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Evaluation of the behavior of murine and human embryonic stem cells in in vitro migration and invasion assays

V. T'Joen, P. Somers*, H. Declercq, M. Cornelissen

Ghent University – UGent, Faculty of Medicine and Health Science, Department of Basic Medical Science – Tissue Engineering Group, De Pintelaan 185 6B3, 9000 Gent, Belgium

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ABSTRACT

Cell migration and invasion are essential processes in a variety of physiological events in the body, but also in several patho-physiological events. In this paper, the behavior of murine and human embryonic stem cells is examined in in vitro migration and invasion models. mESC and hESC were applied as spheroids, also known as patches, and as single cells, to mimic possible cell therapy application strategies. Two known in vitro migration assays, the ECM (extracellular matrix) assay and the Boyden chamber migration assay were selected. These assays revealed that mESC are statistically significantly more infiltrative than hESC. Application as spheroid results in a slightly higher infiltrative capacity compared single cells. The PHF (precultured chick heart fragment) assay was selected as an invasion assay. In the PHF assay a more 3D examination of the infiltrative nature of the ESC can be observed. The mESC showed infiltrative behavior, as spheroids and as single cells. The hESC were infiltrative as single cells but not as spheroids. The results of these assays are mostly complementary and prove the applicability of these assays, which were originally applied in tumor biology, in migratory behavior studies regarding stem cells and their progeny in basic and other conditions.

1. Introduction

An easy and realistic way for obtaining fast tissue regeneration is the local application of cells, a strategy called "cellular based regenerative medicine" or "cell therapy" (Doss et al., 2004; Andersson and Lendahl, 2009). Different cell types including embryonic stem cells (ESC), induced pluripotent stem cells (iPS), bone marrow derived cells (MSC), cord blood stem cells (CB-SC) and other adult stem cells have been suggested as a cell source for these therapies (Smart and Riley, 2008). Several strategies to deliver the cells into the host tissue are being considered, depending on the host tissue in question. Local deposition of cells can be obtained by a single cell injection at the desired site (Smart and Riley, 2008) or by positioning multilayered constructs, also known as cell patches, sheets or aggregates (Stevens et al., 2009). One of the first test steps in a potential cell therapy approach is evaluating the migration/invasion capacity of the cells into the proposed host tissue.

Cell migration and invasion are essential processes in a variety of physiological events such as embryogenesis, wound healing, tissue regeneration, but also in several patho-physiological events such as cancer formation and cardiovascular diseases (Staff, 2001). Cell migration is defined at the movement of individual cells, cell sheets of clusters from one location to another, whilst cell invasion refers to the three dimensional migration of cells (Hulkower and Herber, 2011). In general, it is quite difficult to distinguish between cell migration and invasion as these are closely connected cellular processes which utilize much of the same molecular mechanisms for cell movement. A distinction is sometimes made according to the surrounding through which the cells move. Cell invasion involves the destruction of the basal membrane (BM), whilst cell migration involves only the destruction of the extracellular matrix (ECM) around the cell. This distinction, however, is again not always valid, as the BM can become permeable in some circumstances, e.g. during inflammation, tissue development and tissue repair (Staff, 2001; Valster et al., 2005). The BM is a sheet of specialized ECM, consisting of collagen type IV, heparin sulfate proteoglycans, laminin and sometimes fibronectin that acts as a barrier between tissues. The ECM is a structural component, consisting of collagen (mainly type I), glycoproteins, elastin and proteoglycans that acts as cell support (Albini, 1998).

Several in vitro models are available to study the cell infiltration potential, starting from monolayer co-culture models (Xue et al., 2005; Caspi et al., 2007) to tissue slice cultures (Habeler et al., 2009) and 3D co-culture models (Mareel et al., 1981). The diversity of these assays shows that there is no single superior approach to study cell migration/invasion (Doss et al., 2004; Staff, 2001; Hulkower and Herber, 2011; Valster et al., 2005). In the past, several natural extracellular matrices were used as a migration model, such as bone (Kuettner et al., 1978), cartilage (Pauli et al.,

* Corresponding author. Tel.: +32 9 332 49 19; fax: +32 9 332 38 23.
E-mail address: pamela.somers@ugent.be (P. Somers).

