Characterisation of human skeletal stem and bone cell populations using dielectrophoresis

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Abstract

Dielectrophoresis (DEP) is a non-invasive cell analysis method, which uses differences in the electrical properties of particles and the surrounding medium to determine cellular properties. Once determined, these properties can then be used as a basis for cell separation. The use of skeletal stem cells in the form of cell-based therapies is currently one of the most promising areas for disease treatment for a variety of skeletal and muscular disorders. However, the identification and sorting of these cells remains a challenge in the absence of unique skeletal stem cell markers, preventing the isolation of pure stem cell populations for clinical application.

This study uses a 3D dielectrophoretic well chip device to determine the dielectric characteristic of two osteosarcoma cell lines (MG-63 and SaOS-2) and an immunoselected enriched skeletal stem cell fraction (STRO-1 positive cell fraction) of human bone marrow. The skeletal cells were exposed to a series of different frequencies to induce dielectrophoretic cell movement and a model determined to generate the membrane and cytoplasmic properties of the skeletal cell populations.

Differences were observed in specific membrane capacitance in MG-63, SAOS-2 and STRO-1 enriched skeletal populations. These studies provide evidence of the ability to characterise different human skeletal stem and mature cell populations, based on dielectrophoretic properties providing a parameter that could be exploited at a later stage to separate the cells from heterogeneous populations in a DEP based sorting device in the absence of biochemical labels or bioengineered tags.

These studies provide proof of concept for the use of a DEP-based cell characterisation for use in the detection and isolation of skeletal cell populations from a heterogeneous sample such as human bone marrow.

Introduction

Alternating current (AC) electrokinetics was described five decades ago by Herbert Pohl (1951) as a process which could be used to manipulate microscopic particles; subsequent work showed that the movement of the particles could be used to infer chemical and/or biological properties. Dielectrophoresis (DEP) is an example of an AC electrokinetic technique, wherein the particles subjected to a non-uniform electric field and can be manipulated, separated and analysed in terms of, for example, cellular scale particles, using differences in the electric polarisability of particles and the surrounding liquid (Hughes 2002). The magnitude and direction of the force are frequency dependent; negative DEP is when cells are repelled from high-field regions at the edge of electrodes whilst positive DEP describes the motion of cells towards high electric field regions. Thus, DEP offers a novel method by which cells can be separated based on their unique dielectrophoretic profile, provided there are differences in the DEP response of the two populations. The development of devices that use DEP for the characterisation and later separation of cells has a broad range of applications in biomedical sciences. Typically for a heterogeneous cell population FACS or MACS is used to separate cell subsets, which is reliant on the identification and availability of unique cell surface antigens and their specific antibodies.

The development of a DEP-based separation technique would potentially remove the need for biochemical labels and tags, a particularly relevant issue where these have yet to be identified. The dielectric properties of stem or progenitor cells are of particular interest as their identification and separation from heterogeneous cell populations would allow both the investigation of the biology of these cells, and the potential use of them in regenerative medicine. Skeletal stem cells were originally identified and described as colony forming unit-fibroblastic (CFU-F) (Owen and Friedenstein 1988). The cells are derived from human bone marrow and can be isolated in culture as adherent, clonogenic cells which have the ability to give rise to all cells of the major skeletal tissues, such as cartilage, bone, adipocytes and connective tissue (Robey, Kuznetsov et al. 2007). In order to identify the cells, the cell surface antigen profile has been characterised and it is noted skeletal stem cells do not express the majority of haemopoietic markers, or express a variety of adhesion molecules and co-stimulatory molecules. To circumvent some of the issues around identification and characterization of human bone marrow cell populations, a number of groups have attempted to generate antibodies which recognise these cell subsets including CD105 (SH2/Endoglin), CD73 (SH3/4), CD44, CD90 (Thy-1), CD 71, the STRO-1 antigen and adhesion molecules CD106 (VCAM-1), CD166 (ALCAM), ICAM-1 and CD29 (Simmons and Torok-Storb 1991b; Haynesworth, Barer et al. 1992; Gronthos, Graves et al. 1994; Joyner, Bennett et al. 1997; Pittenger, Mackay et al. 1999; Le Blanc, Tammik et al. 2003; Tare, Babister et al. 2008); (Bruder, Horowitz et al. 1997; Gronthos, Fitter et al. 2007)

