Intranuclear Hemoglobin in Erythroblasts of β-Thalassemia

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The amount and distribution of intranuclear hemoglobin in erythroblasts of normal subjects and patients with homozygous β-thalassemia were studied by microspectrophotometric methods. The mean intranuclear hemoglobin content of the mature erythroblasts represented 33%-40% of the total hemoglobin content both in normal and thalassemic cells. Distinct absorption peaks in transmission scanning lines recorded over thalassemic erythroblasts suggested the presence of intranuclear hemoglobin precipitation in some cells. Similar characteristic absorption peaks of denatured ferric hemoglobin were recorded over large inclusions of the cytoplasm and small intranuclear precipitates. Intranuclear inclusion bodies may be responsible for the disturbance of erythroblast proliferation in thalassemia, thus explaining in part the ineffective erythropoiesis occurring in this disease.

The presence of intranuclear hemoglobin has previously been demonstrated in human as well as other mammalian erythroblasts, using histochemical absorption, microspectrophotometric, and fluorescence techniques. A close association between hemoglobin and nuclear chromatin has been observed in the mature nucleated erythrocytes of the newt, and it has been postulated that hemoglobin may have a feed-back action in switching off further hemoglobin synthesis in the mature cell. It has also been suggested that the hemoglobin concentration may be involved in the regulation of human erythroblastic proliferation. Using a combined microspectrophotometric and microinterferometric method, it has been shown that no DNA-synthesizing normal human erythroblasts occur when the hemoglobin concentration is higher than 22% (MCHC). This hemoglobin concentration was therefore considered to be critical for shutting off further proliferative activity.

In β-thalassemia, hemoglobin synthesis and erythroblastic proliferation are both disturbed. Deficient β-chain synthesis results in a surplus of α-chains, which precipitate intracellularly. Hemoglobin precipitation appears in the basophilic stage of maturation, and the percentage of inclusion-carrying cells increases with progressive maturation, culminating in the mature cells. The maximum load of

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α-chain inclusions in single red blood cells may reach 75% of the total hemoglobin content. Intracellular precipitates may be of importance not only for the shortened red cell survival in this disease, but also for the disturbed pattern of erythroblastic proliferation. So far, inclusions have been described only in the cytoplasm of thalassemic erythroblasts. The purpose of this study was to investigate the possibility of hemoglobin precipitations being present within the nucleus. This might be of importance for the disturbed proliferation of erythroblasts in β-thalassemia.

MATERIALS AND METHODS

Materials

Bone marrow aspirates sampled from four patients with homozygous or double heterozygous β-thalassemia and one hematologically normal person were investigated. Smears for determination of hemoglobin amounts and scanning absorption patterns in erythroblasts (see below) were prepared as described previously. They were fixed in absolute methanol, stored for less than 10 days, and mounted in chemically pure paraffin oil. Smears for investigation of absorption spectra (see below) were kept air dried and unfixed. They were used immediately or less than 1 hr after sampling.

Microrspectrophotometric Investigation of Hemoglobin

Three different types of microrphotometric measurements were made on mapped cells:

1. Determination of the total, nuclear, and cytoplasmic hemoglobin content. The total hemoglobin content of the whole cell and the nucleus were determined separately as the total extinction at 420 nm \( (E_{420}) \) in the rapid scanning integrating microrphotometer with an adjustable measuring field. This wavelength was chosen because it represents the maximal absorbance of the spectrum recorded over a normal human red cell fixed in methanol and mounted in paraffin oil. Corrections were made for unspecific absorption as previously described. Values for the cytoplasmic hemoglobin content were obtained as the difference between the total cellular and the nuclear hemoglobin amounts.

2. Determination of the hemoglobin distribution and the total hemoglobin content from scanning transmission lines. The distribution of hemoglobin was studied on each scanning transmission line recorded over the cytoplasm and the nucleus at 420 nm in a Zeiss UMSP-1. A speed of 40 μm/min was used with a distance of 0.5 μm between each scanning line. This method was used because in addition to the possibility it offers to study the hemoglobin distribution over several areas, it is easier to distinguish the different areas of the cell for more detailed calculations of the integration of the total extinctions. Figure 1 illustrates the transmission differences along one scanning line recorded over the largest

Fig. 1. Absorption scanning line at 420 nm (solid line) with automatically recorded integral of extinction (dash-dot line) over the cytoplasm and the nucleus of a normal erythroblast. (a) + (c) = integrated extinction over the cytoplasm; (b) = integrated extinction over the nucleus.
diameter of an erythroblast. The line across the transmission peaks represents the “integral of extinction” over the corresponding cellular areas. As shown, the difference between nuclear and cytoplasmic areas is easily discerned on the transmission scanning line.

