A Quantitative X-Ray Microanalysis of Iron in Erythrocytes and Hepatocytes: Freeze-Dried versus Conventional Epon Sections —— A Brief Report ——

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ABSTRACT. To confirm and reevaluate the validity of X-ray microanalysis of iron on conventional Epon sections, iron contained in erythrocytes and hepatocytes was analyzed using 500 nm thick freeze-dried and Epon sections. In the freeze-dried sections, an Fe peak could be identified in the elemental spectrum, and significantly high Fe counts were found in erythrocytes and, to a lesser extent, in hepatocyte cytoplasms, as compared with the background count ($p<0.01$). In the Epon sections, however, no Fe peak in the elemental spectrum of erythrocytes could be identified, and Fe counts were significantly high only in erythrocytes. Compared with the freeze-dried sections, approximately 70% of iron in Epon sections may be lost, but the erythrocyte Fe counts are high enough for statistical analysis in 500 nm thick Epon sections.

Key words: X-ray microanalysis — iron — freeze-dried section — Epon section

X-ray microanalysis of cryopreparations is a well-established technique for examining the elemental content of cells.[1] Among numerous elements, iron can be so clearly distinguished in the X-ray microanalytical spectrum that it has been analyzed on conventional Epon sections for pathological diagnosis.[2,3] Because of elemental loss during tissue processing, few quantitative analyses of iron on Epon sections have been attempted.[4] We have previously morphologically studied embryonic liver hematopoiesis,[5,6] and for further investigation of development of the hematopoietic microenvironment, iron X-ray microanalyses appear to hold much promise. The aim of this short study was to confirm and reevaluate the validity of quantitative analyses of iron in erythrocytes and hepatocytes by means of X-ray microanalysis on Epon sections, as compared with freeze-dried sections.

MATERIALS AND METHODS

Two ICR male mice at 100 days of age, purchased from Japan Clea Laboratory (Tokyo, Japan), were used in this study; one for cryopreparations, and the other for conventional Epon-embedded preparations. The mice were anesthetized with chloroform while livers were rapidly dissected out.

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Cryopreparation

Small liver cubes, approximately 1 mm^3, were removed and frozen onto aluminium specimen stubs coated with Tissue Teck O. C. T. compound (Miles, USA) by plunging them into liquid propane (−160°C). Cryosections, approximately 500 nm thick, were cut in a cryochamber of a Super Nova ultramicrotome (Reichert Jung, Austria) at a specimen temperature of −100°C and at a knife temperature of −90°C. The sections were mounted on formvar film-coated copper grids, placed inside a JOEL JEE-4X vacuum evaporator and allowed to freeze dry. Then they were warmed up to room temperature overnight.

Epon-embedded preparation

Liver blocks, 1 mm^3, were immersed in 4% paraformaldehyde with 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 (Karnovsky's fluid) for three hours at 4°C. After washing in the buffer, the tissue blocks were postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for two hours at 4°C. After dehydration in graded ethanols, they were embedded in Epon 812. Using a Leica Ultracut S, 90 nm thick ultrathin sections were cut for ultrastructural observation, and 500 nm thick sections were cut for X-ray microanalysis. The sections were mounted on formvar film-coated single-pored copper grids, and after double staining in uranyl acetate and lead citrate, observations were carried out on a Hitachi H-7100 electron microscope.

