Innovative method for quantification of cell-cell adhesion in 96-well plates

Abraham Zepeda- Moreno, 1, 4 Isabel Taubert, 1 Isabelle Hellwig, 1 Van Hoang, 1 Larissa Pietsch, 1 Vinoth Kumar Lakshmanan, 3 Wolfgang Wagner 1, 3 and Anthony D. Ho 1

1Department of Medicine V; Heidelberg University Hospital; Heidelberg, Germany; 2 Helmholtz Institute for Biomedical Engineering-Cell Biology; RWTH Aachen Medical School; Aachen, Germany; 3 Centre for Nanosciences & Molecular Medicine; Amrita Institute of Medical Sciences and Research Centre; Amrita Vishwa Vidyapeetham University; Kochi, India

Key words: adhesion assay, adhesion, SDF-1, CD44, hematopoietic stem cells, leukemia cell lines

Abbreviations: Ca, calcium; CCK-8, cell counting kit-8; CD, cluster of differentiation; CXCR4, chemokine (C-X-C motif) receptor 4; FBS, fetal bovine serum; FCS, fetal calf serum; hMSC, human mesenchymal stem cells; HSC(s), hematopoietic stem cell(s); Mg, magnesium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline solution; SDF-1, stromal derived factor-1; XTT, sodium-3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)

Cell adhesion is an important part of many complex biological processes. It plays crucial roles in cancer, development and maintenance of stem cell compartment. The measurement of adhesion under experimental conditions might provide important information for cell biology. There are several protocols to measure adhesion, usually based on washing or shaking to remove non-adherent cells. Here, we describe a quantification method based on gravitational force to measure adhesion in a 96-well format. Non-adherent cells are separated and only vital cells are quantified with a colorimetric assay. This assay can be used especially when the “anti-adhesion” effect is present only for a short period of time like is the case of peptides or cytokines since it provides a trap for non-adherent cells in a way that they can not touch again the adherent surface. As examples we provide the quantification of cell-cell interaction with blocking antibodies anti-CD44 in hematopoietic stem cells and the effect of the stromal cell derived factor-1 (SDF-1) in the Jurkat cell line when they are in contact with mesenchymal stromal cells. This method facilitates fast and reliable measurement of cell adhesion in multiwell format for screening assays.

Introduction

The binding of cells to extracellular matrix, other cells or to any other surfaces is called “cell adhesion.” Cell adhesion is a key factor for many processes that include the three dimensional organization of all tissues, embryonic development, inflammation, cellular migration, cell-cell communication, differentiation, cancer and metastasis, as well as many others. This phenomenon is mediated principally by adhesion molecules such as cadherins, integrins, selectins, immunoglobulins, glycoproteins and proteoglycans. 1,3 Interventions in adhesion or its regulatory circuits are of high relevance especially in cancer and for other phenomena like mobilization of normal or cancer hematopoietic stem cells (HSCs). To test possible interventions, several measuring adhesion protocols have been developed, protocols that are mainly based on cell interaction with coated surfaces, cell-to-cell interaction, using washing and shaking steps or centrifugation for separation, which make them technically difficult to execute.

Wagner et al. developed an adhesion assay that is relatively easy to perform and that permits separation and quantification of adherent and non-adherent cells. This method demonstrated the importance of the adhesion molecule such as CD44, 4 and N-cadherin 3 for in the interaction between hematopoietic progenitor cells (HPC) and human mesenchymal stem cells (hMSC). Furthermore, cell-cell interaction can be modified by the stromal derived factor 1/CXCR4 axis. 5 This adhesion assay is based on the gravitational force. Initially, hMSC are plated at the bottom of a chamber and grow until they form a confluent layer, then cells to be tested for adhesion are placed into the chamber to let them interact with the hMSC. The chamber is closed and turned upside down to let non-adherent cells falling down. Pictures from the feeder layer should be taken before and after the inversion so the adherent and non-adherent cells can be quantified. This method has the advantage that it is relatively easy to perform and the quantification is reliable. Nevertheless, counting adherent and non-adherent cells is time-consuming and hampers the application of this assay for large-scale screening. Furthermore it is difficult to consider the cell viability. Therefore, we have developed a new method performed in a 96-well plate format adding the quantification of the non-adherent viable cells with a colorimetric reaction. This new adhesion technique has several...
advantages in comparison with its predecessor: (1) reduction of the time period during the “test” and the “processing” of the results; (2) the experiments can be done in a larger scale; (3) several conditions can be tested at the same time; (4) quantification of viable cells and (5) it is possible to separate the cells which were non-adherent and use them for further examination.

