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ORIGINAL PAPER

Single cell adhesion measuring apparatus (SCAMA): application to cancer cell lines of different metastatic potential and voltage-gated Na^+ channel expression

Christopher P. Palmer · Maria E. Mycielska · Hakan Burcu · Kareem Osman · Timothy Collins · Rachel Beckerman · Rebecca Perrett · Helen Johnson · Ebru Aydar · Mustafa B. A. Djamgoz

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Abstract We have developed a simple yet effective apparatus, based upon negative pressure directed to the tip of a micro-pipette, to measure the adhesiveness of single cells. The "single cell adhesion measuring apparatus" (SCAMA) could differentiate between the adhesion of strongly versus weakly metastatic cancer cells as well as normal cells. Adhesion was quantified as "detachment negative pressure" (DNP) or "DNP relative to cell size" (DNPR) where a noticeable difference in cell size was apparent. Thus, for rat and human prostate and human breast cancer cell lines, adhesiveness (DNPR values) decreased in line with increased metastatic potential. Using the SCAMA, we investigated the effect of tetrodotoxin (TTX), a specific blocker of voltage-gated Na^+ channels (VGSCs), on the adhesion of rat and human prostate cancer cell lines of markedly different metastatic potential. Following pre-treatment with TTX (48 h with 1 μM), the adhesion values for the Mat-LyLu cells increased significantly 4.3-fold; there was no effect on the AT-2 cells. For the strongly metastatic PC-3M cells, TTX treatment caused a significant (~30%) increase in adhesion. The adhesion of PNT2-C2 ("normal") cells was not affected by the TTX pretreatment. The TTX-induced increase in the adhesiveness of the strongly metastatic cells was consistent with the functional VGSC expression in these cells and the proposed role of VGSC activity in metastatic cell behaviour. In conclusion, the SCAMA, which can be constructed easily and cheaply, offers a simple and effective method to characterise single-cell adhesion and its modulation.

Keywords Adhesion · Cell detachment · Cancer · Metastasis

C. P. Palmer · M. E. Mycielska · H. Burcu · K. Osman · T. Collins · R. Beckerman · R. Perrett · H. Johnson · E. Aydar · M. B. A. Djamgoz (✉)
Division of Molecular & Cell Biology,
Neuroscience Solutions to Cancer Research Group,
Sir Alexander Fleming Building, Imperial College London,
South Kensington Campus, London SW7 2AZ, UK
e-mail: m.djamgoz@imperial.ac.uk

H. Burcu · K. Osman
Department of Physics, Imperial College London,
South Kensington Campus, London SW7 2AZ, UK

Introduction

Adhesion and detachment of cells to neighbouring cells and/or the extracellular matrix, and regulation of adhesion/detachment, sometimes in concert, are critical for a variety of biological functions such as embryogenesis, mitosis, morphogenesis, cellular orientation, motility and survival (Gumbiner 1996; Klymkowsky and Parr 1995; Lauffenburger 1996; Lauffenburger and Horwitz 1996; Mitchison and Cranmer 1996). The problem of a correct evaluation of adhesive forces from a great number of individual cells of a given population is also of considerable relevance to the assessment of the adhesive properties of different biomaterials, such as those employed in prostheses, biosensors, etc.

Cellular attachment to the substrate is often mediated by transmembrane glycoproteins, such as integrins, that bind to ligands such as collagens, laminin or fibronectin (Gumbiner 1996; Hynes and Lander 1992). Adhesion is also important pathophysiologically, as in cancer (e.g. Christofori 1984) and inflammatory disease (Kadl and Leitinger 2005) even during addiction (Ishiguro et al. 2006; Weber et al. 2006). In particular, the metastatic cascade is composed of a complex series of cellular behaviours, including initial

detachment of cells from each other within the primary tumour, local invasion faciliatated by degradation of the surrounding tissue, entry into the circulation (blood or lymph), homing, extravasation, re-attachment (usually at tissue specific sites), and finally angiogenesis (Hart et al. 1989; Jiang et al. 1994; Liotta and Stracke 1988). It is now well established that alterations in expression and functioning of cell–cell and cell–matrix adhesion molecules correlate with cancer progression (Christofori 1984; Knudsen and Miranti 2006; Kobayashi et al. 2007; Muller et al. 2002; Wong et al. 2007). In addition, the penetration of anticancer drugs through tumour tissue is affected by cellular adhesion and packing density of tumour cells (Grantab et al. 2006).

There have been various attempts to measure cell adhesion. The simplest adhesion assay is based on surface rinsing to remove weakly attached cells from the substratum and counting remaining cells (Walther et al. 1973). However, this method (termed "static adhesion assay") and other similar methods are qualitative or at best semi-quantitative and may register only gross differences in attachment. The "parallel-plate flow configuration" (Forrester and Lackie 1984) is another common method to study cell adhesion as it is simple to construct and the flow generated within the chamber can be analyzed mathematically. However, as for the static adhesion assay, in these 'global' tests, where populations of cells are involved, a formidable problem is that of the existence of subpopulations of cells with stochastic adhesive expression and of the uncertainty of the various degrees of adhesion of individual cells. This is explicably true for cancer cells due to their inherent heterogeneity, even in the case of given cell lines. Some techniques have been developed to study adhesion at single-cell level (as reviewed in (Missirlis and Spiliotis 2002)). These include micropipette aspiration, magnetic beads, optical tweezers, transistor recording and microcantilevers. However, many of these techniques are not readily available or easily useable because of complexity and/or cost of set-ups.

