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Macrophages Function as a Ferritin Iron Source for Cultured Human Erythroid Precursors

Moshe J. Leimberg,[1] Eugenia Prus,[2] Abraham M. Konijn,[1] and Eitan Fibach[2]*

[1]Department of Human Nutrition and Metabolism, Faculty of Medicine, The Hebrew University, Jerusalem, Israel

[2]Department of Hematology, Hadassah—Hebrew University Medical Center, Jerusalem, Israel

Abstract Iron is essential for the survival as well as the proliferation and maturation of developing erythroid precursors (EP) into hemoglobin-containing red blood cells. The transferrin–transferrin receptor pathway is the main route for erythroid iron uptake. Using a two-phase culture system, we have previously shown that placental ferritin as well as macrophages derived from peripheral blood monocytes could partially replace transferrin and support EP growth in a transferrin-free medium. We now demonstrate that in the absence of transferrin, ferritin synthesized and secreted by macrophages can serve as an iron source for EP. Macrophages trigger an increase in both the cytosolic and the mitochondrial labile iron pools, in heme and in hemoglobin synthesis, along with a decrease in surface transferrin receptors. Inhibiting macrophage exocytosis, binding extracellular ferritin with specific antibodies, inhibiting EP receptor-mediated endocytosis or acidification of EP lysosomes, all resulted in a decreased EP growth when co-cultured with macrophages under transferrin-free conditions. The results suggest that iron taken up by macrophages is incorporated mainly into their ferritin, which is subsequently secreted by exocytosis. Nearby EP are able to take up this ferritin probably through clathrin-dependent, receptor-mediated endocytosis into endosomes, which following acidification and proteolysis release the iron from the ferritin, making it available for regulatory and synthetic purposes. Thus, macrophages support EP development under transferrin-free conditions by delivering essential iron in the form of metabolizable ferritin. J. Cell. Biochem. 103: 1211–1218, 2008.

Key words: erythroid precursors; transferrin; transferrin-receptor; ferritin; macrophages; labile iron pool; exocytosis; endocytosis

Erythroid precursors (EP) are the body's main consumer of iron. Iron uptake by these cells is carried out chiefly by the transferrin (Tf)-transferrin receptor (TfR) pathway [van Renswoude et al., 1982]. Excess iron not utilized immediately for biosynthetic functions is stored in ferritin (Ft). EP development in the bone marrow is closely associated with macrophages (MØ). "Erythroid islands," composed of a central reticulo-endothelial cell surrounded by developing EP, were demonstrated by electron microscopy in the bone marrow of guinea pigs [Bessis and Breton-Gorius, 1962]. It was suggested that in these "islands" Ft iron is provided by the "reticulo-endothelial nursing cell" to the surrounding EP by way of Ft.

We have previously studied iron uptake and metabolism by developing human EP using a two-phase liquid procedure [Gelvan et al., 1996; Vaisman et al., 1997; Meyron-Holtz et al., 1999]. Peripheral blood mononuclear cells were first cultured for 7 days in the presence of various cytokines, not including erythropoietin. During this phase I culture, early erythroid committed progenitors (Burst Forming

Abbreviations used: EP, Erythroid precursors; Tf, transferrin; TfR, transferrin-receptor; Ft, ferritin; MØ, macrophages; FCS, fetal calf serum; PBS, phosphate buffered saline; SCM, serum-containing medium; SFM, serum-free medium; Exo, 12-(4-fluorobenzoylamino)benzoic acid methyl ester; NTA, nitrilotriacetic acid; RPA, rhodamineB-[(1,10-phenanthrolin-5-yl)-aminocarbonyl]benzyl ester; LIP, labile iron pool; MFC, Mean Fluorescence Channel; FITC, fluorescein iso-thiocyanate.

Dr. Leimberg and Dr. Prus contributed equally to this work.

*Correspondence to: Eitan Fibach, Department of Hematology, Hadassah University Hospital, Ein-Kerem, PO Box 12,000, Jerusalem 91120, Israel.
E-mail: Fibach@yahoo.com

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Units-erythroid) proliferated and differentiated into mature erythroid progenitors (Colony Forming Units-erythroid). In phase II, in the presence of erythropoietin, the latter cells continued to proliferate and matured, first into early EP that do not contain hemoglobin (Hb) (proerythroblasts) and then into Hb-containing orthochromatic normoblasts. Serum in this system could be replaced by a Tf-containing serum-supplement with no deleterious effect on EP growth and development. No EP developed when Tf was omitted. Thus, in this in vitro system EP utilize iron mainly in the form of hollo-Tf as they do in vivo. Addition of MØ or Ft during the later stages of the phase II could substitute Tf to some extent [Leimberg et al., 2003, 2005]. In the present report we further studied the role of MØ in the development of EP under Tf-free conditions. Our results show that in the absence of Tf, Ft synthesized and secreted by MØ can serve as an alternative iron source.

