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Label-free profiling of cell dynamics: A sequence of impedance-based assays to estimate tumor cell invasiveness *in vitro*

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ABSTRACT

Dynamic properties of cancer cells, most notably their ability to migrate, have been correlated successfully with their invasive nature *in vivo*. To establish a stronger experimental basis for such a correlation we subjected five different cancer cell lines of well-defined metastatic potential to a sequence of three independent assays reporting on three different aspects of cell dynamics, namely (1) the kinetics of cell spreading, (2) cell shape fluctuations, and (3) cell migration. The sequentially applied assays correspond to different measuring modes of the well-established ECIS technique that is based on non-invasive and label-free impedance readings of planar gold-film electrodes that serve as the growth substrate for the cells under study. Every individual assay returned a characteristic parameter describing the behavior of the cell lines in that particular assay quantitatively. The parameters of all three assays were ranked to establish individual profiles of cell dynamics for every cell line that correlate favorably with the cells' invasive properties. The sequence of impedance-based assays described here requires only small cell populations (< 10.000 cells), it is highly automated and easily adapted to 96-well formats. It provides an in-depth dynamic profile of adherent cells that might be useful in other areas besides cancer research as well.

1. Introduction

The invasiveness of tumor cells has traditionally been studied and categorized by molecular approaches. Most of these assays were directed against those cellular proteins that are involved in cell-cell adhesion and cell migration since detachment from the primary tumor and migration through the extracellular space are hallmarks of the metastasis process [1]. Accordingly, the ability of cells to interact with extracellular matrix (ECM) proteins or with neighboring cells has been studied intensively on the molecular level. In addition to molecular and morphological indicators, the invasiveness of cancer cells has been studied on a more holistic and functional level by probing the cells' actual ability to migrate. The *in vitro* assessment of cell motility is considered as the most integral functional parameter to describe cell invasiveness. It is primarily based on assays that measure the cells' migration through a layer of extracellular matrix or across an endothelial monolayer in so-called Boyden chamber assay [2–4]. Alternatively, a variety of different "wound-healing assays" has been established that provide a measure for a tissue-specific form of motility, *i.e.* collective cell migration [3,4]. In these assays a more or less defined lesion is introduced into a confluent layer of the cells under study and the closure of this 'wound' is quantitatively analyzed by videomicroscopy. However, these assays are usually performed as endpoint assays with no information about the underlying dynamics. Moreover, they require tedious cell counting or image analysis and are hardly applicable to very small cell numbers as, for instance, available from biopsies.

Most recently, several physical sensor devices have been described that provide time-resolved information on cell motility or other measures of cell invasiveness with a considerable automation and throughput. Among these, several assays based on electrochemical impedance measurements have proven their particular usefulness in fundamental and preclinical trials of cancer cell phenotyping. These assays are tailored to monitor a) cell adhesion to extracellular matrix constituents [5], b) collective cell migration [6], c) cell proliferation [7] or d) extravasation across endothelial cell monolayers [8–10]. Thus, several unique features of cell invasiveness can be addressed and quantified. The underlying technology is referred to as *Electric Cell-substrate Impedance Sensing* or ECIS™. ECIS, first described by Giaever and Keese in 1984 [11], is based on growing cells on the surface of planar goldfilm electrodes that are deposited on the bottom

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of a culture dish and monitoring the impedance of those electrodes. When cells attach and spread on the electrode surface, their dielectric cell bodies impede current flow from the electrode to the bulk as they force the current to bypass them at most frequencies using the narrow channels underneath and between adjacent cells. This makes ECIS readings sensitive to changes in (i) electrode coverage and (ii) the 3D shape of the cells on the electrode surface. The time resolution can be tailored to the type of assay: from a minimum of a few milliseconds to days or even weeks. One unique feature of this technology needs to be emphasized: When the impedance of a cell-covered ECIS electrode is studied with high time resolution and instrument sensitivity, a fluctuation of the impedance is observed caused by metabolically driven shape fluctuations of the cells on the electrode. These fluctuations are not synchronized like the beating of cardiomyocytes but more individual and they were named *micromotion* [12]. Micromotion, a unique feature of cell motility and cell shape dynamics, mirror the cells' metabolic activity [13,14] and its dependence on the cytoskeleton provided the basis for a sensitive assay to detect cytoskeletal toxins in very low concentrations [15].

In the present study we have applied three of these impedance-based functional assays sequentially to one cell population in order to probe different aspects of cell dynamics, each of which has been used as an individual indicator for cell invasiveness in the past, namely (i) kinetics of cell spreading, (ii) mircomotion and (iii) the ability for collective cell migration (wound healing).

It was the rationale of this project to use more than one parameter describing cell dynamics as the basis for identifying a possible correlation between cell dynamics and metastatic potential. The three individual impedance-based assays (Fig. 1) have been successfully established before by us and others. But they haven't been applied as a consecutive sequence to one cell population to provide a dynamic cell profile. The mouse adenocarcinoma cell lines LM2, LM3 [16,17] together with the human melanoma cell lines WM35, HT168 and HT168-M1 [18–20] were used as model systems. These cell lines are known to express a diverse character in both, local invasive capacity and spontaneous metastatic potential as indicated in more detail below.