Scanning transmission spectra were recorded on the same individual cells of the normal subject and of one of the thalassemia cases studied with the method described above. The total hemoglobin content of the individual cells has been calculated from the sum of the integral of extinction of all scanning lines recorded over each cell. Corrections for unspecific absorption were made in the calculations of the sum of the integral of extinction from the scanning lines recorded at 310 nm. The hemoglobin content of the cytoplasm and nucleus separately has been obtained as the sum of the integral of extinction of all scanning lines over the individual areas of each cell. The reading of the total extinction over the cytoplasm and the nucleus in one scanning line is shown in Fig. 1.

Identification of Maturation Stage

After the measurements, the smears were stained with the May Grünwald-Giemsa staining technique in order to determine the maturation stage of the mapped erythroblasts.

RESULTS

Amounts of Hemoglobin in the Cytoplasm and Nucleus of Single Cells

In general, the amounts of hemoglobin were higher in the cytoplasm than in the nucleus in both normal and thalassemic erythroblasts of all maturation stages. The mean ratio of cytoplasmic to nuclear hemoglobin in mature nonproliferating late polychromatic and orthochromatic erythroblasts was rather similar in all thalassemic cases, ranging from 1.8 to 2.3, and did not differ significantly from the mean ratio of 1.5 in the normal cells. Similar ratios were obtained with hemoglobin amounts recorded with both scanning microspectrophotometers. The mean intranuclear hemoglobin content of the mature erythroblasts therefore represented 33%–40% of the total hemoglobin content both in normal and in thalassemic cases.

Distribution of Hemoglobin in Nucleus and Cytoplasm

Optical observations at 420 nm. Bone marrow smears were observed in monochromatic light at 420 nm. In normal erythroblasts and most thalassemic erythroblasts, the absorption was relatively homogenous in the nucleus as well as in the cytoplasm. Some thalassemic erythroblasts had spots of greater absorption in the nucleus as well as in the cytoplasm. It was assumed that the strongly absorbing material might represent precipitated hemoglobin.

Hemoglobin distribution as recorded by absorption scanning lines at 420 nm in UMSP-1. Figure 2 demonstrates three transmission scanning lines at 420 nm recorded over a normal and a thalassemic erythroblast, with visible areas of strongly absorbing spots in the nucleus.

A number of small absorption peaks were seen over the nuclear area of the thalassemic erythroblasts, whereas the corresponding absorption line from the normal erythroblasts contained comparatively few irregularities and no distinct
peaks. This suggests that certain areas of some thalassemic nuclei contain a high concentration of hemoglobin, possibly representing hemoglobin precipitation.

**Absorption Spectra**

The mean ratio of the maximal extinction at the Soret peak of the cytoplasm over the nucleus, as obtained from the absorption spectra recorded at 400–500 nm in normal and thalassemic cells without visible precipitations, was 2.1. This is rather similar to the ratio for total extinction at 420 nm of the hemoglobin amount of the cytoplasm over the nucleus. Since the measuring field had an aperture of 1.5 μ, it was possible to make more exact measurements of different areas of the nucleus. Recordings of the ratio of the Soret peak maximum over an inclusion free space of the cytoplasm and an inclusion-like area of the nucleus gave a significantly lower ratio (0.6) as compared to the ratio recorded over an inclusion free space of the cytoplasm and nucleus (2.1). This low ratio indicates the presence of a high concentration of hemoglobin in the inclusion-like area in the nucleus which therefore seems to represent precipitated hemoglobin.

Absorption spectra were also recorded between 500 and 600 nm over the cytoplasm and the nucleus of freshly prepared normal and thalassemic cells. Figure 3 represents such spectra recorded in one thalassemic erythroblast over the following areas: (1) an inclusion-free part of the cytoplasm; (2) a distinct inclusion body in the cytoplasm; (3) a part of the nucleus without highly absorbing material at 420 nm; (4) an area in the nucleus containing highly absorbing material at 420 nm, as shown by a distinct peak on the scanning line recorded with the Zeiss UMSP-I.

The absorption spectra of normal red cells showed two main peaks for oxyhemoglobin at 576 and 542 nm. The 542-nm peak was somewhat higher than the 576-nm
Fig. 3. Absorption spectra from different parts of the cytoplasm and the nucleus of a thalassemic erythroblast, containing inclusion bodies. Note the smaller or absent peak at 576 nm combined with the distinct peak at 542 nm in the inclusions as opposed to the two distinct peaks in the inclusion-free areas.

peak and resembled previous descriptions.⁴ The different heights of these peaks is explained by the base line being somewhat higher at 500 nm than at 600 nm.