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1981) and amnion membrane (Liotta et al., 1980). However, these models lacked homogeneity and needed to be devitalized, making the interpretation of the migration of the cells rather difficult. To overcome these difficulties, other models based on extracellular matrix components were suggested: Matrigel (Albini, 1998; Albini and Benelli, 2007), Humatrix (Kedeshian et al., 1998) and pepsin-extracted or native acid-extracted collagen type I (Sabeh et al., 2009; Vakaet et al., 1991; Wolf et al., 2003). Currently, widely used simple models for cell migration are the ECM assay, also known as the Collagen I assay, and the Boyden chamber assay, also known as transwell chamber assay. The ECM assay consists of a layer of collagen I which is coated onto plates and left for gelation. After gelation, cells are seeded onto the coating and their migratory properties can be evaluated based on cell morphological screening after 24 h. The Boyden chamber assay consists of a filter chamber with an upper and lower compartment. The cells are placed in the upper compartment and left to incubate. Migratory cells will have advanced into the lower compartment of the chamber after 24 h of incubation and can be subsequently visualized by microscopy, fluorescence of colorimetric analysis (MTT). Several modifications, e.g. by addition of coatings, can be done to the Boyden chamber assay to make it more suitable for assessing cell invasion.

These simple in vitro models have the disadvantage that only minor interactions can be examined and that no complex interactions are present, such as in the real situation. More complex models that include cellular compounds are also applied, e.g. BM and ECM are used for studying cell invasion in tumor biology. Other examples are the chicken embryo heart, also known as the PHF assay (de Ridder et al., 2000), the tissue slice culture (Valster et al., 2005) and other 3D co-culture systems (Cretu et al., 2005). In these models, the normal 3D tissue organization is conserved and the in vivo situation is mimicked more accurately.

In this study, we investigated the behavior of ESC using different assays: the ECM assay, which is a native collagen 1 based assay, a Boyden chamber migration assay and a co-culture assay, using precultured embryonic heart fragments of avian origin, the PHF assay. Murine and human ESC were applied as single cells and as multi-cellular spheroids (patches or aggregates), to see if a different application approach had an effect on the infiltrative behavior towards host material. The infiltration capacity was assessed by phase contrast microscopy, optical density measurements and immunocytochemistry.

2. Materials and methods

2.1. Cell culture

2.1.1. Routine mESC and hESC culture

The mouse embryonic stem cell line used for these experiments was obtained from 3.5 days old blastocysts of the B6D2 mouse strain (Tielens et al., 2006). The human embryonic stem cell lines used for these experiments were the commercially available H1 hES cell line (Wicell) and the 2258 hESC cell line (Department of Reproductive Health, UGent, Gent, Belgium). The ESC were cultured on Mitomycin C (Sigma Aldrich, Bornem, Belgium) inactivated MEF's (mouse embryonic fibroblasts) from MF1 mice. The MEFs were treated with 10 mg/ml mitomycin C for 3 h to arrest mitosis and seeded on 0.1% gelatin (Sigma) coated dishes with a density of 4000 cells/cm^2 for mES culture and at a density of 8500 cells/cm^2 for hES culture. The MEF culture medium consisted of DMEM (Dulbecco's minimal essential medium)-Glutamax (Invitrogen, Merelbeke, Belgium) supplemented with 10% FCS (Fetal Calfs Serum, Invitrogen, Merelbeke, Belgium) + 0.1 mM NEAA (non-essential amino acids, Invitrogen, Merelbeke, Belgium) + 0.1 mM β-mercapto-ethanol (Invitrogen, Merelbeke, Belgium). The mESC medium consisted of DMEM-Glutamax (Invitrogen, Merelbeke, Belgium) supplemented with 1000 units/ml LIF (Leukemia inhibitory factor, Chemicon International) + 0.1 mM NEAA (Invitrogen, Merelbeke, Belgium) + 0.1 mM β-mercapto-ethanol (Invitrogen, Merelbeke, Belgium) and 10% FCS (Invitrogen, Merelbeke, Belgium). The hESC medium consisted of 80% DMEM-F12 (Invitrogen, Merelbeke, Belgium) + 20% Serum Replacement (Invitrogen, Merelbeke, Belgium) + 1 mM L-glutamine (Invitrogen, Merelbeke, Belgium) + 0.1 mM NEAA (Invitrogen, Merelbeke, Belgium) + 0.1 mM β-mercapto-ethanol (Invitrogen) and 4 ng/ml bFGF (Millipore, Brussels, Belgium). The medium was changed daily and the ESC grew as colonies. The routine technique for splitting mESC consisted of treating the cells for 3–4 min with Trypsin–EDTA (Invitrogen) and gentle trituration. The routine splitting technique of the H1 and 2258 cell line involved mechanical passaging.

