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Red blood cells: The primary reservoir of macrophage migration inhibitory factor in whole blood

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ABSTRACT

Red blood cells are widely accepted to be inert carriers of oxygen and haemoglobin, but there is growing evidence that they play a much more critical role in immune function. Macrophage migration inhibitory factor (MIF) is a key cytokine in disease with additional oxido-reductase activity, which aids in managing oxidative stress. Although two studies have reported the presence of MIF in red blood cells, no study has quantified the levels of this protein. In this study, freshly isolated plasma, platelets, leukocytes, and red blood cells from healthy individuals were collected and the concentration of MIF was determined using an enzyme linked immunosorbent assay. This analysis demonstrated that MIF in red blood cells was present at 25 μg per millilitre of whole blood, which is greater than 99% of the total MIF and 1000-fold higher concentration than plasma. This result was supported by electrophoresis and Western blot analysis, which identified MIF in its monomer structural form following sample processing. Furthermore, by assessing the level of tautomerase activity in red blood cell fractions in the presence of a MIF inhibitor, it was determined that the red blood cell-derived MIF was also functionally active. Together, these findings have implications on the effect of haemolysis during sample preparation and provide some clue into the inflammatory processes that occur following haemolysis *in vivo*. These results support the hypothesis that red blood cells are a major reservoir of this inflammatory protein and may play a role in inflammation.

1. Introduction

Macrophage migration inhibitory factor (MIF) plays a central role in promoting inflammation [1,2]. This 12.5 kDa cytokine is a key component of the early stage inflammatory response that is associated with innate immunity. To enable this response, MIF is produced at high concentrations and is retained intracellularly for rapid secretion in the active form, a homotrimer [3]. It is secreted by both immune cells (such as macrophages and T cells) and organ specific cells (such as hepatocytes and osteoblasts), and unlike other cytokines, it is also secreted by the pituitary gland in response to infection [4].

Interest in MIF has expanded since its discovery in 1966 [5] and it has been implicated in a range of acute and chronic inflammatory conditions [6–9]. For this reason, MIF has been frequently proposed as a potential disease biomarker [7–11]. However, the involvement of this protein in so many biological processes and the wide range of plasma MIF concentrations in healthy participants (24.7 ± 13.2 ng/mL) [12] makes its use as an independent biomarker challenging [13]. One contributing factor to this variability may be the source of circulating MIF. Levels of MIF are most often monitored in serum or plasma collected from peripheral blood and the source is typically attributed to white blood cell secretion [1,14]. More recently however, platelets have been identified as a previously unrecognised source of MIF which supports the growing literature around the involvement of platelets in inflammation [15,16]. In contrast to white blood cells and platelets, there is minimal literature on the association of with the most common cell type in blood, the red blood cell. Although the conventional view is that red blood cells only function in gas exchange, there is a growing body of literature that suggests that the role of this enucleate cell may be more complex. Proteomic and transcriptomic analyses have identified 2289 unique proteins in red blood cells [17] and transcripts of 1019 different genes [18]. Regarding cytokines, there are also studies that indicates that red blood cells can bind inflammatory chemokines of the C-X-C or C−C family through the Duffy antigen receptor for chemokines (DARC) [19–21].

In 2000, Mizue et al. [22] suggested that MIF is present in red blood cells after seeing an increase in MIF levels which was correlated with the concentration of haemoglobin. Although this study was completed

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without the quantification of the concentration of MIF in red blood cells, it is one of very few reports indicating that there may be a relationship between this inflammatory marker and the most abundant cell type in the human body. In this current study, we sought to confirm the presence of MIF in red blood cells and to quantify the concentration and activity of this protein.

2. Materials and methods

2.1. Blood donation

This study was approved by the Macquarie University Human Research Ethics Committee and by the Northern Sydney Coast Human Research Ethics Committee of NSLHD and CCLHD. Written informed consent was obtained from all participants before enrolment ($n = 20$; female: 14, male: 6). Whole blood was collected from healthy volunteers by venepuncture (19–47 years old). Blood was combined with EDTA (ethylenediaminetetraacetic acid) for anticoagulation (K_2EDTA vacutainers, Becton Dickinson, Franklin Lakes, New Jersey). Blood components were prepared and isolated at room temperature within four hours of collection. All samples were subjected to three freeze-thaw cycles at −80 °C prior to analysis to ensure complete cellular lysis. Samples were stored at −80 °C until analysis.

