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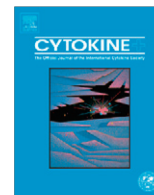

Short Communication

The development of macrophages from human CD34+ haematopoietic stem cells in serum-free cultures is optimized by IL-3 and SCF

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ABSTRACT

The derivation of human macrophages from peripheral blood monocytes remains a convenient method for the study of macrophage biology. However, for macrophage differentiation, a significant proportion of development has occurred prior to the monocyte stage; monocyte subsets also have varying potential for differentiation. Differentiation of macrophages from a less mature precursor, such as CD34+ haematopoietic stem cells, can further inform with regard to the development of macrophage-lineage cells. CD34+ cells were cultured in serum-free medium containing Flt3L, SCF, IL-3, IL-6 and M-CSF. Using differing combinations of growth factors, the effect on cell proliferation and differentiation to adherent macrophage-like cells was determined. The proliferative response of CD34+ cells to M-CSF was determined during the initial phase of cell culture. Thirteen combinations of SCF, IL-3, IL-6 and M-CSF were then compared to determine the optimum combination for proliferation. Adherence was used to isolate mature macrophages, and the macrophage-like phenotype was confirmed by analyses of surface markers, histo-morphology and phagocytosis. This study refines the means by which large numbers of macrophages are obtained under serum-free conditions from haematopoietic precursors.
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1. Introduction

Macrophages (Mφ) are bone marrow-derived cells that reside within tissues and perform essential activities in the steady state and the inflammatory response; dysregulation of these activities exacerbate or perpetuate disease. The monocyte is an intermediate phase between bone marrow precursor and tissue Mφ that is frequently used for the in vitro derivation of dendritic cells (DC), osteoclasts and polarised subsets of Mφ. Despite the ease with which serum-containing medium and M-CSF produce monocyte-derived Mφ (MDM), this technique has several shortcomings. The few circulating monocytes that are capable of proliferation [1] require several days per cell division [2], which limits expansion. Using MDM, Mφ development can only be studied from the monocyte stage onwards, and furthermore some monocyte subsets have matured to a point beyond which they can be differentiated to certain cell types [3]. These deficiencies can be overcome using less mature CD34+ cells, which are capable of producing several haematopoietic lineages.

G-CSF-mobilised blood [4], umbilical cord blood [5] or bone marrow [6] serve as sources of CD34+ cells. Serum-free conditions are commonly used for clinical applications to provide a more defined medium, eliminate batch variation, reduce the risk of infectious agents and the effect of foreign antigens [5,7]. Haematopoietic growth factors (HGF) are used to induce proliferation and differentiation of the stem cells and the more mature cells. Using a multi-step technique, the medium is initially supplemented with cytokines to induce proliferation of progenitors (e.g., Flt3L, SCF, IL-3 and IL-6) [5]. After or during the expansion of the precursors, further differentiation is directed with a different set of cytokines (e.g., GM-CSF, IL-4 and TNFα for DC differentiation) [5]. A final step to isolate a pure population could involve immunomagnetic selection [8].

The rationale of this study was to determine the optimal combination of HGF to expand cell numbers from which Mφ could then be derived. To optimise cell numbers, the effect of HGF that promote the survival and differentiation of Mφ was determined during the phases of culture wherein stem cells and progenitors were most prevalent. Previously we compared CD34+ cell-derived Mφ differentiated using 2 serum-free media [9]. In the current study we measured the effect of M-CSF in the early phase of cell

Abbreviations: Mφ, macrophage(s); DC, dendritic cell(s); MDM, monocyte-derived macrophage(s); PDM, progenitor-derived macrophage(s).

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expansion to determine whether its presence would induce rapid differentiation of progenitors and thereby reduce cell numbers. To determine the effect of HGF on proliferation at an intermediate stage, a comparison of the proliferation rate in 13 serum-free media was performed. After determining the optimal medium in which to adhere and terminally differentiate mature cells, we confirmed their Mφ-like phenotype.

2. Materials and methods

2.1. Isolation and culture of CD34+ cells

G-CSF-mobilized apheresis product was obtained from donors undergoing transplant therapy. The Royal Melbourne Hospital Human Research Ethics Committee approved the study and informed consent was obtained. The apheresis product was diluted in PBS and layered by over Ficoll-Paque (GE Healthcare Biosciences, Australia) to isolate mononuclear cells. CD34+ cells were isolated using a CD34+ cell isolation kit (Miltenyi Biotec, USA). Purity was typically in excess of 95%, as determined by flow cytometry, for all donors.

Cells were cultured in X-VIVO 10 with gentamicin (BioWhittaker, USA) containing 1% Buminate (human albumin, 5% w/v; Baxter, USA) and the following cytokines: Fms-related tyrosine kinase 3 ligand (Flt3L, 50 ng/mL; R&D Systems, USA), stem cell factor (SCF, 200 ng/mL; Amgen, USA), interleukin 3 (IL-3, 10 ng/mL; Amgen), interleukin 6 (IL-6, 10 ng/mL; Chemicon International, USA), macrophage colony stimulating factor (M-CSF, 5000 U/mL; Chiron, USA). Cells were cultured in 24 well ultra-low attachment plates (Corning, USA) at a concentration of 5×10^4 cells/mL. For adhesion experiments from day 20 to 26, non-treated tissue culture plates (Iwaki, Japan) were used, which permit detachment of cells for cell counting and flow cytometry.

