Red Cell Disorders

research paper

haematologica 2002; 87:578-583

http://www.haematologica.ws/2002_06/578.htm

Enhanced macrophagic attack on β-thalassemia major erythroid precursors

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Background and Objectives. In β-thalassemia major (Cooley's anemia), ferrokinetic studies show that 60-80% of erythroid precursors die in the marrow or extramedullary sites. However, study of marrow aspirates does not reveal huge numbers of dead and dying erythroid precursors. We explored this apparent discrepancy with the hypothesis that enhanced phagocytosis of thalassemic erythroid precursors was a likely explanation. Prior studies had reported on an increase in thalassemic marrow macrophages and their enhanced state of activation. Therefore this study explored the characteristics of thalassemic erythroid precursors which might lead to enhanced susceptibility to phagocytosis. We have shown that enhanced erythroid apoptosis parallels the extent of ineffective erythropoeisis in thalassemic patients, and apoptotic cells are rapidly phagocytosed. Thus, increased apoptosis and perhaps other features of thalassemic erythroid precursors might be the cause of their enhanced phagocytic removal.

Design and Methods. Erythroid precursors were isolated from normal and β-thalassemia major marrow, and incubated with uniform cultures of murine macrophages. The extent of phagocytosis was measured and then specific inhibitors were added to identify some of the messages effete erythroid precursors use to signal their condition to macrophages.

Results. β-thalassemia major erythroid precursors are phagocytosed twice as effectively as normal erythroid precursors.

Interpretation and Conclusions. Experiments using inhibitors of phagocytosis showed that enhanced apoptosis is certainly responsible for part of the increased phagocytosis of thalassemic erythroid precursors. Interestingly, normal erythroid precursors are also subject to phagocytosis by qualitatively similar mechanisms.

Key words: phagocytosis, erythroid precursors, β-thalassemia, apoptosis, macrophages.

Marrow aspirates from patients with β-thalassemia major (Cooley's anemia) show very intense erythroid hyperplasia, and ferrokinetic analyses show that 60%-80% of thalassemic erythroid precursors die in the marrow (the normal value is 10-20%) or in sites of extramedullary erythropoeisis.[1] Marrow examination, however, does not show numbers of dying erythroid precursors consistent with a 60%-80% death rate. The exploration of this discrepancy was the aim of our study. Our hypothesis was that there is increased macrophagic removal of β-thalassemia major erythroid precursors. In order to evaluate the possibility of enhanced phagocytosis one must consider the role of macrophage numbers, their state of activation, as well as the possible unique features of the thalassemic erythroid precursors that could contribute to their enhanced removal. Prior studies had shown that there are increased numbers of macrophages in β-thalassemia major marrow[2] and that thalassemic macrophages are activated.[3,4] However, there are no reports exploring special characteristics of thalassemic erythroid precursors that could contribute to their enhanced phagocytic removal. Therefore, the aim of our study was to determine whether the thalassemic erythroid precursors have features that could potentiate their removal and explain the appearance of marrow aspirates.

We have shown that this increased ineffective erythropoeisis is paralleled by an increase in erythroid precursor apoptosis.[2,5] Unlike necrosis, apoptosis results in the expeditious removal of affected cells by macrophages.[6] However, we wanted to discover how macrophages identify and remove apoptotic thalassemic and normal erythroid precursors. Macrophages use several recognition sites to identify cells that are dying from either apoptosis or other causes.[6,7] The phospholipid bilayer of cells undergoing apoptosis is *flipped* so that phos-

phatidylserine (PS) is expressed on the outer membrane leaflet. Macrophages recognize such cells via their CD36 sites.[8,9] Macrophages also have Fc receptors and can recognize cells that have IgG on their outer surface.[3]

Macrophages preferentially engulf red blood cells from patients with β-thalassemia major and intermedia, apparently by recognizing membrane IgG.[3,10] There seems to be an oxidative alteration of membrane protein 3 producing neoantigens, and thus generation and deposition of immunoglobulins.[11] However, these red blood cells are very different from erythroid precursors in that they circulate, traverse the microcirculation and sinusoids, and are very rigid.[12] Therefore this report focuses on Cooley's anemia erythroid precursors which are nucleated, do not circulate and die in large numbers in the marrow.