The aim of the present approach was to build a simple micro-pressure system to effectively measure the adhesion of single cells in vitro. We called this system "single-cell adhesion measuring apparatus" or "SCAMA". A brief communication of work involving this technique was presented to the Physiological Society (Mycielska et al. 2004).

Materials and methods

Basic design and construction of SCAMA

The experimental set-up for SCAMA is depicted in Fig. 1. A glass micropipette (1.0 mm OD/ 0.85 mm ID; Harvard Apparatus 30-0019) was drawn to about 20 μm tip size (range, 17–24 μm) using a Narishige pp-830 puller and connected to a vacuum pump via plastic tubing. The vacuum pump was used to create inside a glass jar ("reservoir") negative pressure which could be applied to the tip of the micropipette at a level controlled by pressing the thumb to the open end of a sealable T-piece. Use of a

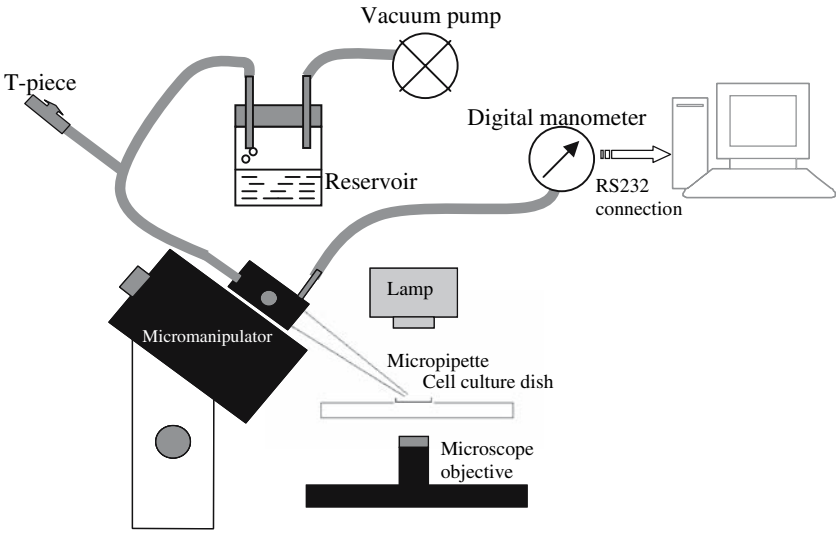

Fig. 1 Schematic representation of the Single Cell Adhesion Measuring Apparatus ("SCAMA"). A 35 mm cell culture dish was placed onto the moveable stage of an inverted microscope. The cells were observed using a 20× objective. Using a micromanipulator, a glass micropipette was positioned at the periphery of a single cell. Upon closing off the T-piece, negative pressure could be generated by a vacuum pump connected to the micropipette via a reservoir. The pressure was measured using a digital manometer connected to a personal computer via a R2232 cable. At the exact moment the cell under investigation was observed to be detached from the culture dish, the pressure was released by opening the T-piece

simple T-piece proved to be highly sensitive and enabled highly consistent data to be produced by a range of operators over time. The reservoir also prevented the medium, bathing the cells, from being sucked into the air pump and buffered the negative pressure being generated (Fig. 1). The negative pressure in the system was measured with a digital manometer (p100 CE Electronics) connected via a RS232 lead to a personal computer and recorded using the digital manometer handheld data recording program (CE Electronics). We could also attach a simple perfusion system (Minipuls3) to the cell culture dish to enable reversible application of different drugs/chemicals to the cells being assayed, if necessary (not shown).

Culture of cell lines

Rat prostate cancer (Dunning) cell lines, AT-2 and MatLyLu (Dunning 1963; Issacs et al. 1986), were cultured in RPMI-1640 media containing 1% fetal bovine serum (FBS) and penicillin-streptomycin (100 μg/ml each). Human breast cancer cell lines (MCF-7 and MDA-MB-231) and human embryonic kidney cell line (HEK) were cultured in DMEM (Gibco) containing 10% FBS and 4 mM glutamine with penicillin–streptomycin. The 'normal' human breast epithelial cell line (MCF-10A) was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 medium (Gibco, DMEM-F12) supplemented with 5% FBS, hydrocortisone (0.5 μg/ml), insulin (10 μg/ml), epidermal growth factor (20 ng/ml), and penicillin–streptomycin. Normal and strongly metastatic human prostatic epithelial cell lines (PNT2-C2 and PC-3M, respectively) were grown in RPMI-1640. The latter was supplemented with 10% bovine calf serum and 2 mM L-glutamine for the PNT2-C2 cell line, whilst the medium for PC-3M cells contained following the additions: 10% bovine calf serum, 4 mM L-glutamine, 1 g/l sodium bicarbonate, 4.5 g/l glucose and 1 mM sodium pyruvate. All cell lines were maintained in a 37°C CO_2 incubator in 100 mm culture dishes. Forty-eight hours prior to use in the SCAMA, the cells were trypsinized and re-plated into 35 mm dishes at a density of 2.5×10^4 cells/ml, in order to obtain separated single cells. Some experiments were performed on cells plated on cover-slips coated with poly-D-lysine. In this case, sterile 20 mm square cover-slips were placed into 35 mm culture dishes containing 1 ml of 25–50 μg/ml of poly-D-lysine. Following incubation at 37°C for 30 min, the cover-slips were washed in growth medium three times, prior to seeding with cells.