STRO-1, a trypsin-resistant cell surface marker, is a commonly used marker to enrich stromal cell populations for an osteoprogenitor phenotype which includes the adherent high growth potential CFU-F (Simmons and Torok-Storb 1991b; Gronthos, Graves et al. 1994). The STRO-1 positive cell fraction has been shown to label approximately 7-11% of the human bone marrow cell population depending upon the method of sorting (Simmons and Torok-Storb 1991b; Tare, Babister et al. 2008), typically MACS (magnetically activated cell sorting) and FACS (fluorescent activated cell sorting) (Chalmers, Zborowski et al. 1998). To improve cell sort strategies, in the last few decades, the concept of 'Lab-on-a-chip' has emerged (Effenhauser and Manz 1994), using several laboratory functions/equipment in single devices in combination with small volumes of sample in order to enhance and efficiently prosecute analysis and processing. The application of microfluidic techniques for isolation and characterisation of individual cells, including skeletal stem cells, offers a potential approach for the selection of pure cell populations for regenerative medicine, as well as offering innovative approaches for characterisation of selected cell populations.

DEP strategies have been used for cell isolation and characterization, thus, CD34+ cells have been enriched from peripheral blood stem cell harvests (Stephens, Talary et al. 1996), and more recently the unique dielectric properties of undifferentiated and differentiated stem cell populations has been determined (Flanagan, Lu et al. 2008). The use of negative DEP and a ring trap system has also been used for the isolation and recovery of single human osteoblast-like cells (Thomas, Mitchell et al. 2010). However, to date there have been few studies looking at the effects of DEP on skeletal stem cells and osteoblast like cells and their characterisation.

In this paper we examine whether populations of skeletal stem cells can be identified based on a unique DEP profile, which would then allow the potential sorting of these cells from heterogeneous populations. In the current study a novel 3-D dielectrophoretic device fabricated from a laminate of copper and polyimide layers, as described by Hoettges et al (Hoettges, Hubner et al. 2008). The design of the three-dimensional electrode is relevant to the 'lab-on-a-chip' systems that are currently being developed for use in research and diagnostics in biological sciences. The capability of this system has previously been shown in determining the effects of different drugs on red blood cells (Hubner, Hoettges et al. 2005), and antibiotic resistance in *E.Coli* (Hoettges, Dale et al. 2007). The DEP profile of MG-63 and SAOS-2 osteosarcoma cell lines (representative of early and mature bone cell populations respectively) was determined by measuring the DEP response of osteosarcoma cell lines as well as the immunoselected STRO-1 positive cells obtained from human bone marrow aspirates, and found that these cells display unique dielectric properties.

Materials and Methods

Cell culture – Two osteosarcoma cell lines MG-63 and SAOS-2 lines were used (obtained from ECACC, catalogue numbers 86051601 and 89050205 respectively), as well as STRO-1 positive cells isolated from bone marrow samples obtained from haematologically normal patients undergoing routine total hip replacement surgery for a fractured neck of femur or as a result of osteoarthritis. The cell lines were cultured using D-MEM supplemented with 10% foetal calf serum (Invitrogen, UK) and were routinely passaged at approximately 70-80% confluence.

STRO-1 positive populations were isolated from primary cultures of bone marrow cells as follows: marrow aspirates were washed in α -MEM (Minimum Essential Medium alpha modified, Sigma-Aldrich Ltd) and the suspended cells were then centrifuged and the immunoselection process continued as previously described (Simmons and Torok-Storb 1991b; Howard, Partridge et al. 2002). Briefly, red blood cells were removed via centrifugation with Lymphoprep (Axis-Shield Diagnostic Ltd) solution and following a blocking step, cells were then incubated with the STRO-1 antibody hybridoma (gifted from Dr J. Beresford, University of Bath). Following washes with MACS Buffer (HBSS (Hank's Buffered Salt Solution) containing 1% BSA (bovine serum albumin) fraction v), cells were incubated with MACS anti-IgM beads (Miltenyi-Biotec LTD, UK), before passing the cell suspensions through a MACS column in the presence of a magnet to give the negative fraction. The column was then washed and in the absence of the magnet, MACS buffer was passed through the column to yield the STRO-1 positive fraction. The culture was maintained in α -MEM supplemented with 10% foetal calf serum and penicillin (5000u/ml); streptomycin (5000 μ g/ml) (Lonza, Switzerland). Cells were cultured in a humidified incubator at 37°C and 5% CO₂ with media changes occurring every third day.

Cell preparation

To prepare the cells for the DEP studies, cells were suspended in an isotonic medium consisting of 8.5% sucrose and 0.3% dextrose buffer (Broche, Bhadal et al. 2007) with the conductivity of the medium adjusted to 10 mS/m using PBS. Cells were trypsinised (0.25% trypsin and 0.05M EDTA, Lonza, Switzerland), centrifuged and washed twice in buffer before final resuspension in 1ml of buffer. The ideal cell concentration found was between 1×10^6 - 1.5×10^6 /ml.