Design and Methods

Materials

Granulocyte-macrophage colony-stimulating factor (GM-CSF) was obtained from Gibco BRL, Grand Island NY, USA; anti-CD36 from BD PharMingen San Diego, CA, USA; and annexin V from R&D Systems Minneapolis, MNB, USA. Human IgG, phosphatidylserine from bovine brain and phosphatidylcholine (PC) from both fresh and frozen egg yolks were obtained from Sigma Corp., St. Louis, MO, USA and CD45 beads and columns came from Miltenyi Biotech Inc, Auburn, CA, USA.

Marrow samples

Marrow of patients with Cooley's anemia being evaluated for allogeneic transplantation in Pesaro, Italy is routinely obtained according to protocols approved by the Pesaro Institutional Review Board. Samples from 10 patients were shipped to Stanford, as previously described,[2] invariably accompanied by *control* samples from patients with other hematologic disorders, or patients transplanted 3-10 years ago who are now free of disease. There were ten such *controls*: 2 with chronic myeloid leukemia, one each with Hodgkin's disease, acute promyelocytic leukemia, myelodysplastic syndrome, monoclonal gammopathy of undertermined significance and aplastic anemia, 2 previously transplanted for Cooley's anemia, and a normal allogeneic donor from the Stanford Marrow Transplantation Program.[2]

Preparation of erythroid precursors

Erythroid precursors were harvested as previously described[2,5] using CD45 antibody linked to magnetic beads (erythroid precursors are predominantly CD45 negative) and magnetic column separation, which allowed erythroid precursors to pass through unhindered.

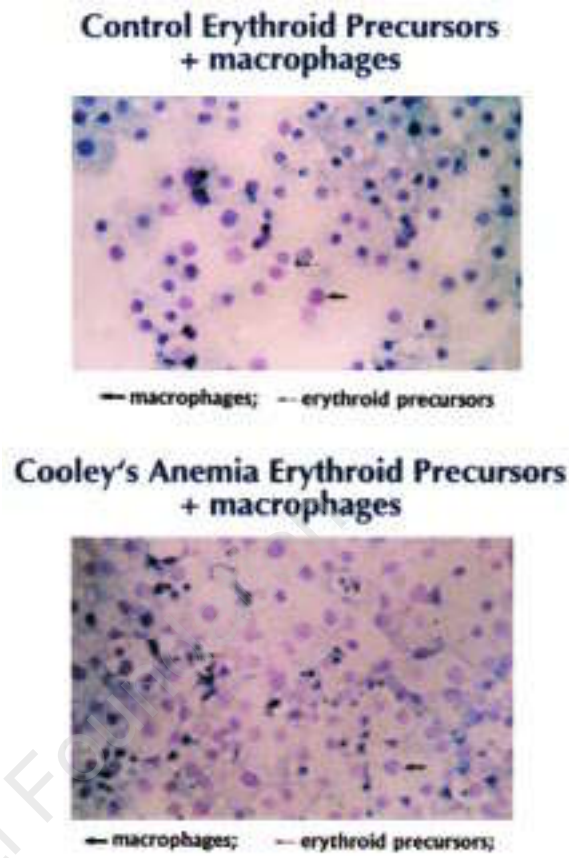

Figure 1. Upper panel. A field of macrophages incubated with normal erythroid precursors. Lower panel. A comparable field of macrophages incubated with thalassemic erythroid precursors.

Macrophage preparation

Both human and murine macrophages were tested and provided comparable results (*data not shown*), but for better reproducibility we used 4-6-day old cultures of C57 murine femoral marrow macrophages. Thus 1×10^6 marrow cells in Iscove's medium supplemented with 10% fetal bovine serum were placed in each well and incubated at 37 C°, 7% CO_2 for 18 hours after which non-adherent cells were washed off. Then 1.0mL of GM-CSF 0.1 μg/mL in 10% fetal bovine serum, Pen/Strep 100U/100 μg/mL in Iscove's medium was added and the incubation was carried out for another 4-6 days at 37°C, under 7% CO_2 (Figure 1).

