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Metallothionein Biosynthesis in Human RBC Precursors

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Key Words

BFU-E • Cord Blood • Glycophorin A^+ cells • Immunohistostaining • MACS • MT-0 • Zinc

Abstract

The *in vitro* biosynthesis of metallothionein (MT) has been investigated in RBC precursors from human cord blood in order to support the hypothesis for the nucleated precursor origin of MT in human red blood cells (RBC). Human RBC precursors are obtained by (i) separating glycophorin A^+ (gly A^+) cells using a magnetic cell sorting (MACS) technique and by (ii) *ex vivo* expansion of precursors BFU-E (burst forming unit-erythroid) on methylcellulose semi-solid culture media from mononuclear cells of cord blood. Biosynthesis of MT is detected at the protein level, by immuno-histochemical staining using a mouse monoclonal antibody (E9) in *ex vivo* expanded RBC precursors obtained from BFU-E. Expression of MT is also detected at the mRNA level by MT specific reverse transcriptase polymerase chain reaction (RT-PCR) both in *ex vivo* expanded precursors from BFU-E and in MACS separated gly A^+ cells. In addition, the expression of the fetal form of MT, MT-0 (also known as MT-1H) at the mRNA level in glycophorin A^+ cells, is also confirmed by cDNA sequencing. With these observations, to our knowledge, MT biosynthesis in human erythroid precursors is reported for the first time. Moreover, the current findings of MT-0 expression at the mRNA level in gly A^+ RBC precursors of hCB has added one more member in the list of cells/organs like fetal liver, human monocytes, non-neoplastic tissues of adenocarcinoma etc., in which the expression of the human fetal form of MT, i.e. MT-0, has also been reported.

Introduction

Metallothioneins (MT), small cysteine-rich metal-scavenging proteins, bind group IB (copper, silver, gold) and IIB (zinc, cadmium, mercury) metal ions with high affinity [1]. Since its discovery [2], MT biosynthesis has been reported in plants, animals and microorganisms.

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Metallothionein occurrence or biosynthesis in human blood cells like lymphocytes [3], monocytes [4], platelets [5], has also been reported. Metallothionein at the mRNA level has been described in reticulocytes by Lambert *et al.* [6], who also have speculated on the possible role of MT in differentiation. However, the inability of erythrocytes to perform protein biosynthesis has asserted several speculations on the origin of MT therein. Nucleated red blood cells (nRBC) or RBC precursors like proerythroblasts have been suggested as a potential site of MT biosynthesis and hence considered as the source of MT in mature RBC [7]. Metallothionein originated in organs like spleen is also suggested to be transferred to RBC [8]. It has also been hypothesized that the source of MT in murine RBC is the early bonemarrow precursors [9]. This was later supported by Huber and Cousins [10] who reported that MT concentrations in erythroid progenitor cells are responsive to dietary Zn intake and status. Extrapolation of this speculation, i.e. MT biosynthesis in nRBC, from mouse to human, has led us to attempt *in vitro* biosynthesis of MT directly in erythroid precursors from human cord blood (hCB). Moreover, the investigation on the origin of MT in human erythrocytes with conclusive answers would give rise to an additional parameter to consider the proposed correlation between the overall metal status and the respective metal bound MT present in erythrocytes. As fetal cell populations are present in cord blood, investigation for the expression of the human fetal form of MT, MT-0 (also known as MT-H) in the nRBC from hCB is also of our particular interest.