MATERIALS AND METHODS

Macrophage and Erythroid Cell Cultures

Peripheral blood mononuclear cells from healthy donors (after giving informed, written consent) were isolated by means of Ficoll-Hypaque centrifugation. For MØ cultures, the mononuclear cells were grown in Iscove's medium supplemented with 10% fetal calf serum (FCS) and 2% human AB serum (Sigma, St. Louis, MO). After 7 days, adherent cells were washed with phosphate buffered saline (PBS) and further grown in Iscove's medium supplemented with 30% FCS for at least 5 days, as previuosly described [Leimberg et al., 2005].

EP were grown in a modified two-phase liquid culture [Fibach et al., 1989]. Mononuclear cells were first cultured in alpha medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% FCS (phase I) as described previously. After 7 days, non-adherent cells were washed and suspended in phase II medium composed of alpha medium, 30% FCS (containing 8 μM iron), 1% bovine serum albumin, 10 μM β-mercaptoethanol, 1.5 mM glutamine, 10 μM dexamethasone and 1 U/ml human recombinant erythropoietin (Cilag AG, Schaffhausen, Switzerland). After 5 days, the cells were harvested, washed and re-cultured in phase II medium containing 30% FCS (SCM) or in serum-free medium (SFM) supplemented with 20 μl/ml Biogro, a serum substitute without Tf (Biological Industries). Some Biogro-containing cultures were supplemented with 0.3 mg/ml human Tf (Kamada Ltd, Rehovot, Israel; containing 8 μM iron). Cells were harvested 10–12 days after the onset of phase II, and Hb-containing EP were counted by benzidine-HCl staining [Leimberg et al., 2005] or lysed and their hemoglobins separated and quantitated using cation-exchange HPLC as previously described [Fibach, 2001].

EP-MØ co-cultures were prepared as follows: MØ grown for 5–7 days in phase II SCM were washed three times with PBS, thus removing all traces of FCS. EP grown in phase II in SCM were washed three times with PBS, suspended in phase II SFM and added to the MØ-containing flasks, as previuosly described [Leimberg et al., 2005].

In some experiments the co-cultures were treated with the following materials: The exocytosis inhibitor 2-(4-fluorobenzoylamino) benzoic acid methyl ester (Exo1) [Feng et al., 2003], the endocytosis inhibitor chlorpromazine [Daukas and Zigmond, 1985], the weak acidophylic base chloroquine [Armstrong and Morgan, 1983] (all purchased from Sigma–Aldrich, Rehovot, Israel), or with anti-human placental Ft derived from rabbit serum [Konijn et al., 1985].

Iron Incorporation and Secretion by Macrophages

MØ were incubated for 3 days with either ^{59}Fe nitrilotriacetic acid (NTA) or Tf/anti-Tf complexes labeled with ^{59}Fe [Cavill, 1971; Esparza and Brock, 1981]. ^{59}Fe-NTA was prepared by complexing ^{59}Fe (New England Nuclear Corp., Boston, MA) to NTA at a molar ratio in accordance with the chelator's dentation (2:1). Tf/anti-Tf complexes were prepared as described previously [Esparza and Brock, 1981] with some modifications: Increasing volumes of anti-Tf serum were added to a fixed amount of radiolabeled Tf prepared as previously described [Cavill, 1971]. The resulting immune complexes were collected by centrifugation, washed with saline (0.9% NaCl) and their radioactivity measured. ^{59}Fe-Tf/anti-Tf complexes and ^{59}Fe-NTA were added to the cultures in amounts that provided 8 μM iron which is the optimal iron concentration in this culture system—equivalent to 0.3 mg/ml culture of di-ferric-Tf. To determine cellular incorporation of iron, MØ were washed, lysed (lysis buffer composition—Tris-HCl, pH 7.4, 50 mM; KCl 80 mM;

Leupeptin 0.2 mg/ml; Aprotinin 0.2 mg/ml; phenyl methyl sulfonyl fluoride 0.2 mM). Samples were heated to 80°C for 10 min in a sample buffer (containing 10% glycerol, 2.3% SDS, Tris-HCl, pH 6.8, 62.5 mM and Bromophenolblue), and separated by non-reducing (5%) SDS–PAGE. To determine secretion of iron-containing compounds, MØ were incubated for 3 days with ^{59}Fe-Tf/anti-Tf complexes or ^{59}Fe-NTA as detailed above, washed and re-incubated in fresh medium for 5 days. The medium was collected, concentrated using a Centricon 50 filter (Amicon) at 5,000*g* and separated by non-reducing (5%) SDS–PAGE. The gels were subsequently dried and exposed to phosphor-imaging. A non-radioactive Ft obtained from human spleen (derived by splenectomy from a thalassemic patient) was used as a standard [Vaisman et al., 1997].