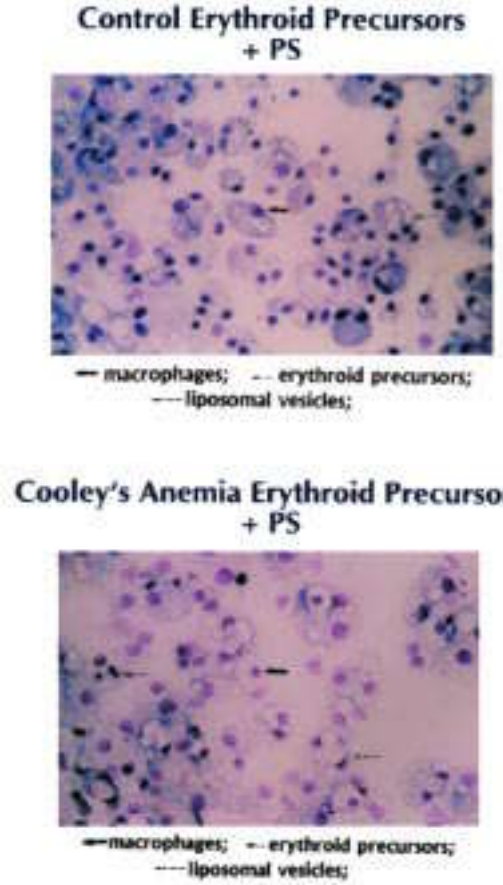

Figure 2. Upper panel. A field of macrophages incubated with normal erythroid precursors after the addition of phosphatidylserine (PS). Lower panel. A comparable field of macrophages incubated with thalassemic erythroid precursors.

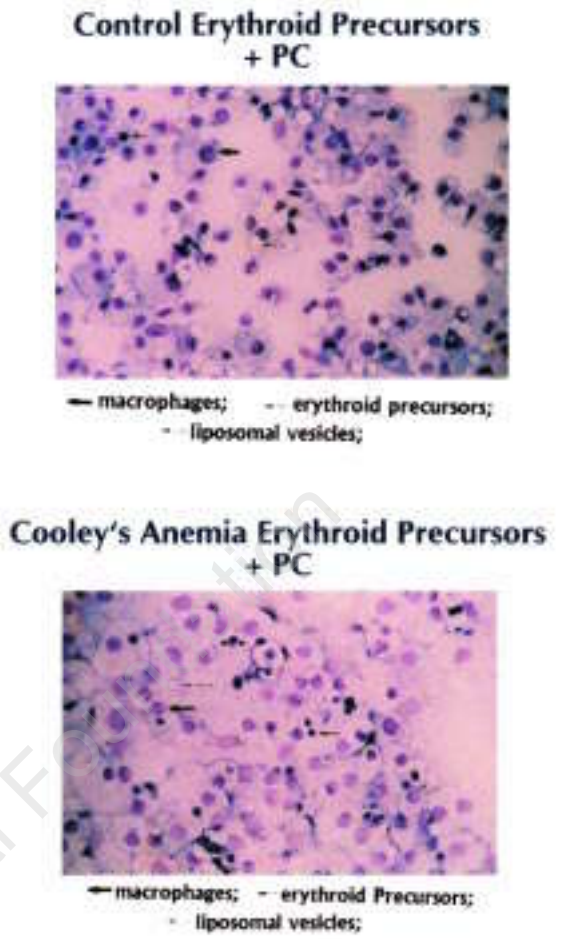

Figure 3. Upper panel. A field of macrophages incubated with normal erythroid precursors after the addition of phosphatidylcholine (PC). Lower panel. A comparable field of macrophages incubated with thalassemic erythroid precursors.

Signal recognition between erythroid precursors and macrophages

PS signaling: to investigate the role of PS signaling in erythroid precursors undergoing apoptosis,[7,8,9] liposomal vesicles composed of PS and phosphatidylcholine (PC) from fresh egg yolk (1:1 PS/PC vesicles) were added and the inhibition of phagocytosis was measured. Controls consisted of vesicles composed of pure frozen egg yolk PC. Stock solutions were vortexed hard to make multilamellar vesicles and these were passed through the Extruder (Lipex Biomembranes Inc.Vancouver BC) six times to produce 100 nm unilamellar vesicles.[13] Next, 32 nmoles of pure PC or 32 nmoles of each of PS/PC were added to the macrophages just before addition of erythroid precursors. Annexin V (AnV) binds to outer membrane PS. Therefore, 50 ng of AnV were added to 1.3×10^6 erythroid precursors and incubated in calcium containing media for 30 min at 37°C. The mixture was washed once, re-suspended in calcium containing Iscove's medium, and then added to the macrophages. Finally, the site on macrophages that recognizes PS on apoptotic cells is CD36. Therefore, 15μg of anti-CD36 were added to the macrophages in each well and incubated for 30 min at 37°C before addition of erythroid precursors.[8,9] (Figures 2 and 3).