Materials and Methods

Separation of nonadherent mononuclear cells from human cord blood

Human cord blood was collected in acid-citrate-dextrose solution from umblical cords of full term newborns and was kept at 4°C for not more than 12 hours before use. Mononuclear cells (MNC) from hCB were separated by density gradient centrifugation on lymphoprep™ (Nycomed AS, Oslo, Norway) according to Bøyum [11]. After washing 3× with PBS, MNC were incubated to remove plastic adherent cells at 37°C for 18-24 hours in RPMI 1640 containing 5% (*v/v*) fetal calf serum, 0.01% (*v/v*) gentamycin, 2mM glutamine (all from GibcoBRL, Life Technologies Ltd. Paisley, Scotland), 1% (*w/v*) glucose (Sigma, St. Louis, MO, USA), 10^{-5} M 2-mercaptoethanol (Fluka, Neu-Ulm, Germany); non-adherent MNC were recovered and washed not more than 3× with PBS.

Ex vivo expansion or purification of nucleated precursors of RBC

Nucleated precursors of RBC were obtained from these non-adherent MNCs in two different ways: (i) by culturing nonadherent MNC in semi-solid methylcellulose culture (MC) media (MethoCult™, StemCell Technologies, Vancouver, BC, Canada). Briefly, non-adherent MNC were mixed at a density of 10^4 cells/mL in MC media supplemented with additional erythropoietin (3U/mL) and interleukin-3 (5ng/mL); 1 mL/well of this cell suspension was incubated in 24 wells tissue culture plates; *ex vivo* expansion of erythroid precursors was carried out at 37°C in high humidity with 5% CO_2 and normal atmospheric O_2; BFU-E colonies were picked up under the microscope at day 12; the purity of the erythroid precursors was checked by May-Grünwald-Giemsa staining; (ii) by separating glycophorin A^+ (gly A^+) cells from non-adherent MNCs following MACS (magnetic cell sorting) using glycophorin A microbeads (reagents and equipments from Miltenyi Biotech, Bergisch Gladbach, Germany) with a positive selection column according to the instructions; purity of the separated gly A^+ cells was achieved by a second pass of positive selection of the separated gly A^+ cells using fresh reagents and another positive selection column; purity was finally checked by flow cytometric analysis; ≥80% gly A^+ cells were taken for further experiments.

In vitro Zn induction

Erythroid precursors obtained either by *ex vivo* expansion or by MACS separation were cultured in liquid cell culture media for the *in vitro* MT biosynthesis in RPMI 1640 (same as above only with the exception of 15% (*v/v*) fetal calf serum) for 48 h at 37°C. *In vitro* Zn induction for MT biosynthesis was carried out in similar cultures of erythroid precursors with 100µM of Zn acetate and hereafter referred as 'induced culture'.

Immunohistostaining

Immunoperoxidase staining was performed with the cytospin preparations of the erythroid precursor cells from control and Zn induced cultures of BFU-E. Briefly, E9 (Dako, Carpinteria, CA, USA) was used as the primary antibody, biotin conjugated rabbit anti-mouse IgG was used as the secondary antibody, finally immune reactivity was observed with peroxidase conjugated avidin-biotin complex using 3-amino-9-ethyl-carbazole plus hydrogen peroxide as substrate.

mRNA extraction, RT-PCR and cDNA sequencing

Poly(A) mRNA was extracted using MICRO-POLY(A)PURE™ (Ambion, Austin, TX, U.S.A.) both from control (without additional Zn) and induced (with additional 100µM of Zn) cell cultures of BFU-E colonies and of gly A^+ cells; cDNA was synthesized with equal amounts of extracted mRNA using the GeneAmp® RNA PCR Core kit (Perkin Elmer, Roche Molecular System, Branchburg, NJ, U.S.A.) according to the manufacturer's instructions. Total MT and MT-0 specific PCR was performed according to Pauwels *et al.* [4]; PCR and subsequent cloning for sequencing was performed using the AdvanTAge™ PCR Cloning kit (Clontech, Palo Alto, CA, U.S.A.); plasmid isolation was performed using High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany) and cDNA sequencing was