**Principle of the Assay**

This assay is designed to quantify living cells that lose adhesion (due to gravitational force) from an adherent cell feeder layer; therefore, the applications of it will be as broad as researchers need and design. All kind of adherent cells can be used for this purpose. Secondly, cell lines or primary cells to be tested are used to measure adhesion. Additional advantages of this method are that cells should not be labeled, transfected or treated in order to be counted and the experiments can be done in a multi-well format. As it is explained in Figure 1, this assay works based on gravitational force, collection and quantification of non-adherent cells. In this way, we avoid washing or shaking steps that can interfere with reproducibility since they might be applied differently between experiments. For experiments in which the agent to be tested has a very short acting period, for example peptides or components that are degraded quickly or the effect is fast, this assay provides a trap for non-adherent cells in which they can not touch again the adherent cell feeder layer (Fig. 1F and G). In other words, in many other adhesion assays, cells to be quantified are always in contact with the adherent surface until they are separated, during this frame time the anti-adhesion effect of some components might be away, that is the case of short acting peptides like hormones and cytokines. In our assay, once the anti-adhesion effect was present, it is quantified.

Another advantage of our assay is the low cost. Additionally, adhesion assays that use fluorescently labeled cells are not considering cell viability or the fluorescence produced by stained death cells or cell debris that are still attached to the adherent surface, cell debris that can be produced by cell handling during staining or even precipitation of the staining material. We skipped these possible essenaries using a colorimetric reaction that is done only by viable cells as is the case for the agents CCK-8 or MTT for example.

The sensitivity of the assay will vary between the different conditions and designs, cell types and the quantification method that are used. Mostly, the sensitivity is depending on the end quantification reagent that is used.

**Examples**

In this section we show some examples of experimental set up and results involving the SDF-1/CXCR4 axis and CD44. As cell feeder layer we used hMSCs. To measure adhesion we used primary HSCs and the Jurkat cell line. We should mention that even if here we only present cells derived from the hematopoietic system, these cells are different and have diverse adhesion properties between them. With this we justify the principle of using an adherent either primary or a cell line as a feeder layer or even a coated surface and another cell type to measure the adhesion. Conditions should be tested by each researcher according to the necessities using the preferred configuration, cell lines or primary cells. The examples presented here were performed and compared with results obtained using similar conditions from the method described by Wagner et al.

**SDF-1 and CXCR4: The Adhesion Pattern of Jurkat Cells to hMSCs**

SDF-1 is an important cytokine secreted by hMSC in bone marrow. The receptor for this cytokine is CXCR4, a G-protein coupled receptor that has been shown to induce cell chemotraction and movement after its activation with SDF-1. As an example of the interaction between hMSC with a leukemia cell line (Jurkat) we performed adhesion assays following the protocol described below (Fig. 2A).

Inducing cell movement and migration is probably one of the most important documented effects of SDF-1. Reduction of the adhesion induced by this cytokine was also found by Faber et al. using HSCs. This effect is as well present in our model in concordance to this. Additionally this experiment demonstrated that this assay is suitable for measuring the effector consequences of chemokines on cell adhesion.
CD44

The cellular microenvironment of the stem cell niche regulates maintenance of HSCs.\textsuperscript{11} This stem cell niche interaction of HSC is, among others, mediated by the multifunctional ubiquitously expressed transmembrane glycoprotein CD44. Using the novel adhesion assay we investigated the effect of A3D8, an anti-CD44 mAb, on the adhesion of HSCs from cord blood to hMSCs (Fig. 2B).