Measurement of cell surface area

The surface areas of cells, presumed to be in contact with the dish was calculated from the average of the long and short axes, measured using a microscope equipped with a graticule. Mean surface areas were calculated from 11 representative units for each cell line (Table 1).

Measurement of single-cell adhesion

A 35 mm dish of cells was placed onto the microscope stage. An individual cell was observed under 100× magnification and the glass micropipette was brought just to the cell periphery using a micromanipulator. Increasing suction was applied to the cell under investigation by gradually closing off the T-piece, which resulted in build-up of increasing negative pressure, displayed/recorded on a personal computer (Fig. 2a). At the exact moment when the cell was observed to detach ('pop') from the culture dish, the suction was released by briskly re-opening the T-piece whereupon the pressure returned to baseline. Thus, the negative pressure precisely required to detach the cell was recorded on the computer as a 'pressure spike' (Fig. 2a). The peak of the spike ("detachment negative pressure"—DNP) was converted to kiloPascals (kPa) and used as a measure of the cell's adhesiveness. Using this procedure, which proved to be very sensitive, several recordings could be made from a single dish within minutes.

In order to account for any obvious difference in cell size, which could affect adhesion via 'surface tension' rather than a molecular mechanism (Akei et al. 2006), the value of DNP could be normalized with respect to cell surface area (A), when necessary, as follows:

$$\mathrm{DNPR} = \mathrm{DNP}/\mathrm{A}$$

Thus, DNPR represented DNP relative to cell surface area where differences in cell size were notable.

Table 1 Cell surface contact areas for eight mammalian cell lines and corresponding DNP and DNPR values. Data are given as means ± standard errors (for each cell line, $n = 11$ for area and 50 for DNP/DNPR)

Cell line	Cell surface contact area (μm^2)	DNP (kPa)	DNPR ($kPa/\mu m^2 \times 10^{-2}$)
MAT-Ly-Lu	163 ± 14	5.5 ± 2.4	3.4 ± 1.5
AT-2	221 ± 25	16.5 ± 4.6	7.5 ± 2.3
PC-3M	334 ± 33	7.7 ± 0.48	2.3 ± 0.3
PNT2-C2	121 ± 15	8.4 ± 0.41	7.0 ± 1.0
MDA-MB-231	116 ± 8	3.2 ± 3.0	2.8 ± 2.6
MCF-7	224 ± 26	8.8 ± 6.5	3.9 ± 2.9
MCF-10A	417 ± 6	33.9 ± 11.9	8.1 ± 2.8
HEK	197 ± 6	2.8 ± 2.6	1.4 ± 1.3

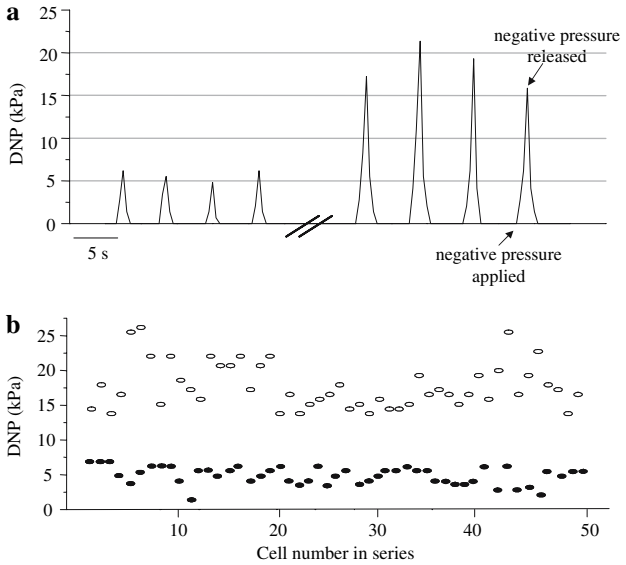

Fig. 2 Measurement of adhesion of two markedly different rat prostate cancer cell lines using the SCAMA. (**a**) Four examples each of recordings from Mat-LyLu and AT-2 cells. The points at which the negative pressure was applied and subsequently released are indicated by arrows on the last recording. (**b**) Sets of 50 measurements each taken from Mat-LyLu (closed circles) and AT-2 (open circles) cell lines, showing the mutual exclusivity of the data. DNP, detachment negative pressure measured in kiloPascals (kPa's)

Static cell adhesion assay

Cultured cells were removed from the dish using 0.5% EDTA in PBS and counted using a haemocytometer. The culture was diluted to 1×10^5 cells/ml with growth media and 1 ml of cell suspension was seeded into quadruplicate wells of a 12-well dish. The cells were placed immediately into a 37°C CO_2 incubator for 1 h. The dishes were taken out and the non-attached cells were removed by adding PBS gently followed by careful aspiration. Subsequently, the cells were fixed using acetone/methanol for 30 min at room temperature and stained using 2% crystal violet for 30 min. The dishes containing the stained cells were submerged in running water for 30 min, drained and dried by tapping onto absorbent paper. The stained cells in each well were resuspended in 200 µl of 1% SDS and the absorbance at 590 nm was measured. As controls, 1×10^5 cells/ml from each cell line were fixed similarly with methanol/acetone, stained with crystal violet and washed five times with PBS in 1.5 ml microtubes. The control cells were resuspended in 1% SDS and the absorbance measured at 590 nm. The latter results were used as the 100% control adhesion value for each cell line. Adhesion values were expressed relative to this control value as a "percentage adhesion factor" (PAF).

