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Original article

mAb C19 targets a novel surface marker for the isolation of human cardiac progenitor cells from human heart tissue and differentiated hESCs

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ABSTRACT

Aims: Cardiac progenitor cells (CPCs) have been isolated from adult and developing hearts using an anti-mouse Sca-1 antibody. However, the absence of a human Sca-1 homologue has hampered the clinical application of the CPCs. Therefore, we generated novel monoclonal antibodies (mAbs) specifically raised against surface markers expressed by resident human CPCs. Here, we explored the suitability of one of these mAbs, mAb C19, for the identification, isolation and characterization of CPCs from fetal heart tissue and differentiating cultures of human embryonic stem cells (hESCs).

Methods & results: Using whole-cell immunization, mAbs were raised against Sca-1+ CPCs and screened for reactivity to various CPC lines by flow cytometry. mAb C19 was found to be specific for Sca-1+ CPCs, with high cell surface binding capabilities. mAb C19 stained small stem-like cells in cardiac tissue sections. Moreover, during differentiation of hESCs towards cardiomyocytes, a transient population of cells with mAb C19 reactivity was identified and isolated using magnetic-activated cell sorting. Their cell fate was tracked and found to improve cardiomyocyte purity from hESC-derived cultures. mAb C19+ CPCs, from both hESC differentiation and fetal heart tissues, were maintained and expanded in culture, while retaining their CPC-like characteristics and their ability to further differentiate into cardiomyocytes by stimulation with TGFβ1. Finally, gene expression profiling of these mAb C19+ CPCs suggested a highly angiogenic nature, which was further validated by cell-based angiogenesis assays.

Conclusion: mAb C19 is a new surface marker for the isolation of multipotent CPCs from both human heart tissues and differentiating hESCs.

1. Introduction

The heart has long been considered to be an organ with very limited regenerative capabilities. This has been challenged by several studies reporting that the heart harbors a resident population of cardiac progenitor cells (CPCs) that might be used to repair the injured heart [1]. A variety of isolation methods, such as cardiosphere formation, and the use of surface markers like c-kit, kinase domain receptor (KDR) and stem cell antigen-1 (Sca-1), have been utilized to enrich for these cells from both adult and fetal hearts, but there is yet to be a consensus on what markers to use to define these cells [2]. In addition, different isolation methods yield distinct binding profiles of stem cell markers, suggesting that they represent different progenitor populations or different stages of pluripotency. CPCs can potentially be used for cell-based cardiac repair, but in order to achieve feasibility and patient safety when applied for cell therapy purposes, there is a need to develop better tools to define a more homogenous population of resident CPCs. Therefore, the aim of this study was to generate a panel of novel monoclonal antibodies (mAbs) targeting surface epitopes on resident human heart-derived CPCs.

The generated antibodies were selected against heart-derived CPCs, which are isolated based on reactivity against mouse Sca-1 antibody, and can be found in both adult and fetal human hearts [3–5]. These

Abbreviations: CPC, cardiac progenitor cell; mAb, monoclonal antibody; hESC, human embryonic stem cell; MACS, magnetic-activated cell sorting; EB, embryoid body; Sca-1, stem cell antigen-1; KDR, kinase domain receptor; TGFβ1, transforming growth factor beta 1; cTnI, cardiac troponin I; cTnT, cardiac troponin T.

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Sca-1 + CPCs are multipotent, and able to generate all three cell types that make up the heart: cardiomyocytes, smooth muscle cells and endothelial cells. They are amenable to expansion in culture, making them an ideal stem cell source for cell-based cardiac repair. In addition, they have been shown to be able to engraft and differentiate in vivo when transplanted into the infarcted mouse heart and improve survival and long-term cardiac function [6]. The anti-mouse Sca-1 antibody has proven to be useful in identifying a homogeneous and robust population of heart-derived human CPCs. However, there is no Sca-1 homologue in the human genome [7]. To date it remains unclear which epitope on human CPCs is recognized by this antibody, thereby significantly hampering a clinical application. A new panel of mAbs raised against surface epitopes expressed by Sca-1 + CPCs will provide human specific alternatives to replace the anti-mouse Sca-1 antibody in the isolation of human CPCs.

Besides heart-derived CPCs, embryonic stem cell differentiation has been used to model heart development, both in mice and human [8,9]. Key stages of human heart development are known, and the transcription and signaling pathways involved in its regulation are well-established from mouse models. However, conflicting results often arise when looking at surface markers expressed on CPCs and comparing these markers between heart development and the differentiation process of human embryonic stem cells (hESCs) towards cardiomyocytes. These discrepancies are typically attributed to differences in maturity and developmental stages of the progenitor cells [10]. Therefore, our panel of CPC-specific mAbs, can be a powerful tool in addressing some of these fundamental questions regarding the similarities and differences between heart-derived CPCs (heart-CPCs), and CPCs isolated from in vitro hESC differentiation (hESC-CPCs).