This example also demonstrates the reliability of our assay when compared with published results obtained by Wagner et al. using blocking antibodies for CD44. In both experimental settings it is shown a reduction of around 15% on cell adherence.\textsuperscript{4}

Materials and Methods

Materials.
- Two 96-well cell culture plates for plate A and plate B (Cellstar Cat. No. 655 180 Microplates, Greiner Bio One, Monroe, NC)
- The appropriate cell culture medium depending on the cell type, with/without red phenol for MTT assays or without it for CCK8 and XTT
- CCK-8 (96992, Sigma, Germany), MTT (M2003, Sigma), XTT (X4626, Sigma) or any other colorimetric cell counting component
- Fetal calf serum (FCS) or fetal bovine serum (FBS)

Equipment.
- Cell culture incubator
- Multichannel pipette
- Laminar flow hood
- Centrifuge with 96-well plate rotor

Instructions. Preparing the 96-well plate. Plate A. Prepare the 96-well plate by seeding adherent cells (for example, hMSC from 10 to 15 thousand) and grow them until there is a confluent layer (overnight is a reasonable period of time).

Adhesion assay. Remember that the design and set of the appropriated controls depends on the necessities of the experiment to be done.

(1) Pre-warm new cell culture medium in a water bath at 37°C.

Plate A. (2) Remove the medium of the adherent cells and add new culture medium together with the cell suspension to be tested (for example Jurkat from 75 to 150 thousand cells per well). Consider that it would depend on what you are testing is the moment to add the component that would interfere with the adhesion; for example, antibodies can be added or pre-incubated with the cell suspension before they are added to each well. Make several repetitions of each condition.

NOTE: One important issue here is that you have in each well from 300 to 350 μl of medium as total end volume, if less or more, the medium will leak or you will have air bubbles in your assay when you turn the plate up-side down.

(3) Incubate the plate at 37°C, 5% CO\textsubscript{2} for at least 1 h in order to let the cells (e.g., Jurkat) seed and make contact with the adherent cells (e.g., hMSC).

NOTE: The cell suspension to be added for quantifying adherence can be prepared depending on your requirements. For example, you can add the cells and the components to be tested in the very same cell suspension or add it later. If you add the component to be tested after the incubation (e.g., SDF-1), do it carefully and incubate the plate for 10 min at 37°C, 5% CO\textsubscript{2}.
reservoirs with different numbers of cells and at least one repetition should be prepared in an end volume of 100 μL.

(7) Centrifuge the plate at 1,500 rpm for at least 5 min.

(8) With the multichannel pipette or with a one channel pipette, discard supernatant till you get a final volume of 100 μL medium in each well [for example if you have 300 μL of total volume you can take 180 μL of supernatant away (20 μL to offset evaporation and losses during the process)]. Be careful to avoid touching the bottom of the 96-well plate since the cells to be quantified are there.

(9) If you would like to use MTT follow the next steps:

(a) Add 10 μL of FCS to each reservoir if you are using serum free media (this is very important).

(b) Add 20 μL of MTT (MTT solution concentration of 5 mg/mL).

(c) Incubate for 3 h.

(d) Centrifuge at 1500 rpm for at least 5 min.

(e) Take all the supernatant with the multichannel pipette.

(f) Add 150 μL of acid isopropanol.

(g) Shake the Plate B for at least 20 min.

(h) Read at 590 nm with a reference of 650 nm.

(10) If you would like to use CCK-8 or XTT follow the recommended protocol of each provider. Nevertheless we recommend not to use red-phenol containing medium if you want to use CCK-8.

Troubleshooting

Insufficient number of adherent cells. It is important that adherent cells cover nearly 100% of the surface of each reservoir. If this cannot be done with the quantity of cells we recommend to use, it is possible to let the plated cells grow until they are confluent enough. Adjust the quantity of cells you need to cover the plate.

Low sensibility of the counting kit. It is possible that you have a very low optical density in the optic reader if the number of non-adherent cells is under the sensibility of the component or you are working with non-viable cells (death). You can make an estimation of the viability of your cells counting them with TRYPAN BLUE and use them when the viability is more than 80% of the total count. Using MTT brings an advantage since living cells are blue stained while death are not.

Air bubbles. Most of the problems of this method can be solved avoiding the presence of air bubbles before turning the Plate A upside down or before quantification with the optic device. If bubbles appear we recommend:

(1) Avoid producing them with a careful handle of the pipette.

(2) If bubbles appear during the adhesion assay before step 3, incubate the plate as it is. During the incubation period, most of them will disappear.

(3) If bubbles are present during step 4 you can:

(a) Break them with a dry tip.

(b) Suck them with a pipette, or

(c) Pump air against them through an adequate tip with a 1,000 μL pipette.