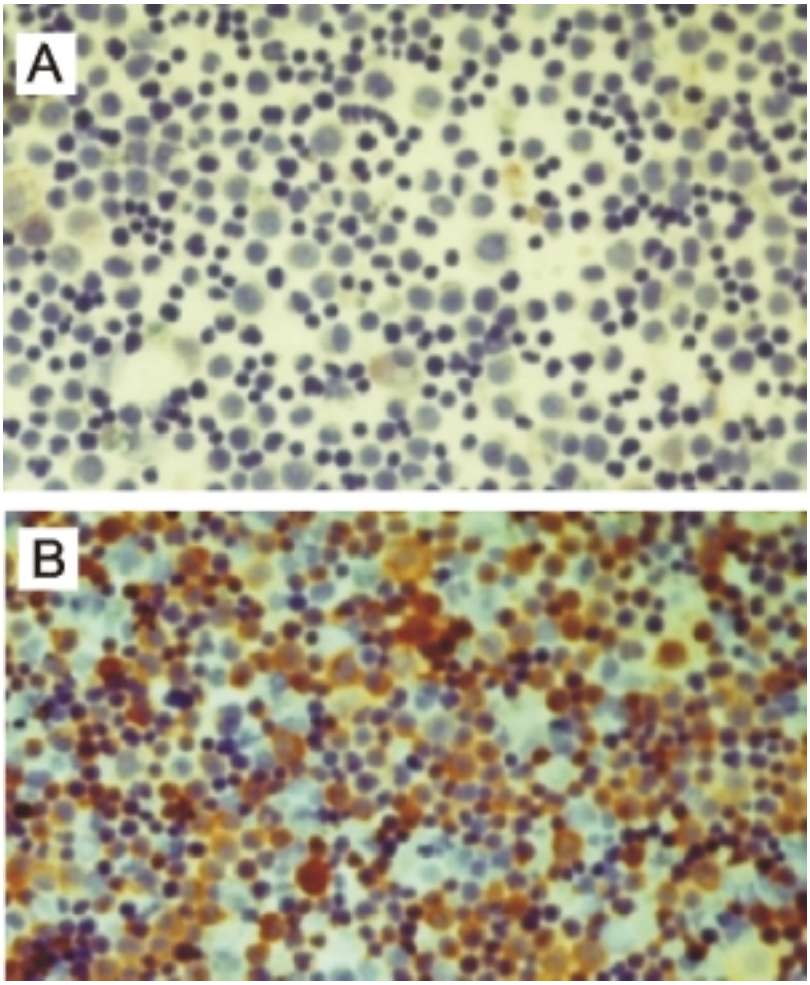

Fig. 1. Immunohistochemical staining of the cytospin preparation of nRBC with mouse anti-MT (E9). Cells are obtained from BFU-E colonies grown by culturing nonadherent MNC from hCB on methylcellulose semi-solid cell culture media for 12 days and then cultured for 48 hours in liquid cell culture media with or without additional 100μM of Zn. (a) Cells from the control culture (left untreated with additional 100 μM of Zn supplement) were found negative for the immune reactivity; (b) on the other hand, cells obtained from the culture with additional 100μM of Zn supplement at 0 hour of liquid cell culture phase were found positive for the immune reactivity (cytoplasm red in color).

performed using the DNA sequencing kit (T7 Sequenase™ version 2.0, Amersham Life Science, Buckinghamshire, UK) according to the instructions.

Results and Discussion

Immunohistochemical staining for the presence of MT expression reveals that MT biosynthesis is inducible at the protein level after 48 hours upon Zn addition in the cell culture of nRBC which was obtained by *ex vivo* expansion of RBC progenitors (BFU-E) in semi-solid MC culture media (Fig. 1). The ample presence (more than 90%) of positively stained nRBC shown in Fig. 1b represent MT containing cells from Zn induced cultures and are obviously the indication of the MT biosynthetic capability of these cell populations. In contrast to the cells from the induced culture, MT containing cells could not be detected in the control culture (Fig. 1a). Consequently inducible expression of MT biosynthesis in the human nRBC is concluded.

