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Magnetic stromal layers for enhanced and unbiased recovery of co-cultured hematopoietic cells

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ABSTRACT

Cell co-culture systems have a long history of application in hematology and hold promise for successful hematopoietic stem and progenitor cell expansion. Here we report that various types of stromal cells used in such co-cultures can be rapidly and efficiently labeled with L-lysine-modified Fe_3O_4 magnetic nanoparticles. Hematopoiesis-supporting activity does not seem to be compromised after magnetic labeling of stromal cells, and the loss of the label by stromal layers during extended culturing is negligible. Magnetic labeling allows for simple and efficient removal of stromal component, yielding unbiased hematopoietic cell populations. When Lin^- bone mouse marrow fraction was co-cultured with magnetic stromal layers and resulting cell populations were harvested by trypsinization, the yields of total nucleated cells, colony forming cells, and phenotypically primitive $Lin^-Sca\text{-}1^+c\text{-}kit^+$ subset were substantially higher as compared with nonadherent cell fractions harvested after conventional stromal co-culture. The advantage offered by the magnetic stroma approach over the traditional one was even more significant after a second round of co-culture and was more dramatic for more primitive hematopoietic cells. We conclude that magnetic stromal layers represent a simple, efficient, and convenient tool for co-culturing and subsequent recovery of sufficiently pure unbiased populations of hematopoietic cells.

Cell co-culture systems are finding increasing application in cell biology and tissue engineering [1,2]. In hematology in particular, their use [3] has a long history, beginning with the pioneering work by Dexter and coworkers on long-term bone marrow hematopoietic cultures [4]. Culturing of hematopoietic cells on different types of primary cells or established stromal cell lines has led to the discovery of a number of proteins that play an important role in support of hematopoietic stem and progenitor cells [5–7]. Stromal co-cultures are now viewed by some researchers as a promising alternative to cytokine-based culturing of hematopoietic stem cells [8–11] because they better mimic the bone marrow environment by providing extra growth factor support and adhesive interactions. Some of the recent studies using stromal co-cultures demonstrate promising results [9,11,12]. However, one of the major technical drawbacks of co-culture systems lies in the extensive presence, even prevalence, of stromal cells after co-culture, which may interfere with many downstream applications and assays. The most widely used approach to this problem so far has been to maintain integrity of stromal layers and collect nonadherent hematopoietic cell fraction only at the possible expense of adherent and under-the-stroma hematopoietic cells. Because the latter might be enriched in more primitive progenitors [13], the quality and representativeness of resulting hematopoietic fractions becomes questionable.

One of the potential solutions to the problem is to render stromal cells magnetic by incubation with ferromagnetic particles. Fe_3O_4 magnetic nanoparticles (MNPs), frequently referred to in the literature as SPIOs (superparamagnetic particles of iron oxide or small particles of iron oxide), are now commonly used for tracing

Abbreviations used: MNP, magnetic nanoparticle; IR, infrared; UV, ultraviolet; APS–MNP, 3-aminopropylsilane-modified MNP; APTES, 3-aminopropyltriethoxysilane; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMF, dimethylformamide; MSC, mesenchymal stromal cell; DMEM, Dulbecco's modified Eagle's medium; DMLG, DMEM/low glucose; FBS, fetal bovine serum; PenStrep, penicillin/streptomycin; DMHG, DMEM/high glucose; IMDM, Iscove's modified Dulbecco's medium; Lin^-, lineage-negative; EDTA, ethylenediaminetetraacetic acid; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; CFU, colony-forming cell; LSK, $Lin^-Sca\text{-}1^+c\text{-}kit^+$.

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distribution and fate of transplanted cells in vivo [14,15]. They are able to be efficiently internalized via endocytosis by various types of cells with [16] and without [17] transfection agents. In some publications, labeling efficiencies approaching 100% have been reported [18]. Most of the studies using various approaches including global gene expression profiling agree that the effects of internalized MNPs are mild and do not affect basic cell properties and physiology [19–21], although some authors report more significant effects of MNPs on cells [22]. Importantly, the internalized MNPs seem to be stably maintained in cells provided that no cell division occurs [23].

In the current study, we tested the suitability of MNPs to prepare magnetic stromal layers for co-culturing hematopoietic cells. We show here that labeling of several frequently used stromal cell types by L-lysine modified MNPs is rapid and efficient and does not appreciably affect their hematopoiesis-supporting properties. Following co-culture with magnetic stroma, sufficiently pure hematopoietic cell populations that contain both nonadherent and stroma-associated cells can be easily and conveniently isolated.

Materials and methods

Preparation and analysis of L-lysine modified MNPs

A scheme of the synthesis is shown in Fig. 1A. Fe_3O_4 nanoparticles (20–40 nm) were produced by the gas condensation method [24] at the Institute of Metal Physics (Ural Branch of the Russian Academy of Sciences). Infrared (IR) spectra in a range of 400–4000 cm^{-1} were recorded on a Nicolet 6700 FT-IR spectrometer (Thermo Scientific) with a Smart Orbit ATR accessory. Spectrophotometric studies in the range of 190–450 nm were performed with a Shimadzu UV2401PC ultraviolet (UV) spectrophotometer. Morphological studies of particles were performed using a Philips CM30 transmission electron microscope.