Data analysis

Data were analyzed by one-way ANOVA. Differences were taken as statistically significant for $P < 0.05$. All the data were expressed as means ± SEMs. "n" represented the number of cells tested. In all cases except where specified the number of dishes tested ("N") was greater than three.

Results

Using the SCAMA (Fig. 1), we were easily able to measure, within about 30 min, the single-cell adhesion of 50–60 cells (48 h post-plating) in given cultures. Measurement of cellular adhesion immediately after plating was attempted and indicated an increase in adhesion over a period of 1 h (data not shown). However, the most consistent recordings, in presumed equilibrated state, were obtained at 48 h post plating and this time was chosen to standardize all experiments.

Adhesion of cancer cells of different metastatic potential and comparison with normal cells

Recordings were made initially from rat prostate cancer, Mat-LyLu cells and AT-2, cells which have been used extensively in our previous electrophysiological studies. These isogenic cells were originally derived from the same tumour but differ markedly in their metastatic potential, inducing secondary tumours in >90% and <10% of cases, respectively, when injected into syngeneic rats (Dunning 1963; Issacs et al. 1986). The results of typical SCAMA recording series are shown in Fig. 2b. Clearly, in spite of the considerable variability within the given data sets observed, the DNP values for the two cell types were mutually exclusive and had averages of 5.5 ± 2.4 kPa (Mat-LyLu) and 16.5 ± 4.6 kPa (AT-2). Thus, the weakly metastatic cells were on average some 3-fold more adherent than the corresponding strongly metastatic cells and this difference was highly significant ($P = 0.02$; $F = 670$; $n = 50$ each). The corresponding DNPR values were 3.4 ± 1.5 kPa/µm$^2 \times 10^{-2}$ (Mat-LyLu) and 7.5 ± 2.3 kPa/µm$^2 \times 10^{-2}$ (AT-2) (Fig. 3a), the difference again being highly significant ($P = 0.03$; $F = 220$; $n = 50$ each). Measurements made on analogous human prostate cancer and normal cells showed a comparable, 3-fold difference in adhesiveness, the DNPR values being: 2.3 ± 0.3 kPa/µm$^2 \times 10^{-2}$ (PC-3M) and 7.0 ± 1.0 kPa/µm$^2 \times 10^{-2}$ (PNT2-C2) ($P = 0.01$; $F = 1020$; $n = 50$ each) (Fig. 3a). For human breast cancer cell lines of varying metastatic potential and normal breast cell lines, the DNPR values obtained were as follows (in kPa/µm$^2 \times 10^{-2}$): 2.8 ± 2.6

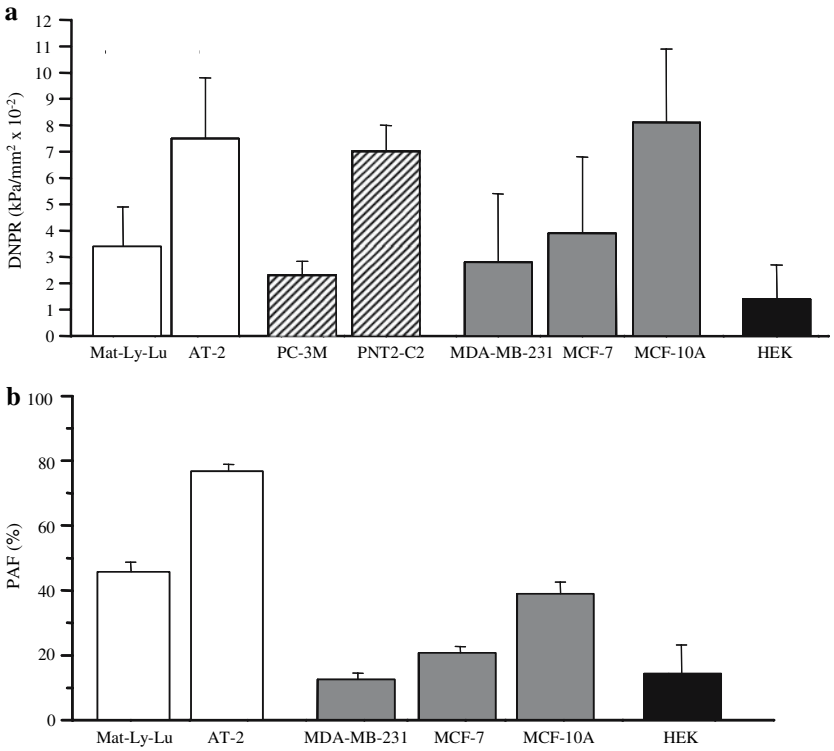

Fig. 3 Adhesion properties of mammalian epithelial cell lines determined by the SCAMA and a static method of measuring cell adhesion. (**a**) The DNPR values (kPa/μm^2) of eight mammalian cell lines: Mat-LyLu, AT-2, PC-3M, PNT2-C2, MCF-7, MDA-MB-231, MCF-10A and HEK-293 (indicated as "HEK"). Each histobar denotes mean + standard error (n = 50 each). DNPR = DNP value divided by the average surface area of the cell. (**b**) The mean adhesion values of six mammalian cell lines Mat-LyLu, AT-2, MCF-7, MDA-MB-231, MCF-10A and HEK-293 (abbreviated as in part a) was determined using a static method of cell adhesion. PAF, percentage adhesion factor (as defined in the Materials and methods)