Iron Incorporation Into Erythroid Cell Heme

MØ treated with ^{59}Fe-Tf/anti-Tf complexes as described above were co-cultured with EP, after which EP were harvested, washed twice with saline, lysed in water and their heme extracted [Thunell, 1965] as following: One milliliter lysate was suspended in 1.5 ml 1 N HCl and then 1.5 ml of an ethyl acetate: acetic acid (3:1) solution added (final concentrations: ethyl acetate 2.87 M, acetic acid 1.63 M and HCl 0.37 M). The mixture was vortexed, centrifuged at 4,000 rpm for 6 min and the resulting upper (organic) heme-containinng phase collected. The lower phase was extracted again, and both organic fractions were pooled. Radioactivity was measured according to a modified version of Thunell's protocol [Gelvan et al., 1996].

Measurement of the Erythroid Cell Labile Iron Pool (LIP)

The effect of MØ on the erythroid cytosolic LIP was measured by co-culturing EP with MØ for 3 h in SFM. The EP were then harvested, washed and incubated in saline at a density of 0.5×10^6/ml with 0.25 mM calcein acetoxymethyl ester (calcein-AM, Sigma–Aldrich, Rehovot, Israel) for 15 min at 37°C. The EP were then collected, washed, suspended in saline and their fluorescence measured immediately thereafter by flow cytometry.

Changes in the EP mitochondrial LIP by MØ were estimated as follows: Iron depleted EP were washed with saline and incubated at a density of 0.5×10^6/ml with 1 μM rhodamine B-[(1,10-phenanthrolin-5-yl)-aminocarbonyl] benzyl ester (RPA, Squarix biotechnology, Marl, Germany) for 20 min at 37°C. Then, they were harvested, washed, suspended in saline and incubated for an additional 10 min at 37°C. Next, the EPs were co-cultured with MØ for 90 min at 37°C, washed and their fluorescence measured by flow cytometry.

These procedures are based on the abilities of calcein-AM to enter viable cells and of RPA to selectively enter their mitochondria. Both compounds become fluorescent upon hydrolysis by esterases [Weston and Parish, 1990, 1992], and their fluorescence is quenched by binding cytosolic or mitochondrial LIP, respectively [Breuer et al., 1995; Petrat et al., 2002]. Thus, the contents of the cellular or mitochondrial LIP are inversely related to the extent of fluorescence.

In the present study, cellular fluorescence was quantified with the FACS-Calibur® flow cytometer (Becton-Dickinson, Immunofluorometry systems, Mountain View, CA). Cells were passed at a rate of 1,000 cells/s, using saline as the sheath fluid. A 488 nm argon laser beam served as the light source for excitation. Instrument calibration and settings were performed using CaliBRITE™-3 beads (Becton-Dickinson). The emission of 20,000 cells was measured using logarithmic amplification for fluorescence and linear amplification for forward light scatter and side light scatter. The latter parameters were used to gate EP, excluding other cells from analysis. The FL1 and FL2 PMTs were used for measuring the cell-associated fluorescence emission of calcein and RPA, respectively. Unstained cells served to determine background fluorescence. The mean cellular fluorescence [the arithmetic Mean Fluorescence Channel (MFC)] of the EP was calculated using the CellQest® software (Becton-Dickinson).

Determination of Transferrin Receptor

EP were harvested, washed and stained with fluorescein iso-thiocyanate conjugated anti-TfR (CD71) antibody (monoclonal, IgG1κ, clone Ber-T9, DakoCytomation, Glostrup, Denmark) at 4°C for 30 min. Then, the cells were washed and analyzed by flow cytometery as described above. Isotype (IgG1κ) control antibodies conjugated with the same fluorochrome (DakoCytomation) were used as control to determine non-specific binding. The fluorescence was measured by the FL1 PMT using logarithmic

amplification and the MFC of the erythroid population was calculated.

RESULTS

Uptake and Secretion of Iron by Macrophages

MØ were incubated with radio–iron either in the form of Tf/anti-Tf immune complexes or bound to NTA. MØ are known to take up particulate iron, such as in the form of the above immune complexes [Cavill, 1971; Esparza and Brock, 1981]. After 3 days, the MØ were washed, lysed and their proteins separated by SDS–PAGE and exposed to phosphor-imaging. Figure 1A shows that both complexes donated radio-iron to intracellular MØ Ft.

