Effect of Surface Digestion and Metabolic Inhibitors on Appearance of Ferritin in Guinea Pig Erythroblasts In Vitro: Evidences for the Production of Apoferritin on the Erythroblast Cell Membrane

By Yasukazu Tanaka and George Brecher

Short-term cultures of guinea pig erythroid cells permitted observation on the micropinocytosis (rhopheocytosis) of ferritin. In the presence of enzymes that attack the cell surface (neuraminidase and trypsin) or of an inhibitor of RNA synthesis (puromycin), accumulation of ferritin in the micropinocytotic vesicle was reduced. No change was seen in presence of lead nitrate, which inhibits transport of iron into hemoglobin-synthesizing cells only slightly. In the presence of potassium cyanide, which inhibits iron transport markedly, ferritin increased two-seven times over controls. The data are compatible with the notion that apoferritin is made on or near the cell surface in response to an excess of iron which accumulates on the cell surface due to failure or overloading of the normal transport mechanism.

Ferritin has been observed in micropinocytotic vesicles of erythroid precursors and the significance of this localization has been studied in man. It has been suggested that some of the iron utilized for hemoglobin synthesis is supplied in the form of ferritin or, alternatively, that excess iron may be eliminated from the erythroblast as ferritin. One of us (Y. T.) has produced experimental evidence that both intake and elimination occurs. Two possible sources for the extraneous iron which may be ingested have been postulated. Ferritin particles may be derived from near-by macrophages or ferritin may be synthesized on the cell membrane of erythroblasts. Both hypotheses are based on electron microscopy of human pathological marrows. Recently, we observed an increase in ferritin laden vesicles and extracellular ferritin in guinea pig erythroblasts during 24-hour incubation in both synthetic media and isologous serum. Because macrophages were rarely observed in this system, the observation suggests the local production of ferritin in erythroid cells. The present study extends the scope of experiments in the same system and describes the effects of puromycin, lead nitrate, cyanide, trypsin and neuraminidase on the production of ferritin in erythroid precursors in vitro.

From General Medical Research, Veterans Administration Hospital, San Francisco, Calif., and Department of Clinical Pathology and Laboratory Medicine, University of California School of Medicine, San Francisco, Calif.
Submitted June 5, 1970; revised August 5, 1970; accepted August 10, 1970.
Yasukazu Tanaka, M.D.: Clinical Investigator, Veterans Administration Hospital and Assistant Professor in Residence, Department of Clinical Pathology and Laboratory Medicine, University of California School of Medicine, San Francisco, Calif. George Brecher, M.D.: Professor and Chairman, Department of Clinical Pathology and Laboratory Medicine, University of California at San Francisco, School of Medicine, San Francisco, Calif.
Fig. 1.—Marrow suspension at 0 hour. The photo was taken from a thick epoxy section stained by alkaline methylene blue. The majority of cells are either granulocytic or erythrocytic elements. A modest number of degenerating elements are present. Macrophages and megakaryocytes are rare. X 1000.

The data support the contention that the ferritin is formed locally on the erythroblast surface.

MATERIALS AND METHODS

The conditions of erythroblast culture were those reported previously.4 Guinea pig femoral marrows were used routinely. In a few experiments, rat erythroblasts were also used.

Several chemicals known to suppress iron absorption and protein synthesis of red cells were tested. In each experiment, marrow suspensions from a single animal were used because the initial number of ferritin particles in the specific micropinocytotic vesicles of erythroblasts differed considerably from animal to animal. One-half of the marrow suspension from one animal was, therefore, incubated in isologous serum containing the chemical to be tested, while the other half of the marrow was incubated in a sample of the same serum without the chemical. In the selection of chemicals and their effective doses we were guided by the work of Jandl et al.,5 except that we used lower doses of cyanide.

Two metabolic inhibitors, 10−3M potassium cyanide and 5 × 10−4M lead nitrate, were tested. Marrow suspensions were incubated for 6–8 hours and the effect of chemicals was evaluated by comparing the experimental samples to the corresponding controls. All experiments were repeated at least twice. Two enzymes, trypsin and neuraminidase, known to affect the iron absorption in reticulocyte rich blood were tested. In the experiments, one half of the marrow suspension from one animal was treated at 37°C for 40–60 minutes with either 0.001 per cent trypsin (once crystallized, salt free, lyophilized; Mann Research Laboratories, New York, N.Y.) in a synthetic medium (CulturSTAT, Eagle’s minimal essential medium, Earle’s Base; BBL, Division of Bioquest, Cockeysville, Md.), or with 5 units of neuraminidase (Vibrio cholera strain Z4; Mann) per milliliter of the same culture medium. After incubation, cells were washed three times with the synthetic medium and resuspended in isologous serum. As control, the other half of the marrow was first kept in the culture medium, then washed and suspended in portion of isologous serum. Marrow suspensions were incubated in the isologous serum containing 10 μg./ml. and 100 μg./ml.
PRODUCTION OF APOFERRITIN ON ERYTHROBLAST CELL MEMBRANE

213

Fig. 2.—An erythroblast after 24-hour incubation.

of puromycin (K. & K. Laboratories, Hollywood, Calif.). A sample of the same marrow suspended in the other half of the same serum served as control.