(MDA-MB-231), 3.9 ± 2.9 (MCF-7) and 8.1 ± 2.8 (MCF-10A) (Fig. 3a). Thus, again, the adhesion values decreased as metastatic potential increased. One-way ANOVA analysis of these values confirmed that the more metastatic cell lines had statistically lower DNPR values than normal cells or the cell lines with a lower metastatic potential. We should note that the mean DNPR value for the MCF-10A cells is probably an underestimate since a significant fraction of these cells (∼24%) could not be detached from the culture dish even after applying the strongest suction (about 60 kPa) that the SCAMA could generate. Therefore, the real adhesiveness of the MCF-10A cell line, in fact, is significantly higher than shown. Finally, human embryonic kidney (HEK) cells were also tested and found to have a DNP value of 2.8 ± 2.6 kPa (DNPR = 1.4 ± 1.3 kPa/μm^2 × 10^{-2}; n = 50) (Fig. 3a).

Comparison with a static method for measuring cell adhesion

A comparison was made between the SCAMA and a static method of cell adhesion (Fig. 3b). The results obtained from the static adhesion method were in broad agreement with the SCAMA data. Thus, again, adhesiveness decreased in line with the metastatic potential for both the rat prostate and the human breast cancer cell lines studied.

We then performed a series of tests to establish the various operational characteristics of the SCAMA. For these, mainly the Dunning AT-2 cells were used since these were relatively easy to culture and had 'large' DNP values that could readily be compared under the different testing conditions.

Heterogeneity and consistency of the SCAMA recordings

Analysis of the distribution of the DNP values for the AT-2 cell line for fifty cells suggested that there were several sub-populations of cells with regards to adhesiveness (Fig. 4a). Similarly for the Mat-LyLu cell line, two clear sub-populations of cells could be visualized, one peaking at 4 kPa and the other at 5.5 kPa (Fig. 4b).

The consistency of SCAMA recordings in relation to possible pipette variability was tested (Fig. 4c). Thus, a number of pipettes of ∼20 μm tip sizes were pulled and three such pipettes were used on a given dish in succession. In each case, 50 cells were tested. As shown in Fig. 4c, there was no significant difference (P = 0.95; F = 0.0031; n = 50) between the three sets of measurements. Increasing the pipette tip size increased the amount of negative pressure required to detach the cells and resulted in more solution being sucked up. On the other hand, pipettes with smaller tips often became blocked with debris. Basically, for tip diameters of 17 and 24 microns, there is no significant difference in the DNP values measured (data not shown). Finally, as regards consistency, we should add that

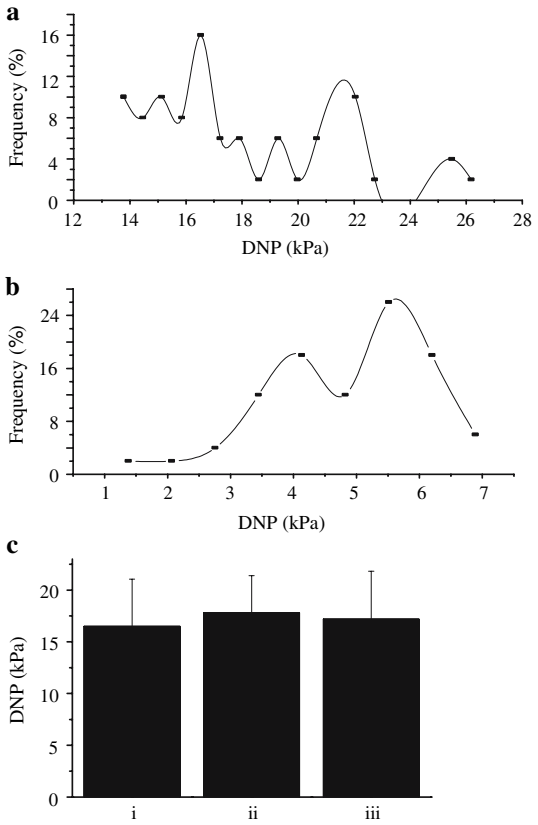

Fig. 4 Heterogeneity of cellular adhesiveness and consistency of the SCAMA measurements. SCAMA was used to measure the adhesion properties of AT-2 (**a**) and Mat-LyLu cells (**b**) plated on glass cover slips. Fifty recordings were made from each cell line. The data are plotted as a percentage distribution showing multiple peaks. (**c**) Repeated SCAMA measurements on a dish of AT-2 cells. Data from three successive sets of measurements (i–iii) are shown (n = 50 each). In between each set, the micro-pipette was changes in order to determine the repeatability of the measurements. Each histobar denotes mean ± standard error (n = 50)

