeated at least three times. At the end, for all experimental groups, a minimum of 3 images ("a", "b", "c", *etc.*, deriving from each independent experiment) at each time point are analyzed.

2. Upload the zip file into the image analysis software which automatically provides by e-mail a spreadsheet summary file containing the experimental data of cell-covered and scratch areas (as percentage) at the selected time points.
   NOTE: The recognition of the leading edge and the gap area is largely based on edge detection method (aimed at identifying points at which the image brightness sharply changes).
3. Save the data and collect them. Normalize all data with respect to the mean value at time zero. Calculate the average value of the normalized data of all replicates at each time point and the relative standard error (SEM). By using two-way analysis of variance (ANOVA), perform statistical analysis with a proper statistics software. Differences between peptide-treated groups and control groups at different time intervals are considered to be statistically significant for a $p<0.05$.
4. Plot the obtained data into a graph as a histogram, which shows the percentage of cell-covered area of all sample groups *versus* the selected time intervals.

Representative Results

This protocol was used to determine the wound healing effect of Esc(1-21) and Esc(1-21)-1c in terms of cell migration activity induced on bronchial epithelial cells expressing the functional CFTR. In this assay, culture inserts were placed in wells of a 12-well plate, and each compartment was seeded with 35,000 cells in MEMg supplemented with 10% FBS. The cells reached complete confluence within 24 h. Afterwards, a 500 µm gap was generated, and each well was filled with MEMg containing the peptide at different concentrations. The experiment was repeated four times. As indicated in **Figure 2**, the almost complete closure of the pseudo-wound field produced in the cell monolayer was induced by the two peptides within ~20 h, at optimal concentrations of 1 µM and 10 µM for Esc(1-21)-1c and Esc(1-21), respectively. This indicates that Esc(1-21)-1c is more efficient in promoting re-epithelialization of a pseudo-wound in bronchial cells.

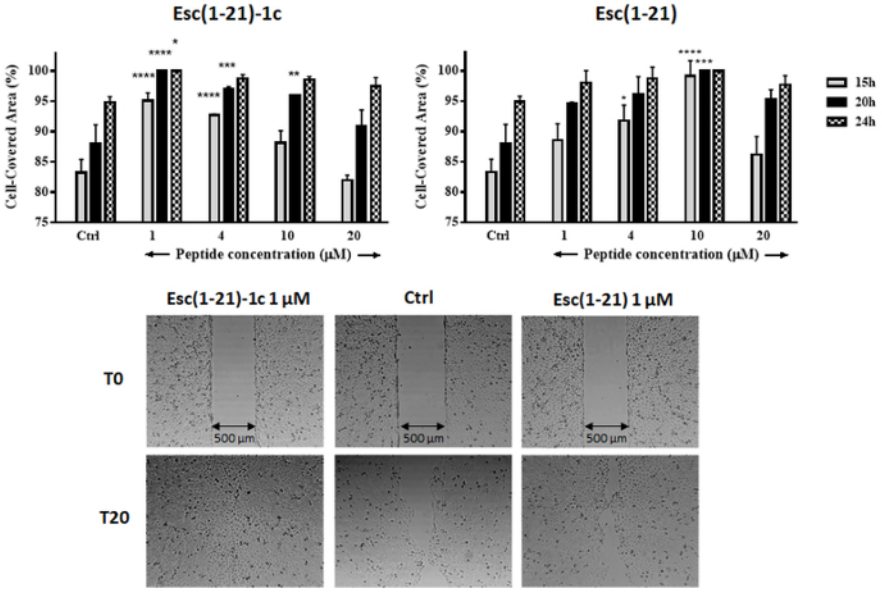

Figure 2: Effect of esculentin-1a derived AMPs on the closure of a pseudo-wound created in a monolayer of bronchial epithelial cells. Cells were seeded in each compartment of the silicone culture insert and, after its removal, cells were photographed for evidence of cell migration 15, 20, and 24 h after the addition of peptides. The percentage of cell-covered area at each time point was calculated compared to the untreated control cells (Ctrl) and is reported on the y axis. All data are the means from four independent experiments ± SEM, and are taken from a previous study[14]. The levels of statistical significance for tests between control and treated samples are: *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$. Micrographs showing representative results before (T0) and 20 h after treatment of cells with each peptide at the concentration of 1 µM, in comparison to the control, are also reported. Please click here to view a larger version of this figure.

To verify whether activation of EGFR played a role in the Esc-induced wound closure, bronchial cells were preincubated for 30 min with 5 µM of AG1478, a selective inhibitor of EGFR, before insert removal. Since the percentage of cell-covered area was significantly reduced at all time intervals compared to the results found when the cells were not pretreated with the inhibitor prior peptide administration (**Figure 3**), this points out that peptide-induced cell migration involves activation of EGFR. However, further studies will be needed to clarify the molecular events triggered by the Esc-peptides (*e.g.,* direct EGFR activation or trans-activation mediated by metalloproteases, which have been shown to cleave membrane-anchored EGFR ligands[19]).