After incubation, marrow suspensions were centrifuged and pellets were fixed with either 0.5 per cent or 1 per cent glutaraldehyde in 0.1 M phosphate buffer at room temperature. Pellets were postfixed with 1.0 per cent osmic acid and embedded in epoxy resin. Thin sections were cut by diamond knives on a Porter-Blum MT-2 microtome. Sections stained with uranyl acetate and lead were examined with an RCA EMU-4 microscope at 50 kV. In all experiments, pictures were taken at the magnification of 7400 times. The image of one erythroblast was projected on slide plates of 3½ × 4 inches. At least 16 erythroblasts were analyzed in each individual experiment and the individual ferritin particles on the plasma membrane and in the micropinocytotic vesicles were counted. The mean values of the ferritin particles in the experimental samples were com-
Fig. 3.—Details of the marked area (*) in Fig. 2 showing ferritin in the specific vesicles (arrows), and extracellular position (double arrow).

pared to those in the corresponding controls. Ferritin particles deposited in intercellular spaces or clustered within the cells were not counted.

RESULTS

General Morphology of Cells Incubated in Plain Serum (Figs. 1–3)

The guinea pig marrow suspensions at 0 hour consisted of a mixture of myelocytes, erythroblasts and red cells. Megakaryocytes were rarely noted (Fig. 1). Fragments of macrophages with a few ferritin particles were occasionally present, but intact macrophages containing large numbers of ferritin particles were never seen. Ferritin particles in membrane invagination and micropinocytotic vesicles were occasionally present. Deposition of ferritin

Fig. 4.—Erythroblasts incubated for 24 hours in isologous serum (Cont), isologous serum with iron dextran (ID) and iron saturated serum (Exp). More ferritin particles are present in individual erythroblasts incubated in the serum from hypersiderotic animals (ID).
The majority of erythroblasts at 48 hours showed advanced signs of cell degeneration and were inadequate for detailed analysis. In suspensions incubated for 24 hours, preservation of cells varied from sample to sample. They contained many intact erythroblasts and a moderate number of degenerating cells. Increased amounts of ferritin in erythroblasts and in the extracellular spaces were
Fig. 6.—Detail of the arrowed area of Fig. 5, showing extracellular ferritin between two adjacent cells.

Fig. 7.—Erythroblasts incubated in lead nitrate containing serum (Exp) and control (Cont). There is slight suppression in two experiments and slight increase in one. The result is inconclusive. The full circles, triangles and squares represent three different experiments, the open circles, triangles and squares their respective controls.

Fig. 8.—Erythroblasts incubated in cyanide containing serum (Exp) and control (Cont). Significant increase in micropinocytotic ferritin is apparent in the cells incubated in the cyanide-containing serum.
Fig. 9.—Erythroblasts treated with trypsin and incubated in isologous serum (Exp) and control (Cont). Significant decrease in appearance of micropinocytotic ferritin in the cells treated with trypsin.

Fig. 10.—Erythroblasts treated with neuraminidase and incubated in isologous serum (Exp) and control (Cont). Moderate decrease in micropinocytotic ferritin in the cells treated with neuraminidase.

Fig. 11.—Erythroblasts incubated in puromycin containing serum (Exp) and control (Cont). Moderate decrease in appearance of micropinocytotic ferritin in the cells incubated in the puromycin-containing serum.

common (Figs. 2, 3). Erythroblasts incubated for 6 hours were well preserved and already showed increased amounts of ferritin. At 12 hours there is a higher incidence of ferritin in the extracellular spaces. A 6–8 hour incubation was, therefore, used in the evaluation of drug effects.

Ferritin Localization in Variously Treated Sera and in Rat Erythroblasts (Figs. 4, 5, 6)

In preliminary experiments, the effect of iron content in the serum was explored because large numbers of ferritin particles were commonly noted in micropinocytotic vesicles of erythroblasts and reticulocytes of hypersiderotic animals. Marrow suspension from two guinea pigs was incubated for 24 hours in (1) plain isologous serum, (2) the same serum with 15 mg./100 ml. of iron as iron-dextran and (3) isologous serum from hypersiderotic animals whose siderophilin was saturated. As shown in Fig. 4, marrow suspension in-
cubated in the serum from hypersiderotic animals showed more ferritin particles in developing erythroid cells. No increase was noted in cells incubated in serum containing iron-dextran. Formation of ferritin was never noted in rat erythroblasts incubated in isologous serum. When guinea pig erythroblasts were incubated, in untreated rat serum, they showed active ferritin synthesis (Figs. 5, 6). This observation suggests that an intrinsic species characteristic of the cell rather than the serum factor is responsible for the absence of demonstrable ferritin synthesis in the rat erythroblasts in vitro.