To determine if this radioiron-Ft is secreted by MØ, following 3 days incubation with ^{59}Fe-Tf/anti-Tf complexes or with ^{59}Fe-NTA as detailed above, the cells were rinsed and re-incubated in fresh medium for 5 days. Next, the medium was collected and concentrated using a Centricon 50 filter (Amicon) at 5,000*g*. This filter does not allow passage of molecules with a molecular weight greater than 50 kDa (e.g., transferrin or ferritin). Radioactivity was detected only in the concentrate, but not in the filtrate, indicating no radioiron-containing compounds smaller than 50 kDa. Separation of the concentrate by non-reducing (5%) SDS–PAGE indicated radioiron solely in ferritin (Fig. 1B). Taken together, the results indicate that not only do MØ take up radio–iron and incorporate it into their Ft, but secrete this Ft as well.

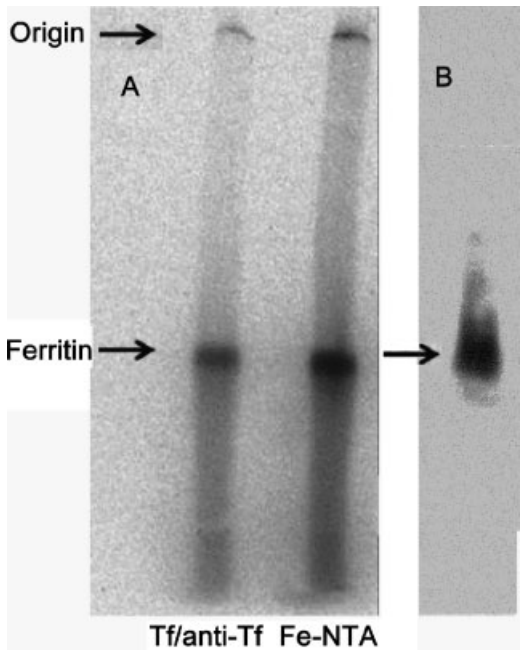

Fig. 1. Incorporation and secretion of exogenous radio-labeled iron by macrophages. Macrophages were incubated for 3 days with ^{59}Fe-transferrin/anti-transferrin immune complexes (Tf/anti-Tf) or with ^{59}Fe-nitrilotriacetic acid (Fe-NTA) in amounts that provided 8 μM iron as described in Materials and Methods. **A**: To determine cellular incorporation of iron, the cells were washed, lysed and their proteins separated by non-reducing (5%) SDS–PAGE. The gels were subsequently dried and radioactivity detected by exposing the gells to phospor-imaging. **B**: To determine secretion of iron-containing compounds, following incubation with radioiron, the cells were rinsed and re-incubated in fresh medium for 5 days. The medium was collected, concentrated using a Centricon 50 filter (Amicon) at 5,000*g* and separated by non-reducing (5%) SDS–PAGE. The gels were subsequently dried and exposed to phosphor-imaging. A non-radioactive human spleen ferritin was run in parallel as standard; its position is marked by arrows. The results of one out of six such experiments are shown. The results indicate that macrophages incorporate radio-iron mainly in their ferritin (A) and that this ferritin can be secreted into the medium (B). No radio-iron with molecular weight lower than ferritin was detected.

Effect of Macrophage-Iron on the Labile Iron Pool of Erythroid Precursors

The labile (chelatable iron pool (LIP) is regarded as the crossroad of intra-cellular iron traffic [Jacobs, 1977]. We examined the effect of MØ on the cytosolic and mitochondrial LIP of EP. EP developing in phase II SCM were harvested on day 5 of phase II, washed and depleted of their iron by overnight incubation in phase II SFM. To measure the effect of MØ on cytosolic LIP, these EP were incubated for 3 h in SFM either with or without rinsed MØ. They were then washed and labeled with calcein-AM. To measure the effect of MØ on mitochondrial LIP, iron-depleted EP were labeled with RPA and then co-cultured with rinsed MØ for 90 min. In both cases the cells were analyzed by flow cytometry as described in Materials and Methods. The results depict the extent of calcein fluorescence (Fig. 2A) or RPA fluorescence (Fig. 2B) of EP grown in SFM with MØ as percent of control EP grown in SFM without MØ (taken as 100%). The fluorescence of EP grown in phase II SCM is also shown for comparison. The results show 55% lower calcein-fluorescence and 75% lower RPA-fluorescence in the presence of MØ ($N = 4$, $P < 0.001$), similar to the fluorescence of EP grown in phase II SCM. Since the extent of fluorescence reflects LIP in an inversely related way, quenching of calcein-AM and RPA fluorescence indicates contribution of MØ to the LIP.