X-ray microanalysis

Sections obtained from the cryopreparations and Epon-embedded preparations, both 500 nm thick, were analyzed in a H-7100 transmission electron microscope fitted with a computerized energy dispersive X-ray microanalytical system, the Kevex Delta Plus Analyzer (Kevex Instruments, USA). Analyses proceeded for 150 sec live time at 100 keV accelerating voltage, and a 2 nA beam current. Spectrums were collected using a square probe giving a probe area of 0.25 μm^2 at a magnification of x 30,000. Fe counts were digitalized by a software QUANTEX installed in the microanalytical system. For Fe counts, analyses were carried out in three different areas in one cell (Fig 1). In erythrocytes, the cytoplasm was divided into three equal parts along its long axis, and, while in hepatocytes, the nucleus was partitioned into three blocks like a pie chart. Fe analyses were carried out at the middle of each partition to obtain mean values. Five to six cells were analyzed in both the freeze-dried and Epon sections. As background Fe counts, five different profiles of sinusoidal lumina were selected on the freeze-dried and Epon sections, and three areas in each profile without cellular elements were analyzed. The data ware given as the mean±SD, and averages of the Fe counts were statistically evaluated by the Student's t-test.

These experiments were approved by the Animal Research Committee of Kawasaki Medical School (No. 00026, 2000) and conducted according to the "Guide for the Care and Use of Laboratory Animals" of Kawasaki Medical School.

RESULTS AND DISCUSSION

Adult mouse livers contained numerous circulating erythrocytes in the sinusoidal lumina of hepatic lobules. The erythrocytes, endothelial cells lining the sinusoidal lumen, and hepatocytes forming cell plates were easily recognized in both the Epon sections and the freeze-dried sections. As shown in Fig 1, the hepatocytes were generally ovoid or polygonal in shape with a large euchromatic round nucleus containing well developed nucleoli. Since the conventional processing methods for Epon embedding extracted lipid and glycogen granules from the cytoplasm of the hepatocytes, the cytoplasm was comprised of two different irregularly shaped regions ; dark regions containing abundant rough endoplasmic reticulum and light regions where glycogen particles were concentrated. Fe analyses were carried out in the dark regions of the hepatocyte cytoplasm containing abundant rough endoplasmic reticulum and free ribosomes (Fig 1).

Fig 2a shows an elemental spectrum obtained from an erythrocyte in a freeze-dried section. Two copper peaks, Cu Kα and Cu Kβ from the grids and a Si peak from instrumentation, were visible, and a sizable iron peak appeared at 6.398 keV. The Fe peak could not be identified in spectrums on nuclei and cytoplasms of hepatocytes and sinusoidal lumina. Table 1 shows the Fe counts

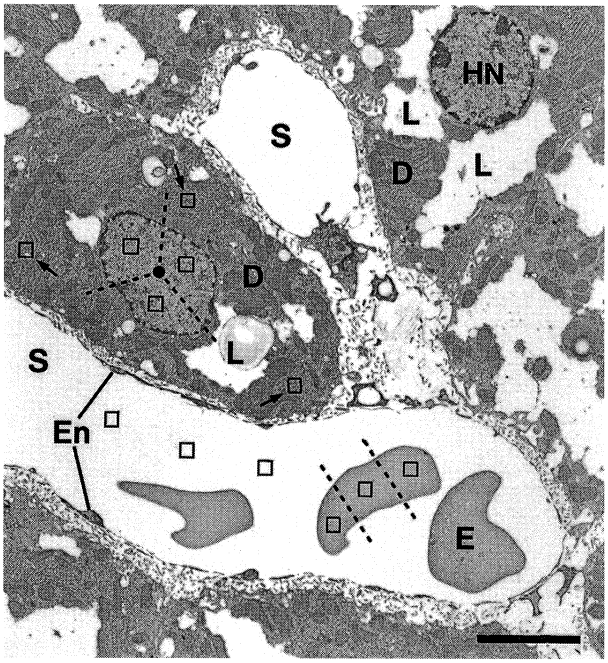

Fig 1. An electron micrograph of adult liver in a 90 nm thick Epon section. Every square indicates actual probe areas, 0.25 μm^2, for iron analysis. An erythrocyte (E) is divided into three equal parts along its long axis and a hepatocyte nucleus (HN) is partitioned into three blocks, like a pie chart. Fe analyses were carried out at the middle of each partition. In hepatocyte cytoplasm, Fe analyses were carried out in the dark regions (D) shown by arrows. As a background Fe count, areas in the sinusoidal lumina (S) without cellular elements were analyzed. En : endothelium of sinusoid, L : light regions of hepatocyte cytoplasm containing lipid and glycogen granules. Bar : 5 μm