**Effect of Metabolic Inhibitors (Figs. 7, 8)**

Cyanide suppresses the iron absorption in reticulocyte-rich blood markedly. In contrast, lead nitrate suppresses iron absorption only slightly. Ferritin synthesis of erythroblasts incubated in isologous serum containing lead nitrate was minimally suppressed in comparison with controls (Fig. 7). In experiments with cyanide, however, erythroblasts synthesized two to seven times as many ferritin particles as controls, both on the membrane and in the micropinocytotic vesicles (Fig. 8). No alteration in cell morphology was noted in either specimen.

**Effect of Surface Digestion (Figs. 9, 10)**

Low concentrations of trypsin are known to inhibit the absorption of iron significantly without affecting the metabolism of erythroid cells. After incubation of erythroblasts in media containing 0.001 per cent trypsin for 40–60 minutes, the number of ferritin particles decreased significantly and suppression of ferritin formation was evident even after an 8-hour incubation (Fig. 9). Production of ferritin was 10 to 30 per cent that of controls. A similar effect was seen in erythroblasts treated with neuraminidase. Ferritin particles numbered 30–50 per cent of controls (Fig. 10). No notable alteration in the erythroblast morphology was seen immediately after enzyme treatment.

**Effect of Puromycin (Fig. 11)**

Erythroblasts incubated in 10 μg./ml. puromycin showed only 30 per cent of ferritin particles of the corresponding controls. The results were consistent in four different experiments. The effect in material incubated in 100 μg./ml. of puromycin was similar.

**Discussion**

Because ferritin micropinocytosis of erythroblasts in the guinea pig marrow is quite similar to that observed in man, it appears warranted to use them as a model system.

Culture conditions do not appear to affect the appearance of ferritin. The amount of ferritin in erythroblasts in culture increased on addition of serum from hypersiderotic animals, and its appearance and distribution was indistinguishable from that in erythroblasts in the hypersiderotic guinea pigs themselves.

The increase of ferritin particles in the micropinocytotic vesicles of guinea pig erythroblasts on incubation of marrow suspensions which are virtually macrophage-free supports the earlier suggestion of the local production of
ferritin in erythroblasts themselves. The suppression of apoferritin synthesis by digestion of cell surface by trypsin and neuraminidase is compatible with the thesis that the synthesis takes place on the plasma membrane. It is likely that the saturation of siderophilin (transferrin) with iron is an important factor in the induction of synthesis both in vitro and in vivo. In guinea pig erythroblasts, ferritin rhopheocytosis can be significantly increased in vivo by injection of iron dextran. Hence, an excess of iron supplied to the cell may similarly provoke apoferritin synthesis in guinea pig erythroblasts. The notion is supported by experiments using iron saturated serum (Fig. 4) and cyanide. Cyanide is known to suppress respiration by its selective conjunction to trivalent iron in cytochrome enzymes. As a consequence, we postulate that iron transport into the red cell precursors is suppressed. Iron accumulates on the erythroid cell membrane and in turn stimulates apoferritin synthesis. Lead nitrate or acetate has been reported to affect transport of iron into erythroblasts and reticulocytes. Lead acetate increases the entry of iron into the ferritin fraction of human and canine marrows. Ferritin synthesis was not measured; hence, the relevance of that observation to our results is not clear. We observed no significant change, possibly because of the semiquantitative nature of the method.

The inhibitory effect of puromycin is in keeping with the dependence of apoferritin synthesis on RNA, even though no ribosome particles were seen near the cell surface.

It is realized that morphologic evidence allows only suggestive conclusions on biochemical processes. Radioautographic experiments are planned to further substantiate the postulated synthesis of ferritin on the erythroblast surface.

A Note About Illustrations

All figures are from guinea pig erythroblasts. Bars indicate 1 μ. In Figs. 4 and 7–11 the means and ranges of ferritin particles in guinea pig erythroblasts under various conditions are indicated on the ordinate.

REFERENCES

Effect of Surface Digestion and Metabolic Inhibitors on Appearance of Ferritin in Guinea Pig Erythroblasts In Vitro: Evidences for the Production of Apoferritin on the Erythroblast Cell Membrane

YASUKAZU TANAKA and GEORGE BRECHER