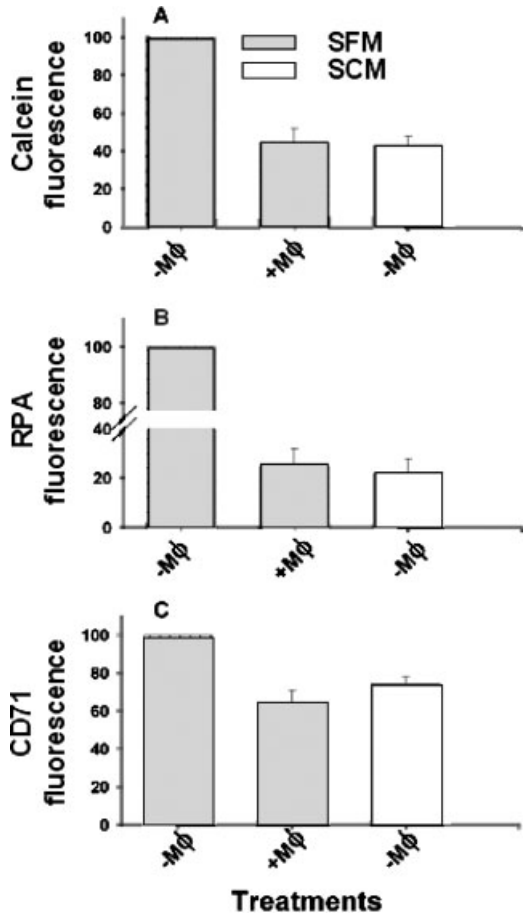

Fig. 2. Effect of macrophages (MØ) on the iron status of erythroid precursors (EP). EP developing in SCM were harvested on day 5 of phase II, washed and depleted of their iron by overnight incubation in phase II—serum-free medium (SFM). To measure the effect of MØ on cytosolic LIP, these EP were incubated for 3 h in phase II SFM either with or without washed MØ. They were then washed and labeled with calcein-AM (**A**). To measure the effect of MØ on mitochondrial LIP, iron-depleted EP were first labeled with Rhodamine B-[(1,10-phenanthrolin-5-yl)-aminocarbonyl]benzyl ester (RPA) and then co-cultured with washed MØ for 90 min (**B**). To measure the effect of on the surface expression of transferrin receptor, washed EP were incubated with MØ in phase II SFM for 18 h, then labeled with fluorescent anti-CD71 antibodies (**C**). In all cases, following incubation with MØ, EP were washed and analyzed immediately thereafter by flow cytometry as described in Materials and Methods. The results depict the extent of cell fluorescence (calculated as the arithmetic Mean Fluorescence Channel) of EP grown in phase II SFM with MØ as percent of control EP grown in phase II SFM without MØ (taken as 100%). The fluorescence of EP grown in phase II serum-containing medium (SCM) are also shown for comparison. The mean ± SD of four experiments performed with cells from different donors are shown. The results indicate lower calcein-fluorescence and lower RPA-fluorescence in the presence of MØ ($N = 4$, $P < 0.001$).

Effect of Macrophages on Transferrin Receptor Expression by Erythroid Precursors

Cell surface TfR expression on EP is sensitive to the iron status of the cell [Rouault et al., 1985; Knisely et al., 1989]. Thus, to evaluate the contribution of MØ to EP iron status, TfR expression was measured. Following iron depletion by incubating EP overnight in phase II SFM, they were co-cultured with MØ in SFM for 18 h. TfR was then labeled with a fluorescein conjugated anti-TfR (CD71) antibody and analyzed by flow cytometry. A 35% decrease in TfR expression of EP cultured with MØ was observed compared to those cultured without MØ (Fig. 2C).

Utilization of Macrophage-Iron by Erythroid Precursors for Heme and Hemoglobin Synthesis

Iron taken up by EP is used predominantly for heme/Hb synthesis. To demonstrate the ability of EP to utilize MØ-derived iron for heme synthesis, MØ were incubated with a particulate radio-iron source (in the form of ^{59}Fe-Tf/anti-Tf) or with soluble ^{59}Fe-NTA) for 3 days as described above. EP were washed on day 5 of phase II, and co-cultured with the iron-labeled MØ for 6 additional days in SFM. The EP were then harvested, rinsed, lysed, their heme extracted and its radioactivity quantified. The results demonstrated the presence of radio-iron in heme extracted from EP co-cultured with MØ pre-incubated with either radiolabeled Tf/anti-Tf or Fe-NTA (903 ± 45 and $1{,}229 \pm 72$ CPM, respectively, as compared to 20 ± 12 CPM in heme extracted from control cells). To verify that MØ were the sole source of radio-labeled iron, EP were cultured in medium used to rinse the MØ after removal of the iron sources. Heme extracted from these EP did not contain radio-labeled iron (results not shown).