Both the consititutive and upregulated transcription of MT mRNA are detected respectively in control and Zn induced cultures of nRBC from BFU-E colonies and of gly A^+ cells. The electrophoretic pattern of equal volumes

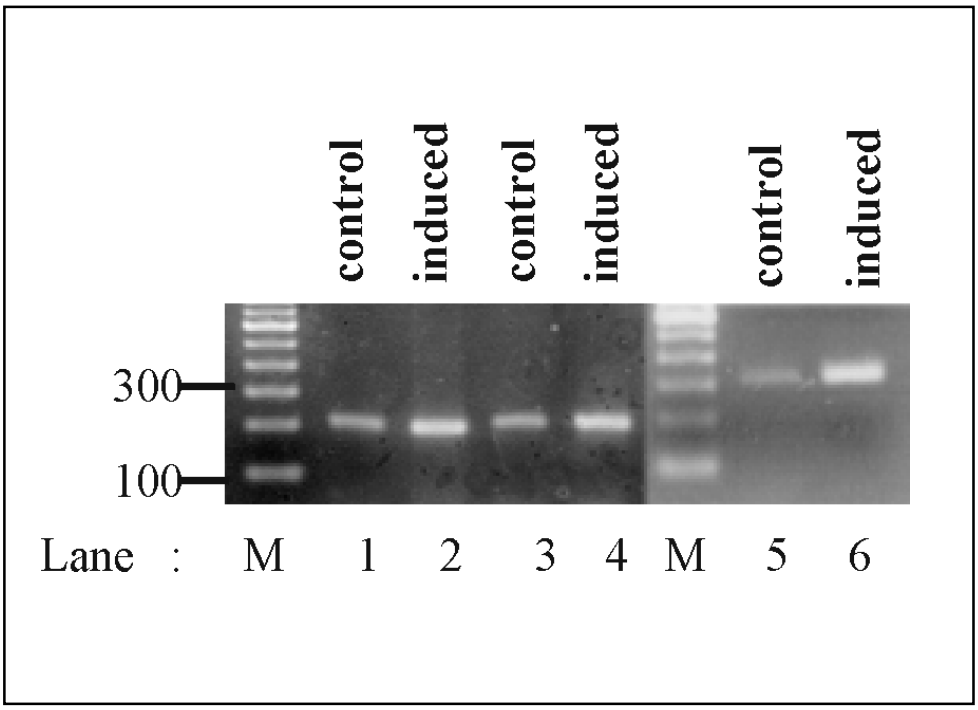

Fig. 2. Electrophoretic profile of total MT or MT-0 specific amplified cDNA pools on 2% agarose gel. Total MT specific amplified cDNA pools from a contol and an induced culture of nRBC from BFU-E are represented in lane # 1 and lane # 2 respectively; similar cDNA pools from a contol and an induced culture of gly A^+ cells are represented in lane # 3 and lane # 4 respectively; MT-0 specific cDNA pools from a control and an induced culture of gly A^+ cells are represented in lane # 5 and lane # 6 respectively; mRNA was extracted from 48 hours old control and Zn induced populations. M, 100 base pair DNA size marker.

of amplified cDNA pools on agarose (2%) gel was observed and photographed under ultra-violet illumination after staining with ethidium bromide (Fig. 2). Total MT specific amplified cDNA pools from a control culture of nRBC from BFU-E colonies and of gly A^+ cells are shown in lane # 1 and lane # 3 respectively. Therefore, the constitutive transcription of MT genes in human nRBC obtained either by *ex vivo* expansion or by MACS selection of gly A^+ cells is demonstrated. The transcription of MT mRNA from Zn induced cultures of nRBC from BFU-E and of gly A^+ cells is also observed and presented with total MT specific amplified cDNA pool in lane # 2 and lane # 4 from the respective cultures.