Membrane-bound IgG

To determine whether β-thalassemic erythroid precursors have increased amounts of membrane-bound IgG, thereby attracting macrophages,[3,10,11] aggregated human IgG was added to see whether this inhibited phagocytosis. A solution of 0.5mg/mL

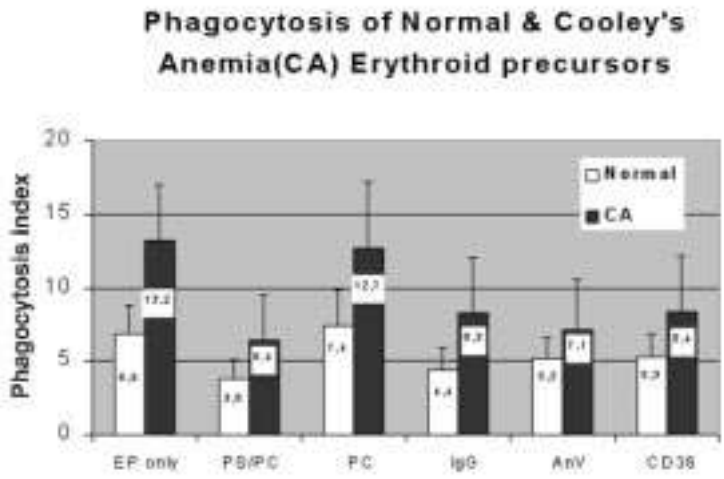

Figure 4. Phagocytic index. The light bars indicate the phagocytic index of normal erythroid precursors and the dark bars of Cooley's anemia (CA) erythroid precursors. The numbers in the bars indicate the mean phagocytic index and the error bars the SD. The statistical analysis is given in the text.

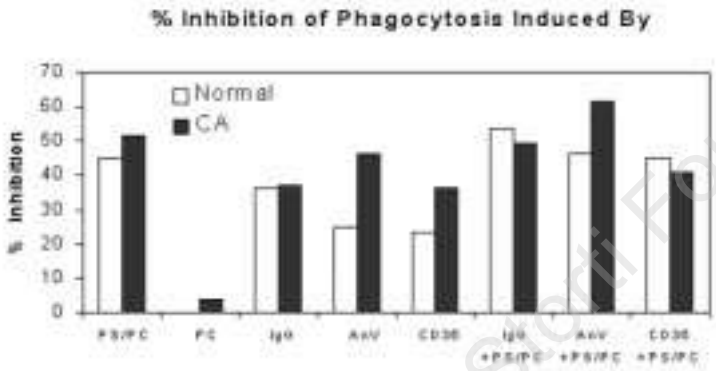

Figure 5. Percent inhibition of phagocytosis of normal (light bars) and Cooley's anemia (dark bars) erythroid precursors.

of human IgG was heated at 60°C for 30 minutes to produce aggregation. Then 25 μg of aggregated IgG were added to each well 10 minutes before addition of erythroid precursors.[10]

Measurement of phagocytosis

After incubation of 1-2×10^6 erythroid precursors with murine macrophages for 4 hours at 37°C the culture dishes were washed three times in PBS and then Wright- Giemsa stained. The number of erythroid precursors actually engulfed by 1,000 macrophages was recorded as the *phagocytic index*.[10] Counts were performed by one person; occasionally a recount was performed.

Results

Twice as many erythroid precursors from 10 patients with Cooley's anemia were phagocytosed as compared with the number phagocytosed from 10 *normal* subjects (Figure 4) p=0.0002.

PC vesicles do not inhibit phagocytosis (Figure 5). However, PS/PC vesicles do, inhibiting phagocytosis of normal erythroid precursors by 40% (p vs. normal control p = 0. 0006) and thalassemic erythroid precursors by 52% (p vs. thalassemic control p = 0.0003).

AnV inhibits phagocytosis of normal erythroid precursors by 25% (p vs. normal control =0.03) and thalassemic erythroid precursors by 46% (p vs. thalassemic control = 0.001).

Anti-CD36 (CD36) also inhibits phagocytosis of normal erythroid precursors by 24% (p vs. normal control = 0.04) and thalassemic erythroid precursors by 38% (p vs. thalassemic control = 0.008). Aggregated IgG (IgG) inhibits phagocytosis of normal erythroid precursors by 35% (p vs. normal control = 0.004), and thalassemic erythroid precursors by 42% (p vs. thalassemic control = 0.007) (Figure 2).

Phagocytosis of normal and thalassemic erythroid precursors was inhibited to the same degree by each class of inhibitors used (Figure 5).