To quantify the effect of MØ on Hb production during co-culture of EP with MØ, EP were iron-depleted, then co-cultured with MØ for 1 day in SFM, washed, lysed and analyzed for Hb by HPLC. Figure 3 shows that such EP cultures contain approximately twice the amount of Hb compared to cultures without MØ. It should be noted, however, that cultures of EP in phase II SCM yielded significantly more Hb.

Mechanisms Involved in Macrophage-Supported Erythroid Development

The above results suggest that MØ support EP in Tf-free medium by supplying iron for their

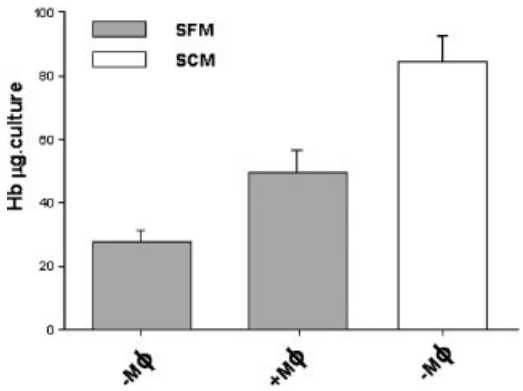

Fig. 3. Effect of macrophages (MØ) on hemoglobin (Hb) production. On day 5 of phase II, erythroid precursors were washed and grown in serum-free medium with (+MØ) or without (−MØ), or in serum-containing medium without MØ (SCM). After 24 h, non-adherent cells were harvested, washed and lysed. The Hb content of the lysates was quantified by HPLC analysis. The mean ± SD of three experiments performed with cells from different donors are indicated. Difference between EP grown in SFM with or without MØ were statistically significant (N = 3, $P < 0.01$).

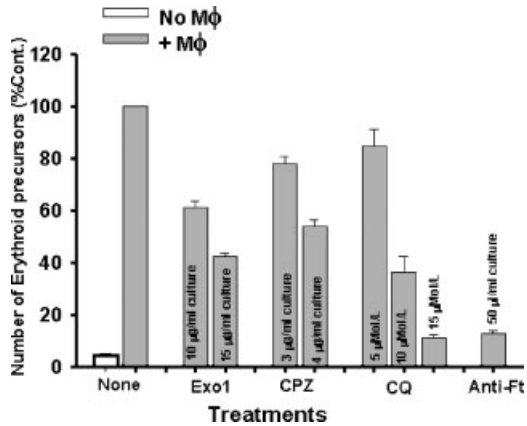

Fig. 4. Effect of inhibitors of exocytosis, endocytosis and endosomal iron release on the macrophage-supported development of erythroid precursors. On day 5 of phase II, erythroid cells were washed, co-cultured with (+MØ) or without (−MØ) macrophages. The cultures were then treated with the indicated concentrations of the exocytosis inhibitor Exo1, the endocytosis inhibitor chlorpromazine (CPZ), chloroquine (CQ), a weak base that neutralizes endosomal pH, thus preventing protein-bound iron release and/or lysosomal enzyme activity, or anti-ferritin (Ft) anti-serum. In the case of Exo1 and anti-Ft, MØ were pre-incubated with these substances for 18 h prior to co-culturing with EP. Five days later, the number of erythroid cells was quantified by benzidine staining. The mean ± SD of three experiments performed with cells from different donors are indicated.

development. To examine some of the mechanisms involved, EP grown for 5 days in phase II SCM were washed and re-cultured with MØ for 5 days in phase II SFM supplemented with various inhibitors. Hb-containing EP were then stained with benzidine and counted microscopically. To examine the role of exocytosis, MØ in phase II SCM were pretreated for 18 h with Exo1, a reversible exocytosis inhibitor [Feng et al., 2003], at concentrations of 10–15 µg/ml culture. EP were, then added and incubated for additional 5 days in Exo1-containing phase II SFM. The results show that Exo1 decreased the ability of MØ to support EP growth by 40–60% in a concentration-dependent fashion (Fig. 4). Exo1 did not affect EP growth when added to Tf-containing phase II cultures (in both cases EP reached about 1.2×10^6 per ml).