DEP Experiments

DEP spectra were recorded using the DEP-well system described elsewhere (Hubner, Hoettges et al. 2005; Hoettges, Dale et al. 2007; Hoettges, Hubner et al. 2008). Briefly, the device itself consisted of a laminate of metal and polymer layers, where consecutive metal layers are connected to opposite phases of a signal generator. The wells are then defined through the

lamine and the resulting structures comprise of thin rings of electrodes which are evenly spaced around the walls of the well (Hoettges, Dale et al. 2007). In the system the high electric field is found at the perimeter of the well and the low electric field found in the centre. Light is passed through the well and MatLab (The Math-works Inc, Nantick MA) captures the images of the well at set time intervals using an AVT Dolphin camera. This is then compared to the initial image taken and the change in light intensity calculated. Cells experience positive DEP when the polarisability of the cells is greater than that of the surrounding medium, this acts to pull the cells toward the electrodes, removing them from the light path and increasing light absorption. In negative DEP the cells are pushed into the light path and there is a decrease in the light absorption. Various MatLab scripts performed all imaging, image and signal processing and data analysis (Hoettges, Hubner et al. 2008).

Data and Statistical Analysis

For each cell line, experiments were repeated a minimum of 3 times, using different cell populations. A total of 24 frequencies were applied, ranging from 1.6kHz to 20MHz at 5 points per decade, with each frequency being run for 1 minute in total. The obtained frequency spectra were analysed using a single-shell model (Irimajiri, Hanai et al. 1979) to derive the dielectric properties using the average summed results. The single cell model assumes that one outer shell consists of the membrane and cell wall, with the inner sphere consisting of the cytoplasm. The 'best-fit model' was found by matching the curve to the measured data and adjusting the dielectric cytoplasmic and membrane parameters until the best match was found.

The entire well was monitored during the course of the experiment, with the well divided into 10 segments, which were monitored separately. For the characterisation analysis, segments 7-9 were analysed exclusively, since the applied field does not reach across the well diameter (Hoettges, Hubner et al. 2008).

The radii of 50 cells were measured and the average calculated using Image J (downloaded from <http://rsbweb.nih.gov/ij/>). All experiments were repeated at least 3 times (unless otherwise stated); values are expressed as mean $\pm$ standard deviation (SD).

Results

Cell Morphology

The MG-63 and SAOS-2 osteosarcoma cell lines were cultured under standard conditions prior to preparation for DEP analysis. MG-63 cells displayed a characteristic polygonal morphology and at

the density seeded remained distinct cell entities in culture. In contrast, SAOS-2 cells, which were similarly cultured in monoculture conditions, displayed an epithelial-like morphology. Both osteoblast populations were allowed to reach 80% confluence before DEP analysis. The premise examined was that an undifferentiated cell phenotype would result in a different DEP profile to an undifferentiated cell type. STRO-1 positive cells in culture displayed a similar fibroblastic morphology to the human bone marrow population from which they were isolated (Figure 1). A 1 μ m difference in cell radii was measured between the MG-63 and SAOS-2 cells.

Dielectrophoretic characteristics of osteoblast populations

Representative graphs obtained using the DEP-well system are presented for each of the cell types MG-63, SAOS-2 and STRO-1 positive cells in Figures 2-4 respectively. The Y-axis shows arbitrary values corresponding to the light intensity change in the well. Values less than zero correspond to frequencies at which cells experience negative DEP while values greater than zero correspond to positive DEP. The data show that the cells exhibited both positive and negative DEP depending on the applied frequency. Typically, cells were observed to exhibit a negative response at the lower frequencies and a positive response at the higher frequencies. In all three cell populations, cell collections (positive DEP) were observed at frequencies of more than 10kHz, with force reducing as the applied frequency exceeded 1MHz. The data were fitted using a best-fit model where the cell electrical parameters were varied until a maximum value of standard error was achieved, using measured values of medium properties and cell size.