*Preparation of 3-aminopropylsilane-modified MNPs (**2**)*

The 3-aminopropylsilane-modified MNPs **2** (APS–MNPs) were obtained similar to the procedure described previously [25]. In a typical experiment, unmodified Fe_3O_4 MNPs (0.200 g) were suspended in 50 ml of 0.05 N NaOH under sonication for 10 min. The suspension was heated to 60 °C in a water bath for 5 h and kept overnight at room temperature. MNPs were then separated using an external magnetic field, washed with water to neutral pH, and evaporated to dryness. For surface modification, 0.200 g of the obtained nanoparticles was suspended in 100 ml of 95% ethanol under sonication for 20 min. 3-Aminopropyltriethoxysilane (APTES, **1**, 117 μl) was added to the suspension, and the reaction mixture was stirred for 16 h. The solvent was then decanted and APS–MNPs **2** were washed with ethanol (2 × 30 ml) and acetone (3 × 35 ml) and then dried under reduced pressure, yielding 0.190 g of APS–MNPs.

*Preparation of N^{α},N^{ε}-di-Fmoc-L-lysine-modified MNPs (**4**)*

APS–MNPs **2** (0.100 g) were suspended in MeCN (50 ml) under sonication for 10 min. The N^{α},N^{ε}-di-Fmoc-L-lysine **3** (0.046 g) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 0.016 g) were then added under continuous stirring. The reaction mixture was stirred for 20 h at room temperature. The solvent was then decanted, and the modified MNPs were washed with acetone (5 × 15 ml) and dried under reduced pressure, yielding 0.085 g of MNPs **4**. Using UV spectroscopy, the resulting MNPs **4** were determined to contain 0.29 mmol of N^{α},N^{ε}-di-Fmoc-L-lysine per 1 g of MNPs.

*Preparation of L-lysine-modified MNPs (**5**)*

MNPs **4** (0.064 g) were suspended in a dimethylformamide (DMF)/piperidine 4:1 mixture (3 ml) under sonication for 10 min. After incubation for 20 min, the solvent was decanted and MNPs were washed with water (3 × 15 ml) to neutral pH and acetone (2 × 15 ml) and then dried under reduced pressure to yield 0.064 g of MNPs **5**. The amount of removed Fmoc groups was determined using UV spectroscopy as described previously [26]. The resulting MNPs **5** were found to contain 0.23 mmol of L-lysine per 1 g of dry substance.

Cell culture

Mouse mesenchymal stromal cells (MSCs) were isolated from C57Bl/6 murine adipose tissues using a protocol developed for human adipose-derived MSCs [27]. Briefly, adipose tissue was minced and incubated in collagenase solution (type IA, Sigma, 2 mg/ml) for 30 min at 37 °C, filtered through a 40-μm cell strainer (Becton Dickinson), and washed twice in Dulbecco's modified Eagle's medium (DMEM)/low glucose (DMLG) by centrifugation at 400*g* for 10 min. Final cell suspension was plated on 10-cm dishes and cultured under hypoxia conditions (5% CO_2, 5% O_2, 37 °C) in DMLG, 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 U/ml penicillin/100 μg/ml streptomycin (PenStrep) until reaching 70–80% confluency.

Human MSCs were cultured under hypoxia conditions (5% CO_2, 5% O_2, 37 °C) in DMLG, 10% FBS, 2 mM glutamine, and PenStrep until reaching 70–80% confluency.

Derivation of J2 clone of murine NIH 3T3 fibroblasts, expressing human Jagged 1 protein and possessing significant murine hematopoietic-supporting activity, was reported previously [28]. J2 cells were propagated in DMEM/high glucose (DMHG), 10% FBS, 2 mM glutamine, and PenStrep. OP9 cells were cultured in α-MEM, 20% FBS, 2 mM glutamine, and PenStrep. MS5 cells were propagated in Iscove's modified Dulbecco's medium (IMDM), 10% FBS, 2 mM glutamine, and PenStrep.

Isolation of Lin^{-} cell fraction

Isolation of lineage-negative (Lin^{-}) fraction of murine bone marrow was performed according to a protocol of Miltenyi Biotec (Bergisch Gladbach, Germany). Bone marrow from femora of C57Bl/6 mice was resuspended in DMHG, 10% FBS, and PenStrep, centrifuged at 300*g* for 10 min, and resuspended in phosphate-buffered solution (pH 7.2) containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 1% bovine serum albumin (BSA). Biotin-conjugated antibodies to Lin antigens and streptavidin-conjugated magnetic particles (both Miltenyi Biotec) were added sequentially to cells, incubated at 4 °C for 20 min, and separated on MS MACS Separation columns. Flow-through fraction was centrifuged at 400*g* for 10 min, resuspended in DMHG, 10% FBS, and PenStrep, and counted.

Preparation of magnetic stromal layers

MNPs were suspended in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/ml, washed twice by adsorption on a magnet, and sonicated in an ultrasonic bath (Bransonic 2510) for 60 min at room temperature. Completeness of dispersion as judged by the absence of visible aggregates was controlled by phase-contrast microscopy.

Cells were resuspended in DMHG, and MNPs were added to a final concentration of 0.1 mg/ml. Incubation was performed at