To study the role of endocytosis and endosomal iron release, MØ-EP co-cultures in SFM were treated with chlorpromazine (3–4 µg/ml culture), an inhibitor of clathrin-dependent receptor-mediated endocytosis [Daukas and Zigmond, 1985], or chloroquine (5–15 µM), a weak base that neutralizes endosomal pH, thereby preventing iron release [Armstrong and Morgan, 1983]. Figure 4 shows a dose-dependent decrease in the number EP in the presence of either compounds, indicating that both processes are important. These results indicate that both endocytosis and endosomal iron release are involved in the MØ-supported EP growth.

Finally, we examined the effect of an antibody to Ft. Addition of 50 µl/ml culture anti-Ft antibody to the MØ-EP co-cultures resulted (Fig. 4) in a >80% decrease in the number of MØ-supported EP compared to untreated cultures. When EP were incubated with anti-Ft antibody in SCM they were unaffected and developed normally (in both cases EP reached about 1.2×10^6 per ml).

DISCUSSION

We have studied previously iron uptake and metabolism in human EP using two-phase liquid cultures [Gelvan et al., 1996; Vaisman et al., 1997; Meyron-Holtz et al., 1999]. In this system, EP that developed following addition of erythropoietin to the second phase of the culture used iron in the form of hollo-Tf. No EP developed in the absence of Tf [Leimberg et al., 2003]. We have demonstrated earlier that under Tf-free conditions both external Ft and MØ are able to function as iron sources for EP [Leimberg

et al., 2003, 2005]. We now studied the mechanism by which MØ support EP development under Tf-free conditions. We initially showed that, similar to observations in rat monocytes [Rama et al., 1988], MØ derived from human peripheral blood monocytes take-up exogenous radio-iron and incorporate it mainly into their Ft (Fig. 1A). This Ft was shown to be secreted by MØ into the culture medium (Fig. 1B), in agreement with a previous report that MØ secrete Ft [Esparza and Brock, 1981]. This "conditioned medium," however, did not reproducibly support EP growth (data not shown), suggesting that the presence of intact MØ is required continuously. We also showed that MØ-derived radio-iron is taken-up by EP and is incorporated into their heme (Fig. 3). Since Ft was the major cellular component in the MØ seen to contain radio-iron, these results suggest that Ft was the iron source for heme synthesis by the EP. Ft was verified as the component in the SFM that supports EP; addition of an antibody to Ft to the MØ/EP co-cultures in phase II SFM resulted in a significant inhibition of EP development.

We next studied the effect of MØ on the "labile iron pool" (LIP) of the EP under Tf-free conditions. LIP consists of various forms of iron bound to low-affinity ligands that vary in composition and quantity under different physiological settings. It is considered the "regulatory" pool of cellular iron [Konijn et al., 1999]. We showed that co-culturing of iron-depleted EP with MØ in phase II SFM increased their cytosolic and mitochondrial LIP within 3 h. This effect is followed by a decreased expression of TfR. Cells regulate their LIP within a range that meets cellular iron requirements by iron-responsive proteins that sense its level, and in turn control the translation of mRNA for TfR and Ft in a compensatory manner [Konijn et al., 1999]. Our results indicate that MØ-iron is taken up and recognized by EP for this pathway.

Inhibition of exocytosis or endocytosis in these co-cultures by Exo1 and chlorpromazine, respectively, or neutralization of endosomal pH—by chloroquine, also reduced EP development. These results suggest that the iron taken up by MØ is incorporated mainly into Ft, which is subsequently actively secreted by an exocytotic process. EP which are in close proximity are able to take up this Ft probably through clathrin-dependent, receptor-mediated endocytosis (as suggested by experiments (Fig. 4) using chlorpromazine, an inhibitor of clathrin-dependent receptor-mediated endocytosis) into endosomes. We have previously suggested the existence of a Ft-receptor specific mainly to H-subunit-rich isoFt on the surface of EP [Meyron-Holtz et al., 1999]. Acidification of the endosomes/lysosomes releases the iron from the Ft by proteolysis and makes it available for regulatory and synthetic purposes. We have also previously shown that chloroquine repression of lysosomal function prevented iron transfer from Ft to Hb [Vaisman et al., 1997].

Taken in toto, our present results show that MØ support EP development under Tf-free conditions by delivering essential iron in the form of metabolizable ferritin.

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REFERENCES

Armstrong NJ, Morgan EH. 1983. The effect of lysosomotrophic bases and inhibitors of transglutaminase on iron uptake by immature erythroid cells. Biochim Biophys Acta 762:175–186.