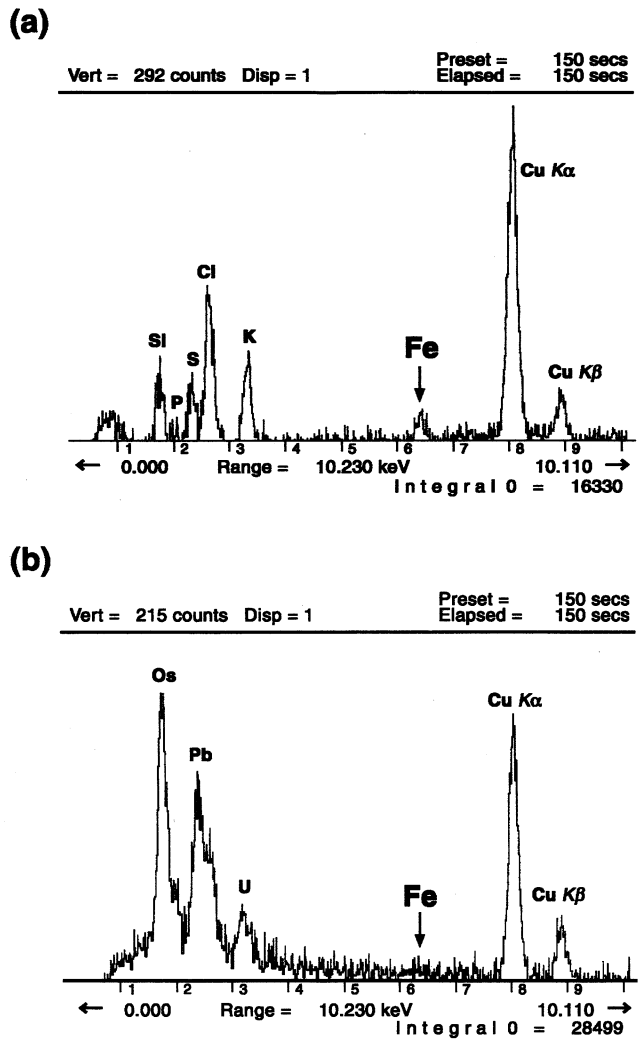

Fig 2. Elemental spectrums of erythrocytes in freeze-dried and Epon sections.
a. Freeze-dried section. Two copper peaks (Cu Kα and Cu Kβ) from the grids and a silica peak (Si) from instrumentation are visible, and a sizable iron peak (Fe) appears at 6.398 keV. b. Epon section. Two copper peaks, an osmium peak (Os), and uranium (U) and lead peaks (Pb) from tissue processing are visible, but no iron peak can be identified.

obtained from each cell element. In the freeze-dried sections, the Fe count of erythrocytes was 353.6 ± 26.8 which was significantly higher ($p < 0.01$) than that of hepatocyte nuclei, 58.8 ± 11.5, and the background Fe count, 56.6 ± 6.8, obtained from the sinusoidal lumina (Fig 3a). Hepatocyte cytoplasms exhibited a high Fe count, 82.9 ± 11.1, and a statistically significant difference ($p < 0.01$) was found between the Fe counts for the cytoplasms and those for the nuclei. In freeze-dried sections, Student's t-values showed that erythrocytes and, to a lesser extent, hepatocyte cytoplasms were significantly high in Fe counts. As is well known, iron is an essential element of hemoglobin, which accumulates in the cytoplasm of erythroblasts during erythropoiesis.[7] Since the erythrons hold