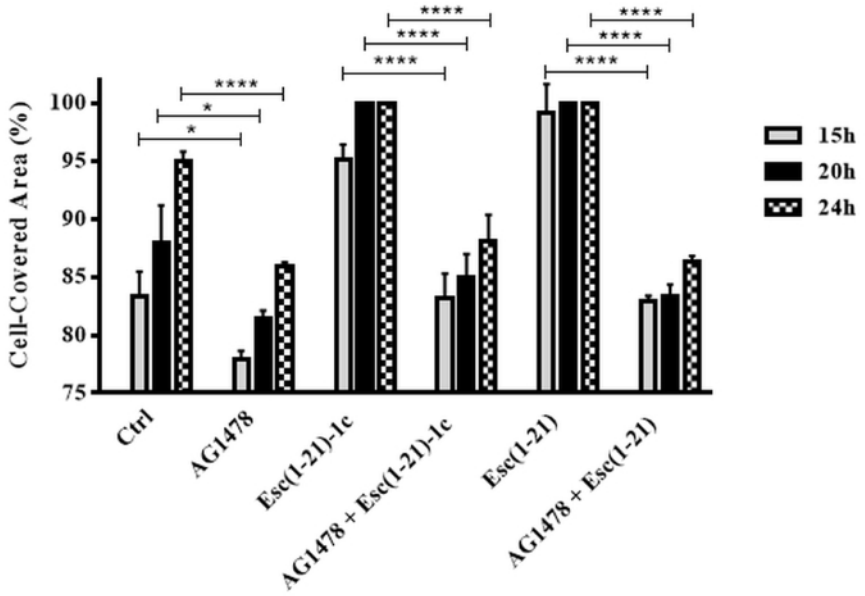

Figure 3: Effect of AG1478 inhibitor on the peptide-induced migration of bronchial epithelial cells. Before insert removal, cells were pre-incubated with 5 µM AG1478 for 30 min and subsequently treated with the peptide alone at the same concentration. All data are the means from four independent experiments ± SEM and are taken from our previous work[14]. The levels of statistical significance for tests between the indicated group samples are: *p<0.05; ****p<0.0001. Please click here to view a larger version of this figure.

Discussion

Cell migration is an essential process in many physiological and pathological events including wound healing, embryonic development, and cancer metastasis. The basic procedure to study cell migration *in vitro* involves: (i) the creation of a cell monolayer, (ii) the production of a pseudo-wound in the confluent layer of cells, (iii) the capture of images at different time intervals until wound closure is reached, and (iv) the analysis of the image sequence in order to quantify the migration speed of the chosen cells.

Our results have shown that the application of culture inserts is an objective and reliable experimental technique for quantitative evaluation of cell migration characteristics. This assay can be performed by any research group with basic laboratory equipment. However, some critical steps of the protocol should be considered to achieve reproducibility. For example, it is important to define, by pilot experiments, the exact number of cells to seed in each compartment in order to get the same extent of confluence in all individual samples. To this aim, having cells with a synchronized cycle phase and avoiding cell clumps before seeding is critical. A possible way to minimize clumping is a gentle passage of cell suspension through a syringe needle. However, increasing the samples size will significantly contribute to decrease variability among the cells.

In addition, the creation of a gap with a well-defined width in all samples is crucial. For this purpose, silicone inserts should not be re-used more than twice. Furthermore, the growth of cells under the silicone separation wall should be avoided. To prevent this trouble, soft pressure on top of the insert, once placed into the dish plate/well, can be manually applied by means of sterile tweezers to increase the adhesion of the insert to the plastic. Cautious removal of the insert will also hamper enlargement of the cell-free surface with disruption of the cell monolayer.

However, once the insert is correctly dislodged and a pseudo-wound is perfectly produced, some cell lines can detach from the plastic surface, especially if they are incubated in media without serum or supplements such as vitamins, amino acids, and growth factors. The absence of these factors in a cell culture medium can be dictated by the fact that they can interfere with the wound-healing promoting activity of test compounds. To circumvent this problem, rational choice of culture medium composition is highly recommended.

Another critical step to consider before setting up this wound healing procedure is the different migration speed that exists among different cell types. Also in this case, preliminary experiments need to be scheduled with the purpose of determining the correct time intervals at which images have to be captured. It is also necessary to consider that one limitation associated with this method is that such culture inserts cannot be employed for studying the migration of cells which grow in suspension (*e.g.,* polymorphonuclear leukocytes).

Despite wound-healing assays performed with the silicone culture inserts are more expensive than the classical scratch assay, they allow a quick and accurate reproduction of data on cell migration activity. Importantly, once the system is set-up, it can be used to expand knowledge on the molecular mechanism underlying the wound healing activity stimulated by either endogenous or exogenous factors. To this end, pretreatment of cell monolayers with specific molecules (*e.g.,* inhibitors) can be carried out before creating the pseudo-wound. An example is given by pretreatment of cells with (i) the cell proliferation blocker mitomycin C to explore whether the closure of the "wounded" field induced by AMPs is influenced by cell division rate[20] or (ii) metalloprotease inhibitors to verify the contribution of metalloproteases in the activation of EGFR-mediated signaling pathway[12].

Importantly, during the wound closure process, samples may be fixed and treated with appropriate stains for cytoskeleton and nuclei detection (phalloidin and DAPI, respectively) to visualize changes in the morphological aspect of cells (*e.g.,* the formation of lamellipodia at the front edge) by fluorescent/confocal microscopy. Moreover, treatment of cells with a fluorescent-labeled AMP may enable the visualization of its cellular

distribution (membrane surface or intracellular/intranuclear localization) at different times, since the beginning of the cell migratory activity. Furthermore, these inserts may be exploited for analyzing wound healing activity on polarized and differentiated epithelial cells (*e.g.*, primary bronchial cells maintained at the air-liquid interface), after having placed them on suitable transwell permeable supports.

In conclusion, the described method has substantial potential to significantly improve the understanding on the migratory features of different types of adherent animal cells/epithelial tissues, as well as on the selection of the most promising wound-healing promoters and/or inhibitors, within a short time and with high accuracy.

Disclosures

The authors have nothing to disclose

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