In this study, we describe the use of mAb C19, one of the antibodies from our panel of heart-CPC-specific mAbs, as a novel surface marker alternative for Sca-1 in the isolation of CPC populations. mAb C19 was found to recognize a glycosylated form of the glucose-regulated protein, 78 kDa (GRP78) found on the cell surface of CPCs. We characterized mAb C19 binding to Sca-1 + CPCs and performed heart tissue microarrays to show its ability to enrich for a multipotent cardiovascular CPC population from both differentiating hESC cultures and primary heart tissue.

2. Methods

2.1. Culture and differentiation of heart- and hESC-CPCs

For human fetal tissue collection and atrial biopsies, individual permission was obtained using standard informed consent procedures and conforms to the Declaration of Helsinki. Prior approval of the ethics committee of the Leiden University Medical Centre was granted. Sca-1 + CPCs isolated from cardiac tissue were used for immunization and antibody screening (Supplementary methods). CPCs isolated from both heart tissue and hESC culture were maintained in culture as previously described [3]. The differentiation of these CPCs was induced with 5 μM 5-azacytidine (Sigma) in the first 3 days of differentiation, followed by transforming growth factor beta-1 (TGFβ1) (Peprotech) stimulation at 1 ng/ml.

2.2. hESC culture and differentiation into cardiomyocytes

The HES-3 (ES Cell International) cell line was used in this study. hESCs were maintained as co-cultures on inactivated mouse feeders as described previously [11]. The directed differentiation protocol follows that described by Lecina et al. [12]. Briefly, HES-3 co-cultures were mechanically dissociated with EZ-Passage to form aggregates in ultra-low attachment plates in serum-free medium supplemented with 5 μM of the p38 MAPK inhibitor, SB203580 (Sigma), for the first 8 days. The medium, including inhibitor, was refreshed every other day.

For continued differentiation after sorting, cells were harvested by incubation with TrypLE (Invitrogen) at 37 °C for 5 min. The single cell suspension was re-aggregated by centrifugation at 100 ×*g* for 5 min in Aggrewell 800 plates (StemCell Technologies) to generate embryoid bodies (EBs) of 5000 cells in size. After 2 days, EBs were transferred to 24-well plates at 80 EBs/well, and allowed to mature with medium change every two days.

2.3. Enrichment of mAb C19 and anti-Sca-1 binding cells with MACS

Magnetic bead separation based on the MACS technology (Miltenyi Biotec) was used to enrich for mAb C19 + cells from differentiating hESCs. Cells harvested on Day 7 of hESC differentiation were incubated at 4 °C with mAb C19 for 30 min, followed by anti-mouse FITC (1:500, DAKO) for 15 min, and finally with anti-FITC microbeads (Miltenyi Biotec) for 15 min, before adding the cell suspension to the magnetic column for cell separation. The flow-through (mAb C19-) and eluted (mAb C19+) fractions were collected for analysis. Heart-CPCs were isolated from heart tissue as previously described ([3] and Supplementary Methods).

2.4. Gene expression and microarray study

Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and cDNA was generated using the Maxima Reverse Transcription system (Thermo Scientific) according to the manufacturer's protocols. Real-time PCR with SYBR green detection was performed using an ABI Prism 7500 Fast thermocycler (Applied Biosystems) with primers listed in Supplementary Table 1. Gene expression was normalized against GAPDH (unless otherwise stated) as the housekeeping gene and samples were run as triplicates. For microarray studies, total RNA was processed with the Affymetrix 3' IVT Express Kit and the resultant labeled cRNA hybridized to GeneChip Human Genome U133 Plus 2.0 Array. The GeneChip Command Console Software was used for acquisition, normalization and analysis, and DAVID [13] was used for bioinformatics analysis and gene ontology classification.

2.5. Angiogenesis assay

In vitro tube formation assay for the sorted cells was conducted with the Angiogenesis Kit (Millipore). Cells were cultured for 20 h at 37 °C/5% CO_2 in EGM-2 (Lonza) supplemented with 50 ng/ml vascular endothelial growth factor (VEGF). Phase contrast images were taken and analyzed using the Angiogenesis Analyzer plugin for ImageJ (developed by Gilles Carpentier, http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ).

2.6. Statistics

Unless otherwise stated, experiments were conducted minimally in triplicates. Unpaired Student's t-test was used to calculate the p-value, of which $p < 0.05$ is deemed to be biologically significant.

3. Results

3.1. Characterization of mAb C19 and its binding to CPCs

A panel of monoclonal antibodies was raised against heart-derived Sca-1 + CPCs (Supplementary Table 2), of which mAb C19 was selected for further investigation. mAb C19 was shown by flow cytometry to bind strongly (>90%) to the CPC line used for immunization, and two other Sca-1 + CPC lines derived from separate patients. In comparison, negligible binding (<5%) towards hESCs (HES-3) and 2 human fibroblast lines (IMR90 and hFF) was observed (Fig. 1A & S1A). Immunocytochemistry staining on Sca-1 + CPC, hESC, and fibroblast lines verified