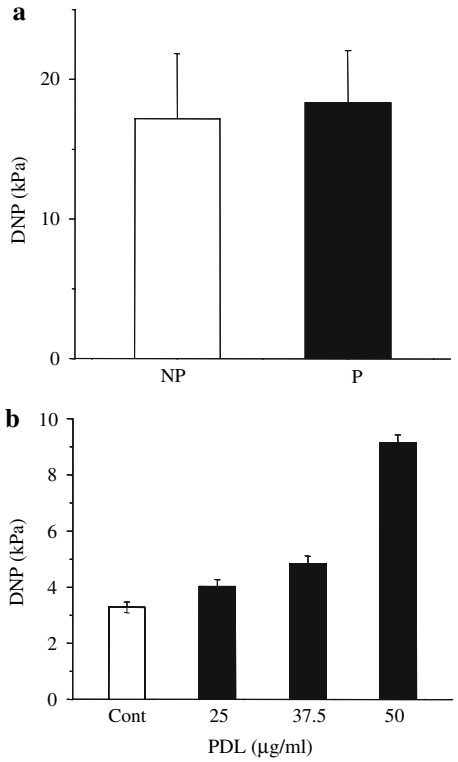

Fig. 5 Effects of perfusion and surface stickiness on SCAMA measurements of AT-2 cell adhesion. (**a**) Cell adhesion (DNP) values were recorded for non-perfused cells ("NP") and cells under perfusion with mammalian physiological saline ("P"). Fifty cells were sampled in each dish. Each histobar denotes mean + standard error (n = 50). (**b**) Measurement of the adhesion of MAT-LyLu cells plated on control glass cover-slips "Cont" and glass cover-slips pre-coated with poly-D-lysine ("PDL") at the concentrations given under each histobar. Fifty recordings were made for each condition. Each histobar denotes mean + standard error (n = 50)

several operators have now used the SCAMA in our laboratory and have obtained very similar measurements from given cell lines.

Lack of effect of non-perfusion

Since one reason for developing the SCAMA was to allow the perfusion of drugs/chemicals onto the cells under investigation, we also sought to determine whether perfusion itself of culture media would significantly alter the cellular adhesiveness, for example, by mechanical disruption. Thus, DNP values were measured for AT-2 cells perfused or not perfused with culture medium. These results indicated that perfusion did not significantly alter the adhesiveness of the AT-2 cell line (P = 0.85; F = 0.003; n = 50) (Fig. 5a). Since the measurement of 50 individual cells would take approximately 20 min at room temperature, we also determined if adhesiveness would change during this period. There was no systematic change in the DNP values measured over 20–30 min (data not shown). Also, we could not detect any difference in cell viability in the cultures being investigated up to 60 min, as determined by trypan blue staining (data not shown).

Effect of surface stickiness

We then questioned whether the SCAMA would accurately measure the increase in the adhesion of cells plated onto normal glass cover-slips versus cover-slips pre-coated with poly-D-lysine, commonly used to increase attachment of tissue sections to glass slides. This test was carried out on Mat-LyLu cells, which normally possess low DNP values. Again, 50 cells were measured on each surface. The results showed, as expected, that Mat-LyLu cells plated onto the cover-slips coated with 50 μg/ml of Poly-D-Lysine had 2.8-fold higher DNP values (9.2 ± 0.3 kPa), compared with

uncoated glass (3.28 ± 0.2 kPa). This difference was highly significant ($P = 0.002$; $F = 312$) and positively correlated with the concentration of the poly-D-lysine used (Fig. 5b).

Effect of tetrodotoxin

As noted in the Introduction, it is well known that adhesion/detachment are important components of the metastatic cascade (Hart et al. 1989; Jiang et al. 1994; Liotta and Stracke 1988). Voltage-gated Na^+ channel (VGSC) activity has previously been shown to be involved in various metastatic cell behaviours, including directional motility (Djamgoz et al. 2001; Fraser et al. 2003, 2005), in which adhesiveness would be expected to play a part (Friedl and Wolf 2003). Therefore, we set to determine whether application of tetrodotoxin (TTX) to selectively block VGSC activity would affect the adhesiveness of the human and rat prostate cancer cells, as measured by the SCAMA. Following pretreatment (48 h) with TTX (1 μM), the DNPR values for the Mat-LyLu cells increased 4.3-fold, from the control value of 4.0 ± 0.9 kPa/μm^2 × 10^{-2} to 17.0 ± 4.0 kPa/μm^2 × 10^{-2} ($P = 0.02$; $F = 876$; $n = 20$ each). There was no effect on the AT-2 cells (Fig. 6a). Interestingly, following the TTX treatment, there was no significant difference between the DNPR values of the MAT-LyLu and the AT-2 cells. For the strongly metastatic PC-3M cells, TTX treatment caused a significant (~30%) increase in DNPR (Fig. 6a), from the control value of 2.3 ± 0.6 kPa/μm^2 × 10^{-2} to 2.8 ± 0.6 kPa/μm^2 × 10^{-2} ($P = 0.02$; $F = 232$; $n = 20$ each). The adhesion of PNT2-C2 ("normal") cells was not affected by the TTX pretreatment (Fig. 6a).

Heterogeneity analysis of the TTX effect was performed on Mat-LyLu and AT-2 cells which are isogenic, i.e. derived from the same original rat tumour cell (Figs. 6b, c). The results showed that TTX affected mainly one of the subpopulations of cells that had the lower adhesiveness, decreasing the relative proportion of this population from ~27% to ~12%. In addition, a new cell population appeared with mean DNPR value of about 24 kPa/μm^2 × 10^{-2} (Fig. 6b). In contrast, there was no such systematic effect on AT-2 cells (Fig. 6c).

