Originals

Insulin-driven erythropoiesis may underlie impairment of erythrocyte deformability in hyperinsulinaemic, hyperglycaemic *ob/ob*-mice

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Summary. The circulating erythrocytes in hyperglycaemic and hyperinsulinaemic obese (*ob/ob*) mice are enlarged with a decreased erythrocyte filtrability and an abnormally low resistance to osmotic stress. These changes probably reflect aberrations of erythropoiesis, as evidenced by enhanced staining for iron in the bone marrow, reticulocytosis, and increased erythrocyte volume fraction. Mature erythrocytes, reticulocytes, and late-phase basophilic erythroblasts were found to have larger diameters than their counterparts in

control mice while myelopoiesis appeared to be unaffected. The average erythrocyte also displayed an increased cell volume and a decreased haemoglobin concentration. It is suggested that the stimulation of the erythroid cell line in *ob/ob*-mice might be a consequence of the hereditary hyperinsulinaemia.

Key words: Erythrocyte, erythropoiesis, insulin, erythrocyte deformability, erythrocyte shape, *ob/ob*-mice.

The flexibility of erythrocytes is important for blood rheology [1]. In human diabetes mellitus the erythrocyte deformability is decreased [2–4]. A similar phenomenon has also been reported to occur in obese-hyperglycaemic (*ob/ob*) mice [5, 6]. The abnormal rigidity of erythrocytes in diabetes may contribute to the development of vascular dysfunctions in capillaries [7] and larger vessels [8].

In non-inbred *ob/ob*-mice the diminished deformability appears to be coupled to an enlargement of the erythrocytes [9, 10]. Both the membrane area and the volume of the circulating erythrocytes are increased. Attention is therefore drawn to the possibility of an abnormal erythropoiesis. As the non-inbred *ob/ob*-mice are not only hyperglycaemic but also markedly hyperinsulinaemic [11] it is noteworthy that insulin is a somatotrophic hormone which stimulates erythroid colony formation in vitro [12, 13]. Against this background we have investigated the bone marrow as well as circulating reticulocytes and erythrocytes in *ob/ob*-mice and their normoglycaemic and normoinsulinaemic lean control counterparts.

Materials and methods

Blood donors

Blood and bone marrow were taken from 7–8-month-old female obese non-inbred Umeå-*ob/ob*-mice and age-matched lean control mice. These *ob/ob*-mice are insulin resistant with a 10–100 fold increase in plasma insulin concentration and a hyperglycaemia that peaks in young animals and then gradually declines [5, 11], and have

been widely used in exploring the mechanisms behind the stimulus-secretion-coupling of insulin [14]. In terms of vascular complications these animals are characterized by certain glomerular lesions [15, 16] as well as arteriolar abnormalities [17]. Mice had access to food and water ad libitum.

Blood samplings

Blood of relatively large volumes, used for Coulter counting and measurements of osmotic resistance was taken from mice anaesthetized by i.p. pentobarbital. Approximately 1 ml of venous blood was aspirated from the right heart ventricle into heparin-treated syringes. Small volumes of blood, for counting and measuring reticulocytes and mature erythrocytes, was taken from the tail-tip (5 µl) of unanaesthetized mice and processed as explained below.

Determination of osmotic resistance

Blood was mixed with solutions of various osmolarities (NaCl, 17.1–145.3 mmol/l), and incubated at room temperature for 30 min. The mixtures were centrifuged at 700 g for 5 min and the Hb which was released to the supernatant was spectrophotometrically (415 nm) determined.

Determination of carbon-monoxide haemoglobin

Blood carbon-monoxide haemoglobin (Hb-CO) was measured on a OSM3 Hemoximeter (Radiometer, Copenhagen, Denmark). The Hemoximeter was calibrated for Hb-CO measurements against a gas chromatography method [18].

Table 1. Bone marrow activity in *ob/ob* and control mice

		Control	<i>ob/ob</i>
Reticulocytes in blood	(%)	3.29 ± 0.30	6.72 ± 1.17 ^a
Erythroid cells in marrow	(%)	19.6 ± 1.50	19.9 ± 1.67
Iron content in marrow	(1–9)	1.63 ± 0.42	5.38 ± 0.65 ^b

The unit (1–9) denotes a semiquantitative scale with increasing reticular iron in the bone marrow. Mean values ± SEM for eight *ob/ob*-mice and eight control mice. ^a $p < 0.02$; ^b $p < 0.001$ for comparison with control mice (two-tailed Student's *t*-test with correction for unequal variance)

Table 2. Diameters (μm) of bone marrow and circulating erythroid cells in *ob/ob* and control mice

	Control	<i>ob/ob</i>
Myelopoiesis stage I	12.8 ± 0.09 (8)	12.6 ± 0.15 (8)
Myelopoiesis stage II	10.7 ± 0.11 (8)	10.7 ± 0.17 (8)
Erythropoiesis stage I	9.43 ± 0.20 (8)	9.59 ± 0.17 (8)
Erythropoiesis stage II	7.84 ± 0.16 (8)	8.22 ± 0.14 (8) ^a
(pooled, <i>n</i> = number of erythroblasts)	7.84 ± 0.10 (200)	8.22 ± 0.09 (200) ^b
Reticulocytes	5.02 ± 0.04 (16)	5.35 ± 0.09 (16) ^c
Erythrocytes	6.27 ± 0.03 (6)	6.51 ± 0.03 (6) ^d

Due to shrinkage the reticulocytes in dried smears were smaller than the suspended mature erythrocytes in suspension. Mean values ± SEM for the number of observations indicated within parenthesis; ^a $p < 0.1$ (not significant); ^b $p < 0.01$; ^c $p < 0.005$; ^d $p < 0.001$ for comparison with control mice (two-tailed Student's *t*-test with correction for unequal variance)

Bone marrow sampling and handling

Bone marrow was taken from the left femur of the cardio-punctured mice. The femur was split and the marrow imprinted on two glass-slides. The dried marrow was fixed and stained by standard methods: May-Grünwald-Giemsa, or Prussian blue reaction for the determination of iron content [19].