2.2. Histology

Cytospins were prepared by centrifuging cells onto glass slides and performing differential staining with a DiffQuik stain set. A digital camera-mounted microscope (Axiovision 25, Carl Zeiss, Germany) was used to take photomicrographs using Axiovision software (version 4.0, Carl Zeiss).

2.3. Flow cytometry

Cells were stained in a 30 μL volume containing the relevant antibody for 25 min on ice, then washed three times with FACS buffer (1% FCS 5 mM EDTA PBS) and analysed using a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, USA). Antibodies used for the determination of CD34+ cell purity and the phenotyping of progenitor-derived macrophages were obtained from Becton Dickinson, USA. Isotype-matched control antibodies were used for all analyses.

2.4. Phagocytosis

Fluorescent latex beads (1.0 μm FluoSpheres® carboxylate-modified microspheres (Molecular Probes/Invitrogen, USA)) were sonicated for 2 min using 30-s pulses, diluted in 1% w/v BSA/PBS (4 μL in 100 μL), incubated for 1 h at room temperature in the dark and vortex-mixed for 1 min. The bead mixture was added to the cultures in which the cells were growing, at a concentration of 10 μL/mL culture medium and the plate was placed in an incubator at 37 °C for 3 h. The cells were then rinsed repeatedly with PBS to remove any unbound beads. Photomicrographs were taken as described in Section 2.2.

2.5. Statistics

Significant differences were determined by Student's *T* test or ANOVA with Newman–Keuls Multiple Comparison post hoc analysis.

3. Results and discussion

3.1. M-CSF modulates proliferation of progenitors – day 0–13

CD34+ haematopoietic progenitors were isolated from aphaeresis material and cultured in serum-free media containing Flt3L (day 0–6), SCF, IL-3, IL-6 ± M-CSF. The presence of M-CSF from day 0 led to a moderate but significant increase in cell number at days 9 and 13 (Fig. 1(A)); adding M-CSF beginning at day 3, 6 or 9 did not lead to a significant difference in cell number at later time points (data not shown). This indicates that the early addition of M-CSF does not reduce proliferation of progenitors as a result of early Mφ differentiation. Previously we have observed that up to 21 days of culture is required for a substantial proportion of CD34+-derived cells to express canonical Mφ surface markers such as CD11b, CD64 and CD14 [9]. An examination of morphology (Fig. 1B(i–iii)) indicated that at day 13 the presence of M-CSF from day 0 resulted in an increase of monocytic and large, Mφ-like cells, compared to cultures lacking exogenous M-CSF. Although HGF modulate the proliferation of monocytic cells in serum-containing medium [10], human peripheral blood monocytes cultured in the HGF-supplemented, serum-free medium used for CD34+ cells had very low viability after 2–3 days (data not shown); the presence of Mφ-like cells at day 13 is thus unlikely to be a consequence of contaminating monocytes at day 0.

3.2. Expansion of cells in 13 media – day 12–20

A priority for the intermediate stage of cell culture was the continued expansion of cell numbers and to determine the influence of the presence or absence of any particular HGF(s). From day 12 to 20, the effect on cell number of 13 combinations of HGF was measured (Fig. 1C); these cells had been cultured from day 0 to 12 in media containing Flt3L (day 0–6), SCF, IL-3, IL-6 and M-CSF. The influence on proliferation of HGF in order of decreasing magnitude was IL-3, SCF, IL-6 and M-CSF, with the presence or absence of IL-3 in cultures being the main determinant of statistical differences in cell numbers (Fig. 1D). Proliferation during this period was highest in the cultures containing SCF and IL-3 (∼12-fold higher than base medium). The presence of M-CSF or IL-6, alone or in combination, did not significantly affect the proliferative response to SCF and IL-3.

In cultures containing IL-3, but no SCF, proliferation was lower (∼8-fold vs base medium) and different combinations of IL-6 and M-CSF (IL-6 ± M-CSF or M-CSF alone) did not significantly reduce this proliferation. In the absence of exogenous IL-3, proliferation was on average lower (less than 6-fold vs base medium); the highest proliferation in IL-3-free cultures was found in the presence of SCF. In the absence of IL-3 or SCF proliferation was <3-fold vs base medium.

With regard to total numbers, the presence of IL-6 and/or M-CSF did not significantly attenuate proliferation in cultures containing IL-3 and SCF, compared to IL-3 and SCF alone. Although cultures containing IL-3, SCF, IL-6 and M-CSF produced the greatest number of cells between day 12 and 20, as our goal was to produce adherent Mφ we then determined the optimal HGF combination in which to adhere the cells.

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