2. Materials and methods

2.1. Cell culture

Breast cancer cells (LM2, LM3) were maintained in Ham's F12 medium (Sigma-Aldrich, St Louis, MO) while melanoma cell-lines (WM35, HT168, HT168-M1) were grown in RPMI 1640 (Sigma-Aldrich, St Louis, MO). Both media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, Carlsbad, CA), 100 µg/ml streptomycin and 100 U/ml penicillin. Cells were kept at 37 °C in an atmosphere of 5% CO_2 and 95% air. Media were replaced every other day. All subculturing of the cells was performed by standard trypsinization (0.25%) at time of confluence.

2.2. Kinetics of cell spreading

Cell spreading kinetics were monitored and analyzed as described previously [5]. Briefly, the interface impedances of the ECIS electrodes (8W1E; Applied BioPhysics Inc., Troy, NY) were stabilized by incubation in 200 µL/well serum-free medium under incubator conditions for several hours. Immediately before cell seeding the whole system was equilibrated in 200 µL/well serum-containing medium until stationary impedance readings were established. Single cell suspensions were prepared from confluent cell monolayers of all cell lines by standardized trypsinization, centrifugation and resuspension in fresh culture medium. The final cell suspensions were then added into each well of the electrode array to establish a cell density app. $3{\cdot}10^5$ cells/cm^2. The dynamics of cell spreading were monitored by repeatedly recording the complex impedance of the electrodes along a frequency range from 25 Hz to 100 kHz using an ECIS 1600R system (Applied BioPhysics Inc., Troy, NY). The reactive part of the complex impedance (*X*), measured at a frequency (f) of 32 kHz, was converted to an equivalent capacitance C

$$C = 1/(2\pi{\cdot}f{\cdot}X). \quad (1)$$

It has been reported previously that the equivalent capacitance C of the system at this frequency is linearly dependent on the coverage of the electrode with cells and therefore mirrors the time course of cell spreading [5]. In order to compare the adhesion kinetics of the various cell lines under study, we analyzed the time-resolved capacitance raw data with respect to the time that is necessary for a half-maximum capacitance drop $T_{A1/2}$.

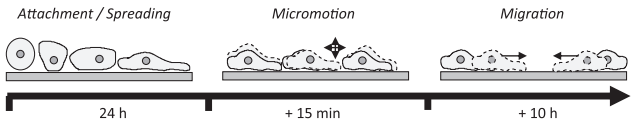

Fig. 1. Three different impedance-based assays are applied sequentially to one and the same cell population in order to generate an individual profile of the cells' dynamic properties: (i) rate of cell spreading; (ii) intensity of cell shape fluctuations (micromotion) and (iii) rate of cell migration.

2.3. Cellular micromotion

The second impedimetric assay to describe the dynamics of a given cell population addresses the metabolically driven cell shape fluctuations referred to as *micromotion*. When cell spreading was completed within 22–24 h after seeding (cf. Fig. 1), the impedance of each electrode was recorded with a time resolution of 500 ms at an AC frequency of 4 kHz for a total of 15 min. The real part of the impedance (resistance) was used to follow micromotion of the cells as it has proven to be the most sensitive indicator of cell shape fluctuations (cf. Fig. S1 in the Supplementary Information) [14,15]. In order to describe the complicated pattern of resistance fluctuations by a single quantitative parameter, we followed the procedures earlier described by Lo et al. [14] to extract the *standard deviation of increments* (SDI) of the fluctuating time series. The numerical recipe is as follows: (i) the original, full-length time series was normalized by dividing each data point of the series by the time average. (ii) We then averaged the increments between two consecutive data points i and (i+1) along the entire dataset and calculated the associated standard deviation of these increments which is then referred to as SDI-2. (iii) The procedure was repeated for increments that were calculated for steps from data point i to (i+3) yielding SDI-4. Data points i to (i+7) provided SDI-8, i to (i +15) yielded SDI-16 and so on. The standard deviations for the different time resolutions quantify cell shape fluctuations as they occur on different time scales. In this study we found that the SDI-64, which mirrors the degree of cell shape fluctuations on a time scale of 32 s, was most sensitive to unravel differences in cell shape dynamics of the different cell lines studied here. In addition to that we calculated the power spectral density (PSD) functions of the micromotion time series by FFT using a MATLAB routine following the procedures described in the original publication about micromotion analysis including a Hanning window function [14].

2.4. Wound-healing / cell migration assay

2.4.1. Automated wound-healing assay using ECIS

After micromotion recordings were completed (cf. Fig. 1), the ECIS-based wound-healing assay was applied to the very same cell population following the established procedures [6]. The ECIS-device was switched back from single frequency recordings to repeated frequency scans (25 Hz to 100 kHz). The cell layers were monitored in this measurement mode for another 10 h before an invasive electric field