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Research Article

Retinal differentiation of human bone marrow-derived stem cells by co-culture with retinal pigment epithelium *in vitro*

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ABSTRACT

The goal of this study was to assess the *in vitro* differentiation capacity of human bone marrow-derived stem cells (hBMSCs) along retinal lineages. Mononuclear cells (MNC) were isolated from bone marrow (BM) and mobilized peripheral blood (mPB) using Ficoll-Paque density gradient centrifugation, and were sorted by magnetic-activated cell sorting (MACS) for specific stem cell subsets ($CD34^+CD38^+$/$CD34^+CD38^-$). These cells were then co-cultured on human retinal pigment epithelial cells (hRPE) for 7 days. The expression of stem cell, neural and retina-specific markers was examined by immunostaining, and the gene expression profiles were assessed after FACS separation of the co-cultured hBMSCs by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Furthermore, *in vitro* functionality of the differentiated cells was analyzed by quantifying phagocytosis of CY5-labeled photoreceptor outer segments (POS). After 7 days of co-culture, hBMSCs adopted an elongated epithelial-like morphology and expressed RPE-specific markers, such as RPE65 and bestrophin. In addition, these differentiated cells were able to phagocytose OS, one of the main characteristics of native RPE cells. Our data demonstrated that human $CD34^+CD38^-$ hBMSC may differentiate towards an RPE-like cell type *in vitro* and could become a new type of autologous donor cell for regenerative therapy in retinal degenerative diseases.

Abbreviations: hBMSC, human bone marrow-derived stem cells; BM, bone marrow; mPB, mobilized peripheral blood; hRPE, human retinal pigment epithelium; AMD, age-related macular degeneration; POS, photoreceptor outer segments; FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting.

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Introduction

The retinal pigment epithelium (RPE) is a monolayer of pigmented cells that forms part of the outer blood retinal barrier and plays various roles in maintaining retinal function [1]. Thereby, the RPE provides essential support for the long-term preservation of retinal integrity and visual functions by absorbing stray light, regenerating visual pigment, supplying nutrients, secreting growth factors, and phagocytosing the shed photoreceptor outer segments. Furthermore, RPE cells display a rather unusual feature of plasticity [2]. A dysfunctional RPE causes impairment and death of the photoreceptor cells, leading to deterioration or a central loss of vision. These mechanisms play an important role in the pathogenesis of age-related macular degeneration (AMD).

AMD is the leading cause of irreversible blindness in the industrialized world [3]. More than 8 million people have age-related macular degeneration, and the overall prevalence of advanced AMD is projected to increase by more than 50% by the year 2020 [4]. Current therapeutic strategies for the disease include pharmacological treatment, microsurgery, and cell replacement. Autologous translocation of the RPE has been also employed. However, outcomes are variable, and such manipulation is technically difficult and prone to adverse side effects. More importantly, this surgery is unable to regain lost central vision [5,6]. On the other hand, partial restoration of visual function was reported in humans, mainly after autologous RPE layer transplantation [7].

Recently, stem cell-based therapy for retinal degeneration has been proposed with the development of stem cell technology [8] and has been tested in animal models for several retinal degenerative diseases [9]. Additionally, several clinical trials using SC-derived RPE cells as a treatment option for AMD are under way [10]. New findings have recently contradicted central dogmas of commitment of adult SC including bone marrow-derived stem cells (BMSCs) by showing their plasticity to differentiate across tissue lineage boundaries, irrespective of classical germ layer designations [11]. In this regard, there is evidence that the bone marrow contains subsets of cells, which are capable of multi-lineage differentiation into cells with non-hematopoietic capabilities [12]. Furthermore, Li et al. showed in the mouse system that BMSC could be induced into the RPE lineage *in vitro* [13]. Thereby, RPE co-culture provided microenvironment and cues for such directed differentiation. One factor involved could be pigment epithelium-derived factor (PEDF), which is released by the RPE and known for its neurotrophic/neuroprotective and anti-angiogenic actions that support the growth/differentiation of photoreceptors and neural retinal cells [14].