Table 1 shows a summary of the cellular characteristics as derived by the modeling of the data. Conductivity is a measure of the ability to conduct an electric field whilst the permittivity is a measure of the cytoplasm or membranes ability to transmit an electric field. The MG-63 cells exhibited a cytoplasmic conductivity (σ_{cyto} , reflecting the ionic concentration) of 0.23S/m (the lowest of the three cell types), and the highest specific membrane capacitance (C_{spec} , reflecting membrane morphology) at 16.0 mF m⁻². The SAOS-2 cells exhibited the highest cytoplasmic conductivity of 0.52 S/m and a specific membrane capacitance of 13.6 mF m⁻². Finally, the STRO-1 positive fraction of human bone marrow cells exhibited the lowest cytoplasmic conductivity of 0.34 S/m and a specific membrane capacitance of 10.7 mF m⁻². Thus the C_{spec} activity was 60% higher in the MG-63 cell population than the STRO-1 positive population, and 30% higher than the SAOS-2 cell population. The smallest cells were measured to be the SAOS-2 cells, which were also determined to have the highest cytoplasmic conductivity. Table 2 shows a percentage differences in cellular characteristics of the three cell types. The greatest difference in cytoplasmic conductivity and C_{spec} was seen between MG-63 and SAOS-2 cells of 77.33%, and 13.27% respectively. The greatest difference in G_{spec} was seen between MG-63 and STRO-1 cells which showed a 39.70% difference.

Discussion

The studies performed here have demonstrated the importance of harnessing the dielectrophoretic properties of cell populations and the potential of a DEP-based cell characterisation device for use in the detection and isolation of skeletal cell populations from a heterogeneous sample such as human bone marrow. Specifically, the current studies have shown that the specific membrane conductance (G_{spec}) could be used as a discriminating factor to separate mixed MG-63 and SAOS-2 populations, or, equally a mixed population with STRO-1 cells. The dielectric parameters were determined by finding the best-fit combination of values corresponding to the membrane and cytoplasmic properties of the cell as well as the (measured) medium properties and cell radius, using a single-shell dielectric mathematical model. The relative permittivity of the medium was assumed to be 78, equal to that of water (Huang, Wang et al. 1995) and medium conductivity set at 10 mS/m. The best-fit model was determined by matching the curve to the measured data, altering the dielectric parameters of the membrane and the cytoplasm until a best match was found. Inconsistency in the cellular profile was observed occasionally and may have been due to dead cell populations in the solution, or cells exposed to the electric field multiple times giving scattered data points at the highest plateau in the curve. Previous studies have also looked at changes in membrane properties between apoptotic and healthy cells which clearly differentiate viable cells versus cells undergoing apoptosis (Labeed, Coley et al. 2006).

The smallest cells were measured to be the SAOS-2 cells, which were also determined to have the highest cytoplasmic conductivity. This could be explained by the cytoplasm of the SAOS-2 cells being more concentrated than that of the other two cell types. Although the difference in radius of the SAOS-2 cells compared to the MG-63 cells is small, the 30% reduction in volume may have a significant influence on the conductivity of the cytoplasm. A similar reduction in cell size was observed in K562 (human myelogenous leukaemia) cells following treatment with drugs that induce apoptosis. DEP was used to detect early apoptosis in these cells, with a low σ_{cyto} measured in the apoptotic cell population. A possible mechanism proposed for this was that throughout the apoptotic process, cells become smaller, causing the cytoplasm to become concentrated with the remaining ions as the water is effluxed in an attempt to restore the osmotic balance, increasing the conductivity of the interior (Chin, Hughes et al. 2006). Typically a very low σ_{cyto} value (approaching that of the suspending medium) is indicative of necrosis, as the membrane permeabilizes and cell interior equilibrates with the medium (Chin, Hughes et al. 2006). Due to limitations on the higher voltages that could be reached by the signal generator available, a more accurate representation of the cytoplasmic properties of the cells could not be obtained.

Cspec was 60% higher in the MG-63 cell population than the STRO-1 positive population, and 30% higher than the SAOS-2 cell population. In addition, the smallest cells were measured to be the SAOS-2 cells, which were also determined to have the highest cytoplasmic conductivity. The relationship between cell size and cytoplasmic conductivity has previously been discussed in relation to healthy and apoptotic cells (Labeed, Coley et al. 2006). No statistically significant difference was observed in the Gspec values across the cell types. However, the differences observed in the Gspec of the cells could potentially be used as a means of separation should the cells be placed in a heterogeneous mixture, particularly in the case of MG-63 versus SAOS-2 cells and MG-63 versus STRO-1 positive cells.

