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Research Article

Revealing non-genetic adhesive variations in clonal populations by comparative single-cell force spectroscopy

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ABSTRACT

Cell populations often display heterogeneous behavior, including cell-to-cell variations in morphology, adhesion and spreading. However, better understanding the significance of such cell variations for the function of the population as a whole requires quantitative single-cell assays. To investigate adhesion variability in a CHO cell population in detail, we measured integrin-mediated adhesion to laminin and collagen, two ubiquitous ECM components, by AFM-based single-cell force spectroscopy (SCFS). CHO cells generally adhered more strongly to laminin than collagen but population adhesion force distributions to both ECM components were broad and partially overlapped. To determine the levels of laminin and collagen binding in individual cells directly, we alternatingly measured single cells on adjacent microstripes of collagen and laminin arrayed on the same adhesion substrate. In repeated measurements ($\geq$60) individual cells showed a stable and ECM type-specific adhesion response. All tested cells bound laminin more strongly, but the scale of laminin over collagen binding varied between cells. Together, this demonstrates that adhesion levels to different ECM components are tightly yet differently set in each cell of the population. Adhesion variability to laminin was non-genetic and cell cycle-independent but scaled with the range of α6 integrin expression on the cell surface. Adhesive cell-to-cell variations due to varying receptor expression levels thus appear to be an inherent feature of cell populations and should to be considered when fully characterizing population adhesion. In this approach, SCFS performed on multifunctional adhesion substrates can provide quantitative single-cell information not obtainable from population-averaging measurements on homogeneous adhesion substrates.

Introduction

Despite sharing a common origin and function, cells in clonal populations are often surprisingly heterogeneous in different cellular properties [1,2], such as cell size [3], multiplication rate [4] or protein expression [5]. Frequently, population variability increases when cells are transferred from their natural surrounding into *in vitro* cell culture [4,6,7], but the underlying molecular

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mechanisms are largely unknown. Likewise, the biological significance of many *in vivo* cell-to-cell variations is still unclear [2]. Nevertheless, cell populations are usually stable and function reliably despite the inherent heterogeneities in many aspects of cell behavior. In some cases cell-to-cell variations may well be beneficial by increasing population diversity and by enhancing survival in the face of changing environmental conditions [8]. During development, cell variability may also be advantageous for robust cell line expansion [4]. Cell variability may also confer drug-resistance to populations [9].

Better understanding the mechanisms and consequences of cell variability requires suitable assays for analyzing single cell behavior [10]. Phenotypic variation linked to morphological changes, such as variations in cell size or spreading area can usually be assessed by standard light microscopy, while variation in protein expression levels between individual cells can be analyzed by fluorescent microscopy or flow cytometry. In contrast, determining functional properties that do not involve morphological changes are usually more difficult to measure. As a consequence, less is known about functional heterogeneities, including adhesive or mechanical variations within populations. Many established cell adhesion assays, such as washing assays, generate only population-averaged adhesion data. As a consequence, subtle variations in adhesion between individual cells, which may be of potential biological significance, are usually impossible to detect with these assays. At the same time, carefully analyzing adhesive properties of individual cells may be crucial for better understanding the behavior of the entire population. For instance, in a cancer cell population extreme adhesive properties of a single aberrant cell may be sufficient to lead to dissemination and metastasis [11,12]. Furthermore, conventional adhesion assays usually permit only testing cell adhesion to a single type of ECM component at a time, whereas information about differential adhesion of an individual cell to two or more different types of ECM may be desirable.

Over the last years new single-cell assays have become available that provide quantitative adhesion information of individual cells with a force resolution reaching down to the single-molecule level. Some of these single-cell methods have been specifically applied to quantify cell-ECM protein interactions, including optical [13] and magnetic tweezers [14,15], the biomembrane force probe [16], and atomic force microscopy (AFM)-based single cell force spectroscopy (SCFS) [17,18]. Among these techniques AFM-based SCFS provides the most versatile force range (~10 pN–100 nN). In addition, the precise positioning system of the AFM allows excellent control over the contact conditions (interaction force, time and position). So far SCFS has been mainly used to measure cell adhesion to homogenous substrates coated with a single type of ECM component. Comparative measurements, however, able to analyze adhesion of a single cell to different surfaces, require multifunctional adhesion substrates. Advances in microcontact printing (μCP) over the last two decades have been instrumental in producing microstructured adhesion substrates [19,20], and abundant ECM proteins, such as fibronectin, laminin, vitronectin, and collagen have all been used in this technique. While many established microcontact printing techniques are capable of fabricating single ECM protein patterns backfilled with a non-adhesive material, such as polyethylene glycols (PEG) [21], multiple ECM protein printing techniques have only recently been emerging [22].

Here we use SCFS on bifunctional adhesion substrates to investigate adhesion variability in a CHO cell population. We find substantial variability in adhesion to collagen and laminin between individual cells. Furthermore, we show that adhesion variability is non-genetic and cell cycle independent but that it scales with the variation of integrin receptor expression within the population. Using bifunctional adhesion substrates we also demonstrate that adhesion to different ECM components is independently regulated and that differential adhesion strength at short contact times correlates with differential long-term spreading behavior. Adhesive variations caused by varying adhesion receptor levels therefore appear to be an inherent feature of cell populations cultured *in vitro* and should be considered when comprehensively characterizing population cell adhesion.

Materials and methods

Cell culture

All cell culture reagents were purchased from Invitrogen (http://www.invitrogen.com) except when specially mentioned. Chinese hamster ovary (CHO-K1) cells were obtained from the ATCC (CCL-61) and cultured in α–MEM containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C and with 5% CO_2. Cells were passaged every 2–3 day or before reaching confluency. Prior to SCFS experiments, cells were first adapted to serum-free Gibco® CO_2-Independent Medium (http://www.lifetechnologies.com) supplemented with 4 mM L-gluta mine, 100 IU/ml penicillin, and 100 μg/ml streptomycin for 1 h. Subsequently, cells were rinsed with D-PBS containing no Ca^{2+} and Mg^{2+} and trypsinized for 3 min. Trypsin was inactivated by adding soybean trypsin inhibitor to a final concentration of 1.3% (http://www.sigmaaldrich.com). After centrifugation at $100 \times g$ for 5 min, the supernatant was removed and cells were resus pended in fresh serum-free CO_2 Independent Medium. For integrin-blocking experiments, cells were pre-incubated in ser um-free CO_2 Independent Medium containing 100 μg/ml YIGSR peptide or 5 μg/ml rat integrin α6 blocking antibody (clone GoH3) or 5 μg/ml of a rat isotype control antibody at 37 °C for 30 min in suspension prior to performing SCFS in the same solution. Subclones were obtained from the original CHO cell population by serial dilution. Subclones were expanded from single cells and used for adhesion measurements after 3 passages. To investigate cell spreading, cells were seeded on bifunctional substrates, incubated at 37 °C for 16 h and paraformaldehyde-fixed.

Preparation of laminin/collagen bifunctional substrates

Laminin stripes were produced by a modification of the lift-off method [23]. Polydimethylsiloxane (PDMS) cuboids with a top surface area of ~1 cm^2 were covered with 250 μl of a solution containing 20 μg/ml natural mouse laminin (http://www.invitrogen.com) and 2 μg/ml Alexa594-conjugated goat anti-human IgG (http://www.invitrogen.com) as a fluorescent marker. After incu bation in a humidified chamber at 37 °C for 1 h, the PDMS cuboid was rinsed twice with distilled water and briefly dried under nitrogen flow. The cuboid was then pressed shortly onto a structured silicon wafer and transferred onto a freshly