(4) It is important to check the presence of air bubbles before continuing, if they are present, they must be broken or extracted. Take the plate with both hands, and quickly (but not violently) turn the plate up-side down and incubate it at 37°C, 5% CO₂ for at least 1.5 h (see Fig. 3A–D).

(5) Place a new 96-well plate in the laminar flow hood (Plate B). Take the Plate A together with the cover as it is (upside down) and place it in the laminar flow hood (also upside down). Repeat the next order of steps as often as it is necessary:

(a) Place the necessary tips in the multichannel pipette.

(b) Take the Plate A without lid and tilt it to a position between 45–70 degrees (Fig. 3E and F).

(c) Place the tip of every pipette channel at the middle of the depth of each reservoir, avoid touching the bottom (where the adherent cells are) of the 96-well plate (Fig. 3F). Take off the hole supernatant row by row and transfer it to the corresponding wells in Plate B.

(d) Carefully put Plate A back into its corresponding top (upside down) or keep it in your hand (Fig. 3E).

(e) Discharge the tips of the multichannel pipette.

(f) Repeat these steps for the next row of reservoirs.

Colorimetric reaction and measurement of non-adherent cells. Plate B. (6) Prepare reservoirs for a standard curve. Several...
**High optical density in controls.** Usually all negative controls should have a very low optical density but in some cases they are too high or even denser than the testing wells. If you are using MTT please check if you have added FCS to all your tests and control wells. If you are using CCK-8 or XTT, please consider that in our experience and even if the provider claims that red phenol is not interfering with the optical density, it is so. Therefore we recommend using red phenol-free culture medium for this adhesion assay when CCK8 and XTT is used. You might also consider contamination or degradation of the component if it is old or has been frozen several times.

**Acknowledgments**

We would like to thank Helga Olinger, Caroline Berger and Tobias Jochum from the Institute for Toxicology and Genetics of the Karlsruher Institute of Technology, Germany, for invaluable support. Special thanks to Anke Diehlmann for all the technical support she gives to our group. This work was done with the support of CONACyT (National Council for Science and Technology) Mexico, DAAD (German Academic Exchange Service) and the German Federal Ministry of Education and Research in frame of the grant “START-MSC2” No. 01GN0940.

**References**

1. Silvestre J, Kenis PJ, Leckband DE. Cadherin and integrin regulation of epithelial cell migration. Langmuir 2009; 25:10092-9.
2. Ley K, Kansas GS. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. Nat Rev Immunol 2004; 4:325-35.
3. Wein F, Pietsch L, Saffrich R, Wächter P, Walenda T, Bork S, et al. N-cadherin is expressed on human hematopoietic progenitor cells and mediates interaction with human mesenchymal stromal cells. Stem Cell Res 2010; 4:129-39.
4. Wagner W, Wein F, Roderburg C, Saffrich R, Diehlmann A, Eckstein V, et al. Adhesion of human hematopoietic progenitor cells to mesenchymal stromal cells involves CD44. Cells Tissues Organs 2008; 188:160-9.
5. Faber A, Roderburg C, Wein F, Saffrich R, Seckinger A, Hiesch K, et al. The many facets of SDF-1alpha, CXCR4 agonists and antagonists on hematopoietic progenitor cells. J Biomed Biotechnol 2007; 2007:26065.
6. Caillerie F, Lavery R. Cadherin mechanics and complexation: the importance of calcium binding. Biophys J 2005; 89:3895-903.
7. Miller BJ, Banisadr G, Bhatracharya BJ. CXCR4 signaling in the regulation of stem cell migration and development. J Neuroimmunol 2008; 198:31-8.
8. Lapidot T, Dar A, Kollet O. How do stem cells find their way home? Blood 2005; 106:1901-10.
9. Aberle H, Schwartz H, Kemler R. Cadherin-catenin complex: protein interactions and their implications for cadherin function. J Cell Biochem 1996; 61:514-23.
10. Shintani Y, Fukumoto Y, Chaika N, Grandgenett PM, Hollingsworth MA, Wheelock MJ, et al. ADH-1 suppresses N-cadherin-dependent pancreatic cancer progression. Int J Cancer 2008; 122:71-7.
11. Yin T, Li L. The stem cell niches in bone. J Clin Invest 2006; 116:1195-201.