Three to six studies were performed using combinations of two inhibitors (Figure 5). The limitation of numbers of erythroid precursors, particularly normal erythroid precursors, prevented further experiments. Therefore the results could not be statistically analyzed. Combining PS/PC vesicles and either AnV or anti-CD36 produced the same extent of inhibition of phagocytosis of normal and thalassemic erythroid precursors as PS/PC vesicles alone. This was expected since these agents act on the same signaling mechanism. However, combining PS/PC vesicles with IgG also produced no additive inhibition, perhaps because the signaling involves the same mechanisms or because our assay was not sensitive enough to detect small changes.

Discussion

The extent of phagocytosis of Cooley's anemia erythroid precursors is about twice that of control precusors (Figure 4) as indicated by the phagocytic index which is an arbitrary time-limited measurement useful for detecting signals and making comparisons while using identical populations of unstimulated macrophages. Likely, the difference would have been greater had we used patient macrophages that are very activated and increased in number.[2,3,4] However, our aim in this study was to identify those features on the erythroid precursors *per se* that lead to their identification and removal by macrophages. That aim required that we used a single population of unstimulated macrophages in order to compare the cellular characteristics of normal and thalassemic erythroid precursors.

There are differences between thalassemic and normal erythroid precursors, which probably account for the enhanced phagocytosis of thalassemic precursors. One difference is the approximately four-fold increase in apoptosis[2,5] in thalassemic erythroid precursors, which results in surface expression of PS, an important signal to macrophages.[7,8,9] The inhibition of phagocytosis by PS/PC vesicles, AnV, and anti-CD36 (Figures 4 and 5) supports this idea. Another consideration is our prior observation showing that there is a different distribution in levels of maturity between thalassemic and normal erythroid precursors with thalassemic marrow erythroid precursors being represented by more basophilic erythroblasts and fewer orthochromic erythroblasts.[2] Our prior studies indicate that while apoptosis in normal subjects occurs exclusively in orthochromic erythroblasts, in severe β-thalassemia apoptosis also occurs prominently in orthochromic erythroblasts, but sometimes occurs in polychromatophilic erythroblasts as well.[5] It is not clear whether the different levels of erythroid maturity affect our current results.

Normal and thalassemic erythroid precursors send qualitatively similar messages to macrophages (Figure 4). PS exposure caused by apoptosis, is important since phagocytosis is inhibited strongly by PS/PC vesicles, AnV, and anti-CD36 (Figure 5). Phagocytosis is also inhibited by IgG suggesting that the formation of neoantigens may occur in erythroid precursors, as in β-thalassemic red blood cells.[10,11]

The enhanced phagocytic removal of thalassemic erythroid precursors is likely a three-fold composite of: (i) their increased apoptosis, which sends messages such as PS, and perhaps IgG, to macrophages, (ii) the increase in the numbers of thalassemic marrow macrophages,[2] and (iii) the fact that these β-thalassemic macrophages are very activated.[3,4] We propose that the biology of the disease explains why marrow aspirates do not show large numbers of dead and dying cells.

Contributions and Acknowledgments

EA, SS and GL contributed equally to this work and were primarily responsible for it, from conception to submitted manuscript: they should be considered as the principal authors. The remaining authors qualified for authorship according to the World Association of Medical Editors (WAME) criteria, and have taken specific responsibility for the following parts of the content: FC, collection of clinical data; MSB, FC, HB and LM, laboratory experiments; LM, statistical analyses. Order of authorship: authors are listed according to a criterion of decreasing individual contribution to the work, with the following exceptions: the first and last authors contributed equally to this article.

Funding

These studies were supported by grant NIH RO-1-DK- 13682 to SLS and a grant from the Berloni foundation against Thalassemia to FC.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Carlo Brugnara, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Dr. Brugnara and the Editors. Manuscript received November 16, 2001; accepted April 11, 2002.

What is already known on this topic

Ineffective erythropoiesis due to increased apoptosis, and premature demise of erythroid precursors in the marrow is a characteristic feature of homozygous β thalassemia. However, it is not clear what pathways are involved in the recognition and removal of erythroid precursors by macrophages.

What this study adds

By studying the process of phagocytosis *in vitro*, the study shows that β thalassemia major erythroid precursors are phagocytosed twice as effectively as normal precursors. The process is most likely mediated by the abnormal surface expression of phosphatidylserine on the thalassemic erythroid precursors.

Potential implications for clinical practice

Enhanced removal of erythroid precursors explains the absence of a large number of dead, apoptotic cells in bone marrow aspirates of patients with homozygous β thalassemia.

Carlo Brugnara, Deputy Editor