Outside the inclusion body, the cytoplasm and nucleus of the thalassemic erythroblast yielded similar spectra with peaks at 542 and 576 nm. The 576-nm peak was lower than the 542-nm peak, as previously described for the cytoplasm of thalassemic erythrocytes.⁴ This pattern of two peaks at 542 and 576 nm is characteristic for oxyhemoglobin, and the slight shift to a higher 542-nm peak in thalassemic cells has been interpreted as indicating the presence of some hemoglobin in the form of ferrihemoglobin A.⁵ The spectra from inclusion bodies in the cytoplasm showed a much greater height difference between the 576- and the 542-nm peak than the spectra from inclusion-free areas. In most of the inclusions which were large enough to cover the measuring aperture of the phototube, the 576-nm peak was shoulder-shaped; this shoulder suggests a mixture of oxyhemoglobin and denatured ferric hemoglobin ("Low Spin" hemichrom)⁶ characteristic for the precipitated hemoglobin forming Heinz bodies in the cytoplasm. A similar picture was obtained over the nuclear areas with large amounts of absorbing material at 420 nm. This seems to indicate that precipitation of hemoglobin occurs in the nucleus of the erythroblasts in β-thalassemia.

DISCUSSION

Before discussing the results, it is necessary to exclude the possibility that by the methods applied we have not been measuring truly intranuclear hemoglobin but cytoplasmic one over or underlying the nucleus. We believe that this is not so on the following grounds:
(1) It seems very unlikely that about 30%–40% of the total hemoglobin could be trapped in the areas over and under the nucleus, since the area of the cell surface covered by the nucleus is approximately that large; were it so, one would have to assume that cytoplasmic thickness in a smeared cell is the same over the entire surface irrespective of the space occupied by the nucleus.

(2) By the scanning transmission lines, a rather steep transition from cytoplasm to the nucleus is observed, which would not be present if the cytoplasm in the fixed cell was surrounding the upper and lower surface of the nucleus.

(3) Assuming that hemoglobin overlies the nucleus, one would expect the lowest amount to be present over the most central part of it (maximal thickness of the nucleus); in that case the ratio of the maximal extinction at the Soret peak region in that particular area of the nucleus (1.5 μ in diameter) over that of an area of the cytoplasm would differ according to the nuclear area examined. However, in most erythroblasts this ratio was constant irrespective of the nuclear area tested.

The observation of hemoglobin precipitates within the nucleus of β-thalassemic normoblasts by electron microscopy is a further argument in favor of the above. Therefore, from the data presented it seems unlikely that any significant amount of cytoplasmic hemoglobin is included in the calculations of the amount of intranuclear hemoglobin of a smeared and fixed erythroblast.

Intranuclear hemoglobin has been observed in other species. A characteristic rather irregular distribution was seen, and the amounts of hemoglobin in cytoplasm and nucleus were approximately equal. Studies with a fluorescence technique for the localization of heme have demonstrated a uniform distribution of fluorescence between the cytoplasm and the nucleus in human erythroblasts of all maturation stages; however, no estimates of the exact ratio of hemoglobin amounts in cytoplasm and nucleus were reported. In this study we found that a considerable amount of hemoglobin is localized within the nuclei of normal human and thalassemic erythroblasts up to 40% of the total cellular hemoglobin content. Intranuclear hemoglobin appears to be demonstrable also on electron micrographs, and a direct continuity of hemoglobin between the nucleus and the cytoplasm was observed in normal and thalassemic erythroblasts. It thus appears well documented that the erythroblast nucleus contains hemoglobin.

In homozygous β-thalassemia, the deficient β-chain production results in an excess of α-chains, which are precipitated intracellularly in both erythroblasts and erythrocytes. The present results suggest that precipitations occur not only in the cytoplasm but also in the nucleus of the thalassemic erythroblasts; this conclusion is based on finding distinct areas with high absorbancy at the Soret band region over the nucleus. The high absorbancy was manifested as distinct peaks at this wavelength on a scanning absorption line over the nucleus. Furthermore, the absorption spectra in the 500–600-nm region in these high absorbing nuclear areas were similar to those recorded from inclusion bodies in the cytoplasm of thalassemic erythroblasts and different from that of soluble hemoglobin.

The precipitation of intranuclear hemoglobin in thalassemic erythroblasts may be connected with the disturbed proliferation observed previously. A large population of early polychromatic erythroblasts was found to accumulate in the G1-phase of the cell cycle, indicating that mitotic activity had ceased at a stage earlier than normal.
In normal erythroblasts, no further DNA synthesis takes place above a hemoglobin concentration of 22% MCHC. This concentration was considered to be critical for the cessation of further DNA replication in these cells. As the MCHC of the early polychromatic cells of thalassemic patients was significantly lower than the normal MCHC limit of 22%, the mechanism for the accumulation of thalassemic cells in the G phase may be different.

Whereas the role of intranuclear proteins in the initiation of DNA replicative activity in certain mammalian cells is rather well documented, their role in switching off DNA synthesis remains speculative. The excess α-globin chains found in the nucleus of thalassemic erythroblasts may either have been produced within the nuclei, or they may have been synthesized in the cytoplasm and been included within the nuclear membrane after the completion of mitosis. The latter possibility might account for the accumulation of erythroblasts in the postmitotic phase. Thus intranuclear precipitation may interfere with yet unknown mechanisms, that are critical for the initiation of DNA synthesis.

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