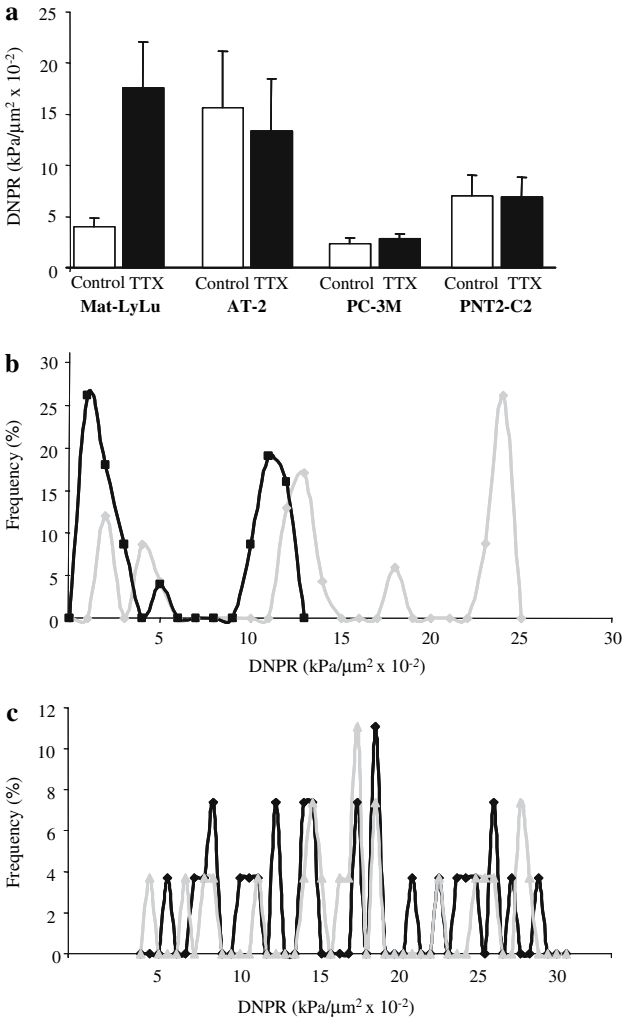

Fig. 6 Effect of tetrodotoxin on cellular adhesiveness. (**a**) Rat and human prostate cancer cell lines were used. Strongly metastatic: Mat-LyLu and PC-3M; weakly metastatic: AT-2 and PNT2-C2, respectively. Pretreatment with 1 μM TTX for 48 h had no effect on the weakly metastatic cells but the adhesion of the strongly metastatic counterparts was increased significantly. SCAMA measurements were made on 20 cells in each of three dishes for each cell line. Each histobar denotes mean + standard error ($n = 60$ cells in total). White bars, control data; dark bars, data obtained from TTX-pretreated cells. Histogram (%) distributions of DNPR values of Mat-LyLu (**b**) and AT-2 (**c**) cells (from a) obtained under control conditions (dark) and following TTX treatment (light)

Discussion

Adhesion is an important, dynamic characteristic of cells under both physiological and pathophysiological conditions. However, the currently available adhesion methodologies, such as static adhesion assays, parallel flow chambers and atomic force microscopy all have limitations for obtaining single-cell data. The SCAMA could overcome many of these, offered several distinct experimental advantages, and readily produced highly consistent data at the level of single units.

The SCAMA was designed to satisfy the following requirements: (i) to measure adhesion properties of single cells; (ii) to be readily constructible; (iii) to be inexpensive; (iv) to enable perfusion of drugs/chemicals onto cells either as a whole or individually; and (v) to test the reversibility of pharmacological effects on cellular adhesiveness. The

operational characteristics of the system and the data obtained would suggest that these criteria were achievable.

Although within a cell line there was a marked distribution of adhesion, the SCAMA was able to differentiate between the adhesive properties of several different cell lines. The measurement of adhesion by SCAMA was found to be reproducible as long as the conditions were kept constant and the pipette tips were pulled to the same size. We have noticed some operator variability in the absolute values of DNP/DNPR values measured, presumably due to subjective differences in the positioning of the pipettes and application of negative pressure. Importantly, however, several operators have consistently and quantitatively replicated the results concerning (a) differences in adhesion of metastatic vs. non-metastatic cells and (b) effects of key agents like TTX in increasing adhesiveness.

One drawback of the SCAMA, in its present form, is that we cannot measure the absolute force required to remove a cell from the dish; what we measured is the negative pressure in the system corresponding to cell detachment. This is not a serious limitation for doing relative measurements, however, comparing given cells under different experimental conditions, or cells of different functional characteristics. If necessary, SCAMA could enable measurements of the adhesion of several hundreds of cells in a given experiment lasting minutes. Furthermore, analysis of the recordings obtained from AT-2 and Mat-LyLu cells indicated that there were various sub-populations of cells as regards their adhesiveness. It is not yet known if such heterogeneity corresponds to the cells' established electrophysiological variability (Grimes et al. 1995) and/or cell–cell or cell-substrate adhesion molecule (CAM) expression characteristics. It may be interesting in the future to isolate these sub-populations and analyse the factors which could contribute to their differential adhesion. We should also note that, although cells had to be plated at sufficiently low density in order to obtain single cells for measurement, density itself can affect the cells' metastatic character (Kuwano et al. 2004).