Several subsets of SCs have been found in the bone marrow (BM) including hematopoietic stem cells, the source of all blood cells, and mesenchymal stem cells, adherent, non-hematopoietic stromal cells that differentiate into mesenchymal tissues [15]. Thereby, the expression of the CD34 membrane antigen provides a useful tool to discriminate the hematopoietic stem cell population [16]. Moreover, CD34-enriched populations of HSC and blood progenitor cells were also used in experimental setups for clinical use [17]. However, the $CD34^+$ population is heterogeneous and includes differentiating cells, committed progenitor cells, early multipotent progenitors and stem cells. Recent studies have shown that the $CD34^+CD38^-$ population is highly enriched for stem cells but depleted of lineage-committed precursors [16].

In this study, the differentiation potential of human bone marrow-derived $CD34^+CD38^-$ stem cells toward RPE cells was investigated by immunostaining and by studying the gene expression of retina-specific markers. Further, the pre-differentiated BMSCs were functionally characterized by an *in vitro* phagocytosis assay to quantify this crucial RPE characteristic.

Materials and methods

Isolation of stem cells

Human bone marrow (BM) was obtained with informed consent from T-cell acute lymphatic leukemic patients by aspiration from the posterior iliac crest and collected in heparin-containing tubes. Alternatively, peripheral blood (PB) was collected from non-Hodgkin lymphoma patients by leukapheresis after the standard mobilization protocol. All procedures were approved by the local ethics committee and in accordance with the tenets of the Declaration of Helsinki.

BM or PB mononuclear cells (MNCs) were isolated by Ficoll-Paque™ PLUS (1.078 g/ml; GE Healthcare, Freiburg, Germany) density-gradient centrifugation according to the manufacturer's protocol. In a first step, enrichment for $CD34^+$ immature hematopoietic precursor cells was performed by immunomagnetic separation using the CD34 MultiSort kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). Subsequently, separation of primitive $CD34^+CD38^-$ stem cells and $CD34^+CD38^+$ common progenitor cells was performed using CD38 MicroBeads (Miltenyi Biotec) also according to the manufacturer's instruction. Briefly, the $CD34^+$ cells were indirectly magnetically labeled with CD38-biotin and anti-biotin MicroBeads. The $CD34^+CD38^+$ cells were depleted on a MACS column in the magnetic field of the MACS separator. The non-magnetic fraction contained the $CD34^+$ $CD38^-$ cells. The purity of the sorted cell subsets was determined by FACS analysis (FACS LSR II, BD BioSciences, Allschwil, Switzerland). Data were analyzed with FlowJo software (Version 7.6.3; TreeStar Inc., Ashland, OR, USA).

RPE preparation

Human RPE cells were prepared from donor eyes after approval by the local ethical committee. Connective tissues were removed, and eyes cups were rinsed with 2% dispase (Invitrogen, Carlsbad, CA, USA —supplier for most of the *in vitro* consumables) in Dulbecco's Modified Eagle Medium (DMEM) at 37 °C for 8–10 min and then incubated in trypsin for 37 °C for 60 min. RPE cells were gently peeled mechanically with a rounded glass stick and triturated gently ten times with DMEM plus 10% fetal bovine serum (FBS) using a glass Pasteur pipette. The cells were centrifuged at 1200 rpm for 5 min and resuspended in DMEM with 10% FBS. The cells were then plated onto fibronectin-coated (10 µg/ml) culture dishes and cultured in DMEM, 10% FBS, and gentamicin. The medium was changed twice a week until the cultures reached confluency, which usually occurred by 2–3 weeks. The cultures were then passaged using 1×10^6 cells per 75 cm^2 culture flask. Meanwhile, cells were routinely stained for the RPE markers RPE65 and bestrophin (see below) so that their epithelial origin could be proven.

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