2.2. Spheroid and suspension preparation for the experiments

For obtaining spheroids, mESC were detached from the falcon using a cell scraper and hESC were detached by addition of 1 mg/ml Collagenase IV solution (Invitrogen, Merelbeke, Belgium) for 40–50 min. Next, the cells were cultured in medium on a gyrotory shaker (120 rpm, 37 °C, 5% CO_2) during 2 days to get stem cell spheroids. For obtaining single cells, the mESC were trypsinized for 5 min (Trypsin–EDTA, Invitrogen, Merelbeke, Belgium) and the hESC were treated with a mild Cell Dissociation solution (Sigma Aldrich, Bornem, Belgium) for 10–20 min. The ESC was centrifuged at 1000 rpm for 5 min. After trituration, a concentrated single cell suspension was obtained by diluting the cells in 0.2 ml of medium.

2.3. Cell characterization

2.3.1. mESC characterization

mESC pluripotency was determined every 10 passages by immunostaining for Oct-4 and SSEA-1 and characterized enzymatically with alkaline phosphatase staining. For Oct-4 and SSEA-1 staining, colonies were fixed in 4% paraformaldehyde for 10 min, washed with PBS and blocked with blocking serum (PBS/1% BSA/5% normal goat serum or rabbit serum/0.2% Tween). Primary antibodies mouse monoclonal anti-Oct-4 (1/50) and mouse monoclonal SSEA-1 (1/50) were incubated for 2 h. After washing, the secondary antibodies (biotin-labeled rabbit anti-mouse IgG or biotin-labeled goat anti-mouse IgG) were incubated for 30 min. Visualization was done using streptavidin–HRP and DAB. The slides were mounted with mounting medium. Alkaline phosphatase staining was done using BCIP/NBT (BCIP/NBT Liquid Substrate System by Sigma Aldrich, Bornem, Belgium) according to manufacturers recommendations. Evaluation was done by light microscopy on the JENA Jenaval microscope (ProgressCapture Pro 2.7.7 software).

2.3.2. hESC characterization

hESC pluripotency was determined every 10 passages by immunostaining for SSEA-3, SSEA-4, TRA 1-60, TRA 1-81 and Oct-4 (isoform 4A) (antibodies from Santa Cruz Biotechnology Inc., Heidelberg, Germany) and with alkaline phosphatase staining (BCIP/NBT Liquid Substrate System by Sigma, Bornem, Belgium). For the immunostaining, colonies were fixed in 1% paraformaldehyde for 10 min, washed with PBS (Phosphate Buffered Saline) and blocked with blocking serum (PBS/5% normal rabbit or goat serum/0.2% Tween) for 30 min. The primary antibodies which were used are rat monoclonal anti-SSEA-3 (IgM 1/200), mouse monoclonal anti-SSEA-4 (IgG 1/500), mouse monoclonal anti-TRA 1-60 (IgM 1/100), mouse monoclonal anti-TRA 1-81 (IgM 1/50) and mouse monoclonal anti-Oct-4 (IgG 1/100). After 1 h of incubation, the cells were washed with PBS and incubated with the secondary antibody (FITC-labeled goat anti-mouse IgM 1/200 or biotin-labeled rabbit anti-mouse IgG) for 30 min. In case a