2.2. Plasma and blood cell isolation

Collected cell populations (whole blood, red blood cells, white blood cells, and platelets) were isolated and frozen immediately for analysis of cell lysates or were used fresh for storage experiments. Concentration and purity of whole blood, red blood cell, white blood cell, and platelet populations were determined using a haematology analyser (Coulter AcT Diff, Beckman Coulter, Brea, California). Plasma was isolated from anticoagulated whole blood by centrifugation (1500*g*, 10 min, 23 °C). For isolation of the blood cells, whole blood was added to a high molecular weight dextran solution (6% w/v dextran, 450–600 kDa, in 0.15 M sodium chloride) at a 2:1 ratio (blood to dextran). This solution was gently mixed and left upright for 60 min at room temperature for red blood cell sedimentation. After sedimentation, two layers formed and were collected and transferred into individual tubes. The lower, red blood cell dense layer, was washed once in phosphate buffered saline (PBS) and the purified red blood cells were collected. The upper white blood cell rich fraction was washed once in PBS and any contaminating red blood cells were removed by hypotonic lysis. Subsequently, isotonicity was restored and remaining cells were washed again in PBS. For isolation of platelets, platelet rich plasma (PRP) was prepared by centrifugation of whole blood (100*g*, 20 min, 23 °C). The PRP was collected and contaminating cells were removed by two subsequent cycles of centrifugation (100*g*, 20 min, 23 °C). Platelets were then isolated and washed twice in PBS. The cytosolic fraction of red blood cells was isolated by separation from red blood cell membranes from the frozen lysates. The cell membranes were isolated by centrifugation (16,000*g*, 15 min, 4 °C) after which the upper, cytosolic fraction was collected and analysed. To prepare the membrane fraction, the isolated cell membranes were washed once in PBS in the presence of Roche cOmplete protease inhibitors (16,000*g*, 15 min, 4 °C) then re-suspended in PBS at the equivalent of 1.25×10^6 cells/mL. This suspension was then sonicated to release any bound MIF (4 × 30 s cycles at 70% with 1 minute on ice between cycles) after which this fraction was collected and analysed.

2.3. Protein detection

Concentrations of MIF in blood components were measured using a MIF ELISA (R&D Systems, Minneapolis, Minnesota) which was run according to the manufacturer's instructions and the ideal dilution for each sample type was user optimised. The absorbance data for the MIF ELISA were collected with a Synergy 2 plate reader (BioTek, Winooski, Vermont) at 450 nm with absorbance correction at 570 nm. For Western blot analysis, red blood cell lysates were prepared by suspending 500,000 cells in 2% sodium dodecyl sulfate (Sigma-Aldrich, St. Louis, Missouri) and heated for five minutes. These lysates were then reduced and alkylated by the addition of 10 mM tributylphosphine (Sigma-Aldrich) and 10 mM acrylamide (Bio-Rad, Hercules, California) followed by incubation at room temperature for 1 h. These samples were then diluted 1:1 in Laemmli sample buffer (Bio-Rad) and the prepared samples were then loaded onto a Criterion TGX gel 4–15% (Bio-Rad) for electrophoresis. Following this, the proteins were blotted onto a Westran S PVDF membrane using the semi-dry method of Khyse-Anderson [23]. Membranes were blocked in 5% non-fat milk in PBS-Tween and were probed with rabbit anti-human MIF pAb (1:500 dilution, Abcam, Cambridge, United Kingdom, cat. # ab65869), followed by goat anti-rabbit IgG conjugated with alkaline phosphatase (1:15000 dilution, Sigma-Aldrich, cat. # A3687), and finally an alkaline phosphatase detection system (Alkaline Phosphate Conjugate Substrate Kit, Bio-Rad).

2.4. Recovery efficiency

Recombinant MIF (rMIF, final concentration of 2 ng/mL, R&D Systems) or the corresponding volume of diluent (control) were spiked into red blood cell lysates immediately prior to analysis of samples by MIF ELISA. The recovery efficiency of recombinant MIF was calculated using the following equation:

$$\frac{[MIF]\ in\ control\ lysates + [rMIF]}{[MIF] in\ rMIF\ spiked\ lysates} \times 100 = Percentage\ recovery$$

2.5. Immunophenotyping

The immunophenotype of the purified red blood cell population was determined by antibody labelling of the cells with the antibodies listed in Table 1. Flow cytometry of these cells was performed on a FACS-Calibur flow cytometer with a 488 nm argon laser (Becton Dickinson), data was collected using CELLQuest Pro software (ver. 5.2.1) and analysed using Flowing Software 2 (ver. 2.5.1, Finland).

2.6. MIF tautomerase activity

Red blood cell derived MIF was tested for dopachrome tautomerase activity as previously described [24]. Briefly, *L*-dopachrome methyl ester was prepared immediately before use by adding 100 µL of *L*-3,4 dihydroxyphenylalanine methyl ester (12 mM; Thermo Fisher Scientific, Waltham, Massachusetts) and 100 µL of sodium periodate (24 mM, AnalaR, VWR International, Radnor, Pennsylvania) to 3800 µL of reaction buffer (50 mM Bis-Tris, 1 mM EDTA, pH 6.2). Immediately prior to analysis, 200 µL of this solution was added to 20 µL of 40 nM MIF solution, or assay blank, in a 96-well plate. Absorbance was monitored at 475 nm over 10 min. For analysis of enzymatic activity, the following

Table 1
Monoclonal antibodies for immunophenotyping of red blood cells.

Antibody	Fluorochrome	Clone
Mouse anti-human CD31	APC	WM59
Mouse anti-human CD44	PE	515
Mouse anti-human CD45	FITC	HI30
Mouse anti-human CD74	FITC	M-B741
Mouse anti-human CD235a	APC	HI264
Mouse IgG1,κ Iso Control	PE	MOPC-21
Mouse IgG1κ Iso Control	FITC	P3.6.2.8.1
Mouse IgG2a κ Iso Control	FITC	G155-178
Mouse IgG2a κ Iso Control	APC	MOPC-173