The expression of the fetal form of MT, i.e. MT-0, attracts our special interest during this investigation while cord blood was used as the source of nRBC. Therefore, the mRNA expression of this isoform was determined. The constitutive transcription of MT-0 mRNA is observed in control cultures by the presence of an amplified cDNA pool after total MT-0 specific RT-PCR and is represented in lane # 5 (Fig. 2). The upregulated transcription of MT-0 mRNA from Zn induced cultures is also observed and presented by the MT-0 specific amplified cDNA pool in lane # 6 (Fig. 2). Furthermore, out of 10 clones of the MT-0 specific amplified cDNA pool, 3 randomly selected ones have been sequenced and the nucleotide sequence of the human fetal form of MT (MT-0) was confirmed by sequence similarity search using the basic BLAST [12].

Though not quantitative, yet the difference in band intensity of the amplified total MT/MT-0 specific mRNA extracted from the control and the Zn induced cultures either of nRBC from BFU-E colonies or of gly A^+ cells, clearly represents upregulation of MT mRNA upon Zn addition (Fig. 2).

Among all the speculations on the origin of MT in human RBC, biosynthesis in nucleated precursor cells is the most plausible one. The expression of MT mRNA in control cultures of *ex vivo* expanded nRBC and of gly A^+ cells and the increase of this expression with additional Zn treatment in similar cultures of respective cell populations from hCB demonstrates the MT biosynthetic capability of human nucleated precursors of mature RBC. With these observations, to our knowledge, constitutive and upregulated transcription of MT mRNA in human erythroid precursors is reported for the first time. The presence of MT at the protein level is detected in the Zn

induced cultures but not in the control cultures of earlier forms of nRBC obtained from BFU-E colonies (Fig. 1). This observation confers the absence of constitutive MT expression at the protein level in nRBC from hCB. Perhaps, it might be the aftermath of Zn starvation for 12 days in Zn free MethoCult medium. However, control of the MT expression at the transcriptional level could also be possible as at least weak signal of MT mRNA is observed in the control cultures.

While the possibility exists of getting trace amounts of mRNA from cells of other erythroid lineages which are present <15% in the MACS separated gly A^+ cells, the isoform specific expression of MT-0, in such cell population needs further clarification, which is currently under investigation.

However, the kind of experimental proofs provided in this communication does not exclude other possibilities like, transfer of MT into the mature RBC, while there is at least one report so far to our knowledge in which the existence of an MT receptor in human astrocytes has been described [13]. However, considering the possibility of having both the exogenous and endogenous source of MT in mature RBC, quite a number of emerging questions including various functional aspects of MT, like heavy metal homeostatis and detoxification, in nucleated precursors of mature RBC are yet to be resolved.

The inverse relationship of cadmium-MT concentrations between spleen, the major erythropoietic organ, and RBC, suggested possible transfer of cadmium-MT from spleen to RBC. In addition, MT induction by $CdCl_2$ in phenyl hydrazine treated anemic mice and prior injection of erythropoietin in $CdCl_2$ treated polyemia mice also revealed the effect of erythropoiesis on MT induction [8]. The present study is therefore, expected to provide the commencement of the emerging studies on the correlation between erythropoiesis and MT biosynthesis. Besides, the possibility of MT expression in nRBC in circulation, perhaps in adults too, can also be assumed from these findings. While Zn-MT in erythrocytes has been suggested as an index for the Zn status [14, 15, 16], it is important to know the origin of Zn-MT in erythrocytes so that a proper correlation between Zn status and RBC Zn-MT can be made.

The expression of MT-0 has been reported in Zn induced human monocytes [4] and also in nonneoplastic tissues from adenocarcinoma [17]. Therefore, the detection of the MT-0 expression at the mRNA level in erythroid precursors, a part of the whole blood cell population from hCB, has once more extended the growing list of the cells/organ(s) in which the expression of the human fetal form of MT (MT-0) has been observed. The possibility of the expression of other isoforms of MT in cord blood nRBCs and even the overall expression of MT in other cell types in hCB is still to be investigated.

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