Membrane capacitance (Gspec) is the measure of the membrane that acts as a barrier toward, and can accumulate, ionic charges in the suspending medium in response to the applied electric field; membrane conductance (Cspec) reflects the net transport of ionic species across the membrane and through pores and ion channels (Wang, Becker et al. 2002). No significant difference was observed in the Cspec of the cell types, however the cytoplasmic conductivity and Gspec showed differences that could potentially be exploited for the purposes of cell separation. Three possible explanations may account for the differences observed in the specific membrane capacitance values for the different cell types, namely, differences in the thickness, composition, and folding of the membrane (Gascoyne, Wang et al. 1997b). Since an increase in the Cspec equals an increase in the membrane surface area, which could be the result of more folding taking place in the membranes of these cells (Labeed 2004). Membrane capacitance is proportional to cell surface area (Holevinsky and Nelson 1998), as no significant changes have been observed in the membrane capacitance of the cells it can be implied that the cell surface areas of the cells are comparable. Membrane capacitance is an important technique for studying exocytosis, endocytosis, and stimulus secretion coupling mechanisms in a variety of cells. Changes in the membrane capacitance provide a direct and quantitative record of the time course of cytoplasmic granule insertion into the plasma membrane during exocytosis (Holevinsky and Nelson 1998).

Exploiting the differences in the Gspec values and altering the conductivity of the media used could enable separation of the mixed cell populations, since the conductivity of the surrounding media affects the membrane conductance under the influence of the electric field. Membrane conductance reflects the transposition of charge carriers across the membrane through membrane channels and pores. If the suspension conductivity is high enough, it can effectively suppress the influence of membrane conductance on cell electrokinetic responses. As yet there are no examples in the literature that have used differences in the specific membrane conductance of the cells as a cell sorting strategy although the idea was suggested by Gascoyne and Vykoukal. In a study by Thomas et al (2010) the isolation and recovery of specific cells was demonstrated using

dielectrophoretic ring traps. Of particular relevance to the studies performed here, the target cell population was the osteoblast-like cell line MG-63 cells. Using the ring traps, the authors were able to recover low numbers of cells illustrating the potential of such a dielectrophoretic device for cell isolation from heterogeneous populations although this technique still relied on the determination of sufficient surface antigens to enable identification of stem cells with fluorescent markers.

Conclusion

These studies have provided proof of concept for the potential of a DEP-based cell characterisation device for use in the detection and isolation of skeletal cell populations from a heterogeneous sample such as human bone marrow. In these studies, differences in the specific membrane conductance of a mixed population of MG-63 and SAOS-2 cells could be exploited in order to develop the sorting strategy. The development of the DEP well device to perform characterisation and sorting in a sterile environment would provide a 'lab-on-a-chip' tool that would provide a cheap and quick way of identifying cells of interest in a heterogeneous cell population.

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MG-63

SAOS-2

Human Bone Marrow

**STRO-1 positive selected cells
from HBM**

Figure 1: Cells in culture. MG-63 cells show a typical polygonal morphology whilst SAOS-2 cells are epithelial-like. Both human bone marrow cells and STRO-1 positive isolated cells display a fibroblastic morphology. Scale bars represent 100 μ m.

Figure 2: Light intensity (arbitrary units) DEP spectra for MG-63 cells, together with a ‘best-fit’ model from which the dielectric properties were determined. The data was obtained by measuring the change in light intensity across the well after 60s of exposure to frequencies between 1.6kHz-20MHz. Error bars shown depict the error across the well as analysed by the MatLab script. Data drawn from one population.

Figure 3: Light intensity (arbitrary units) DEP spectra for SAOS-2 cells, together with a ‘best-fit’ model from which the dielectric properties were determined. The data was obtained by measuring the change in light intensity across the well after 60s of exposure to frequencies between 1.6kHz-20MHz. Error bars shown depict the error across the well as analysed by the MatLab script. Data drawn from one population.

Figure 3: Light intensity (arbitrary units) DEP spectra for STRO-1 cells, together with a ‘best-fit’ model from which the dielectric properties were determined. The data was obtained by measuring the change in light intensity across the well after 60s of exposure to frequencies between 1.6kHz-20MHz. Error bars shown depict the error across the well as analysed by the MatLab script. Data drawn from one population.

	MG-63	SAOS-2	STRO-1
Cell Radius μm	7.4	6.4	7.7
Cytoplasm Conductivity (σ) S/m	0.23 (0.05)	0.52 (0.11)	0.34 (0.28)
Cspec (mF m^{-2})	16.0 (7)	13.6 (3.3)	10.7 (3.2)
Gspec	408.9 (74.2)	358.0 (70.4)	391.6 (162.5)

Table 1: Cellular characteristics as determined by the DEP well chip and the model applied to the data. Values shown averaged over 3-4 repeats of separate populations, with SD given in brackets.

	Cytoplasmic Conductivity (σ) S/m	Gspec	Cspec (mF m^{-2})
MG-63 vs SAOS-2	77.33	16.22	13.27
SAOS-2 vs STRO-1	41.86	23.87	8.96
MG-63 vs STRO-1	38.60	39.70	4.32

Table 2: Percentage difference in cellular characteristics of three cell types.