Adhesion of cancer cells of varying metastatic potential and normal cells

As expected, cell lines (MDA-MB-231 and Mat-LyLu) of highest metastatic potential possessed the lowest adhesion factors, while cell lines which are weakly metastatic (MCF-7 and AT-2) were found to have higher DNP values. "Normal" cell lines (e.g. MCF-10A) adhered relatively most strongly. On the other hand, for non-isogenic cell lines with large differences in surface area (e.g. PNT2-C2 and PC-3M cells), no immediate difference in adhesion was observed in relation to metastatic potential. However, when surface area was taken into account and DNPR values were calculated, the same relationship could clearly be seen between adhesion and metastatic potential. In fact, the DNPR values calculated for all the cell lines used correlated inversely and proportionately with metastatic potential. Use of DNPR (rather than DNP) would also be necessary when testing the effects of pharmacological agents on adhesion. This was done for TTX (Fig. 6) which was shown previously to alter the morphology of rat prostate cancer cells (Fraser et al. 1999). Finally, we should note that whilst a clear inverse relationship between DNP/DNPR values and metastatic potential could readily be demonstrated for cells 'at rest', this is likely to be a dynamic relationship. In particular, adherence of metastatic cells would be expected to increase later in the metastatic cascade, as cells would extravasate and lodge at secondary tumour sites (Hart et al. 1989).

Control of cancer cell adhesion by VGSC activity

For both rat and human prostate cancer cells, treatment with TTX increased the adhesion of the strongly metastatic cell lines (Mat-LyLu and PC-3M, respectively). There was no effect on the corresponding weakly/non-metastatic AT-2 and PNT2-C2 cells, consistent with the latter not expressing any functional VGSC (Grimes et al. 1995; Laniado et al. 1997). The effect of TTX on Mat-LyLu and PC-3M cells is consistent with the proposed role of VGSC in enhancing metastatic cell behaviour (Djamgoz et al. 2001; Fraser et al. 2003; Grimes et al. 1995; Laniado et al. 1997; Mycielska et al. 2003). Thus, blocking VGSC activity increased adhesion and the difference between strongly and weakly metastatic cells became much less, and disappeared in the case of the isogenic rat cells (Dunning 1963). The fact that the effect of TTX appeared less on PC-3M vs. MAT-LyLu cells could be due to a relative difference in the proportions of cells expressing functional VGSCs, $\sim$20% vs. >50%, respectively (Grimes et al. 1995; Laniado et al. 1997).

Although, at present, the mode of VGSC action in controlling cellular adhesiveness is not known, two possibilities may be considered. First, VGSC activity may control transcriptional expression of CAMs, consistent with the time (48 h) taken for the effect of TTX to occur. Such a mechanism has been seen in cultured neurones where specific patterns of VGSC activity had a significant effect on expression of specific CAMs (Itoh et al. 1997). Second, the auxillary β-subunit(s) of the VGSC complex itself may behave as a CAM (Isom 2002). Thus, if VGSC expression is under auto-control (Brakenbury and Djamgoz 2006; Sherman and Catterall 1984), blocking its activity with TTX could affect β-subunit expression and thus

influence adhesion. Further work is required to evaluate these possibilities. The effect of TTX in increasing adhesion was unlikely to be due to its reported effect on area, at least for the Mat-LyLu cells for which data are available, since the reported effects of TTX on cell size were typically ~20% (Fraser et al. 1999), much more subtle than the effects on adhesion reported here (>3-fold).

Concluding remarks: possible improvements and further applications

Possible improvements to the SCAMA include the following: (1) Incorporation of a U-tube to enable fast application of drugs just to the single cell being measured within the experimental dish (e.g. Suzuki et al. 1990). (2) A 'picospitzer' ('puffer') can be used to apply expensive drugs (e.g. antibodies) from a pipette onto single cells, at least for studying short-term effects (e.g. Djamgoz 1983). (3) It would be possible to include a cell trap in the design to enable the collection of measured cells for subsequent single-cell RT-PCR analysis (Wagatsuma et al. 2005). (4) A solenoid can be incorporated to control the T-piece electronically, although we should emphasize that use of thumb proved to be sensitive and quick. In fact, we have previously used a similar manual T-piece arrangement in an atomizer (Djamgoz 1983) and for injecting specific volumes of isotonic solution into *Xenopus* oocytes (Bryan-Sisneros et al. 2003). (5) The theoretical basis of the measurements can be made more rigorous so as to enable calculation/comparison of the molecular forces controlling adhesion. Further characterizations of this system should include determining the adhesion of different cancer cell lines plated onto different surfaces to exclude an influence of the surface viscosity on these comparative measurements.

As regards possible future applications, first, it should readily be possible to modify the system so as to enable measurement of cell–cell (rather than cell-substrate) adhesion (Walther et al. 1973). Basically, one of a pair of coupled cells would be held 'steady' whilst SCAMA suction would be applied to the other.

Second, HEK-293 cells can be used for transfection of proteins relevant to adhesion, including CAMs, mechanosensitive ion channels and specific substrates, such as E-selectin, fibronectin or collagen can be used (e.g. Bershadsky et al. 2006). Such studies can elucidate structure–function relationships for the protein of interest and associated signalling mechanisms in quantitative approaches. Given the fact that SCAMA can measure changes in adhesion for a cell line and that we can easily perfuse drugs on the cells in question, it should be possible to test the ability of various drugs/chemicals to modulate cellular adhesion. Indeed, SCAMA was used as a tool to investigate the effect of Sigma receptors on β1-integrin mediated adhesion using a β1-integrin blocking antibody which drastically reduced the adhesion of MDA-MB-231 cells (unpublished data). Finally, it should similarly be possible to study other cell types, including neurones, fibroblasts, lymphocytes, endothelial cells, even bacteria, adhesion characteristics of which would also be important to their physiology and pathophysiology.

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