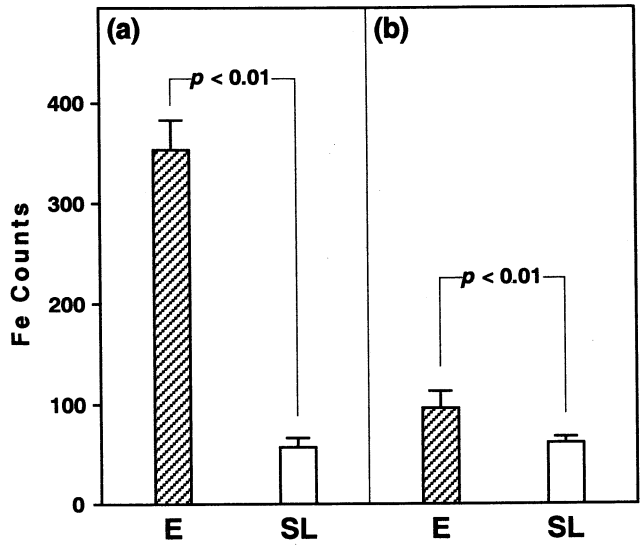

Fig 3. Fe counts of erythrocytes and sinusoidal lumina in freeze-dried (a) and Epon sections (b). Fe counts from Epon sections were much lower than those from cryosections, but each erythrocyte Fe count (E) were significantly higher than the background Fe counts obtained from the sinusoidal lumina (SL).

the major fraction of body iron as heme compounds (especially hemoglobin), high Fe counts and iron peaks in the elemental spectrum of erythrocytes could be considered to correspond to the amount of hemoglobin binding iron. Hepatocytes are known to contain two kinds of storage iron: ferritin and hemosiderin.[8] Ferritin is found scattered in the cell sap and also gathered within lysosome membranes; i.e., siderosomes, while hemosiderin is deposited of degraded protein and coalesced iron and is located with secondary lysosomes.[9,10] Ghadially[11] reported characteristic Fe peaks in the X-ray microanalytical spectrum in the hemosiderin in the secondary lysosomes of macrophages. Although hepatocyte cytoplasms did not show any Fe peaks in the spectrum, it is reasonable to assume that the high Fe counts could be mainly due to ferritin iron in the cell sap.

In the Epon-embedded 500 nm thick sections, the elemental spectrum of erythrocytes did not show any clear Fe peak (Fig 2b), but, as shown in Table 1, the Fe count of erythrocytes was 95.8 ± 3.4, which was significantly higher ($p < 0.01$) than the background Fe count, 60.9 ± 3.4 (Fig 3b). The average Fe counts of hepatocyte nuclei and cytoplasms in Epon sections were 61.1 - 68.7, and no significant differences between the hepatocyte components and the sinusoidal lumen were found in the counts (Table 1). In the Epon sections, high Fe counts were found only in the erythrocytes, and neither a clear Fe peak of erythrocytes nor high Fe counts of hepatocyte cytoplasms could be identified. Since alcohol dehydration, buffer rinses and post-osmication during conventional processing methods for Epon embedding cause depletion of iron in erythrocytes, these differences were mainly the result of an elemental outflow during preparation.[12] Compared with freeze-dried sections, approximately 70%

TABLE 1. Fe counts of freeze-dried and Epon sections

	Freeze-dried section	Epon section
Erythrocyte	353.6±26.8	95.8±14.6
Hepatocyte Nucleus	58.8±11.5	61.1± 2.4
Cytoplasm	82.9±11.1	68.7± 6.8
Sinusoidal Lumen	56.6± 6.8	60.9± 3.4

Values are shown as Mean±SD.

of iron may be lost in Epon sections. However, in spite of the loss, the erythrocyte Fe counts were high enough in 500 nm thick Epon sections for statistical analysis (Fig 3b). Although human hemoglobin concentrations in normal erythrocytes are known to fluctuate around 30%,[13] an X-ray microanalysis on thick conventional Epon sections appears to be quite useful for both qualitative and quantitative evaluation of iron in fetal livers during a rise and fall of embryonic erythropoiesis.

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