Bessis MC, Breton-Gorius J. 1962. Iron metabolism in the bone marrow as seen by electron microscopy: A critical review. Blood 19:635–663.

Breuer W, Epsztejn S, Cabantchik ZI. 1995. Iron acquired from transferrin by K562 cells is delivered into a cytoplasmic pool of chelatable iron(II). J Biol Chem 270: 24209–24215.

Cavill I. 1971. The preparation of 59 Fe-labelled transferrin for ferrokinetic studies. J Clin Pathol 24:472–474.

Daukas G, Zigmond SH. 1985. Inhibition of receptor-mediated but not fluid-phase endocytosis in polymorphonuclear leukocytes. J Cell Biol 101:1673–1679.

Esparza I, Brock JH. 1981. Release of iron by resident and stimulated mouse peritoneal macrophages following ingestion and degradation of transferrin-antitransferrin immune complexes. Br J Haematol 49:603–614.

Feng Y, Yu S, Lasell TK, Jadhav AP, Macia E, Chardin P, Melancon P, Roth M, Mitchison T, Kirchhausen T. 2003. Exo1: A new chemical inhibitor of the exocytic pathway. Proc Natl Acad Sci USA 100:6469–6474.

Fibach E. 2001. Cell culture and animal models to screen for promising fetal hemoglobin-stimulating compounds. Semin Hematol 38:374–381.

Fibach E, Manor D, Oppenheim A, Rachmilewitz EA. 1989. Proliferation and maturation of human erythroid progenitors in liquid culture. Blood 73:100–103.

Gelvan D, Fibach E, Meyron-Holtz EG, Konijn AM. 1996. Ferritin uptake by human erythroid precursors is a regulated iron uptake pathway. Blood 88:3200–3207.

Jacobs A. 1977. Low molecular weight intracellular iron transport compounds. Blood 50:433–439.

Knisely AS, Harford JB, Klausner RD, Taylor SR. 1989. Neonatal hemochromatosis. The regulation of transferrin-receptor and ferritin synthesis by iron in cultured fibroblastic-line cells. Am J Pathol 134:439–445.

Konijn AM, Tal R, Levy R, Matzner Y. 1985. Isolation and fractionation of ferritin from human term placenta—A source for human isoferritins. Anal Biochem 144:423–428.

Konijn AM, Glickstein H, Vaisman B, Meyron-Holtz EG, Slotki IN, Cabantchik ZI. 1999. The cellular labile iron pool and intracellular ferritin in K562 cells. Blood 94:2128–2134.

Leimberg JM, Konijn AM, Fibach E. 2003. Developing human erythroid cells grown in transferrin-free medium utilize iron originating from extracellular ferritin. Am J Hematol 73:211–212.

Leimberg JM, Konijn AM, Fibach E. 2005. Macrophages promote development of human erythroid precursors in transferrin-free culture medium. Hematology 10:73–76.

Meyron-Holtz EG, Vaisman B, Cabantchik ZI, Fibach E, Rouault TA, Hershko C, Konijn AM. 1999. Regulation of intracellular iron metabolism in human erythroid precursors by internalized extracellular ferritin. Blood 94:3205–3211.

Petrat F, Weisheit D, Lensen M, de Groot H, Sustmann R, Rauen U. 2002. Selective determination of mitochondrial chelatable iron in viable cells with a new fluorescent sensor. Biochem J 362:137–147.

Rama R, Sanchez J, Octave JN. 1988. Iron mobilization from cultured rat bone marrow macrophages. Biochim Biophys Acta 968:51–58.

Rouault T, Rao K, Harford J, Mattia E, Klausner RD. 1985. Hemin, chelatable iron, and the regulation of transferrin receptor biosynthesis. J Biol Chem 260:14862–14866.

Thunell S. 1965. Determination of incorporation of ^{59}Fe in hemin of peripheral red blood cells and of red cells in bone marrow cultures. Clin Chim Acta 11:321–333.

Vaisman B, Fibach E, Konijn AM. 1997. Utilization of intracellular ferritin iron for hemoglobin synthesis in developing human erythroid precursors. Blood 90:831–838.

van Renswoude J, Bridges KR, Harford JB, Klausner RD. 1982. Receptor-mediated endocytosis of transferrin and the uptake of Fe in K562 cells: Identification of a nonlysosomal acidic compartment. Proc Natl Acad Sci USA 79:6186–6190.

Weston SA, Parish CR. 1990. New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. J Immunol Methods 133:87–97.

Weston SA, Parish CR. 1992. Calcein: A novel marker for lymphocytes which enter lymph nodes. Cytometry 13: 739–749.