Blood cell counts

Erythrocytes and leucocytes were counted by means of a Coulter counter (Model S-plus, Coulter Electronics, Inc., Hialeah, Fla., USA). The "Unopette microcollection system" (Becton-Dickinson, Rutherford, NJ, USA) was used by which 44.7 μl of whole blood was collected in a pipette and suspended in 10 ml Isoton II medium (Becton-Dickinson). Leucocyte and erythrocyte counts, haemoglobin, erythrocyte volume fraction (EVF, haematocrit), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were measured or calculated.

Reticulocyte counts

Whole blood, 5 μl, was gently mixed with 5 μl 1% brilliant cresyl blue, incubated for 10 min at room temperature and smeared. Approximately 700 erythrocytes were counted on each slide and the proportion of reticulocytes recorded.

The mouse tail-tips were fixed in 10% formalin, embedded in paraffin, sectioned, stained with haematoxylin/eosin, and studied by light microscopy. The distal piece, about 5 mm, of the tail was not found to hold any bone marrow that could disturb the blood mea-

surements. The reticulocyte-count did not vary with the handling procedure during blood collection; any squeezing of the tail did not result in altered cell counts (data not shown).

Morphological measurements

Bone marrow cells, reticulocytes, and mature erythrocytes were measured by means of an image analysis device (MOP-Videoplan, Kontron Bildanalyse, Munich, FRG) which consisted of a Zeiss microscope, equipped with a bright field 100/1.25 oil objective lens. The microscope was optically connected, via a horizontal tube, to a digitizer with cursor. The outer contour of the cells was followed by the cursor, and the projected cell area, perimeter, and perimetrical shape factor calculated by the computer. The diameter of a circle corresponding to the cell area was also automatically obtained and is here presented as a measure of the cell diameters. Calibration was performed with a Zeiss stage micrometer. All cells were measured on coded slides without knowledge of their identity.

Reticulocytes were analysed in dried smears of blood whereas mature erythrocytes were measured in a Hepes buffered Krebs-Ringer solution (NaCl, 135 mmol/l, KCl, 4.7 mmol/l, KH₂PO₄, 1.2 mmol/l, MgSO₄, 1.2 mmol/l, and CaCl₂, 2.56 mmol/l, glucose, 5 mmol/l, bovine serum albumin, 0.2 g/l, with the pH adjusted to 7.40 by addition of NaOH), after sedimentation to the bottom of a counting chamber. On an average the material consisted of 35 reticulocytes in each of 16 *ob/ob*-mice and 16 control mice, and 50 suspended erythrocytes in each of six *ob/ob*-mice and six control mice.

The myelopoietic as well as the erythropoietic cells were sub-grouped into two stages of maturation. In practical terms, the early myelopoietic phase consisted of cells with rounded nuclei corresponding to myeloblasts and promyelocytes. The later phase comprised cells with more irregular or doughnut-shaped nuclei. The early erythropoietic cells were large proerythroblasts including early basophilic erythroblasts, whereas the later phase consisted of more mature basophilic erythroblasts [20]. The material consisted of 25 cells of each group in eight *ob/ob*-mice and eight control mice.

The percentage of erythropoiesis to myelopoiesis was measured by counting, on an average, 750 bone marrow cells, randomly selected in each slide. Iron content was evaluated on the specially stained slides by a semiquantitative method graded from one to nine with increasing amounts of stained iron.

Statistical analysis

Results are given as mean values ± SEM. Significances for group differences and correlation coefficients were evaluated by unpaired two-tailed Student's *t*-test, F-test, or analysis of variance. Significance was defined as $p < 0.05$, except when otherwise stated.

Results

Bone marrow activity

The *ob/ob*-mice exhibited a markedly augmented number of reticulocytes as compared with control mice (Table 1). The variance in the reticulocyte counts was also increased ($F = 15.5$, $p < 0.001$). Four out of 16 *ob/ob*-mice had more than 10% reticulocytes but were within the interval + 0.8 SD to + 2.1 SD. The median was 4.9%, as compared with 3.1% in control mice samples. Although reticulocytes were more numerous in *ob/ob*-mice, no sign of an increased proportion of erythroid-to-myeloid cells was found in the bone marrow (Table 1). There was no abnormality observed for the megakaryoblasts or the megakaryocytes in

Fig. 1. Osmotic resistance of erythrocytes. Haemolysis was measured as a function of dilution in 12 *ob/ob*-mice (●) and 8 control mice (○). The concentration of NaCl at which 50% of the erythrocytes had lysed was also calculated by interpolation and indicated with horizontal bars of standard error. Mean $\pm$ SEM. * $p < 0.02$

ob/ob-mice (data not shown). The iron content in the marrow was substantially greater in the *ob/ob*-mice as compared with the control mice (Table 1).

Bone marrow morphology

Table 2 shows the diameters, calculated from measurements of projected cell areas, of bone marrow cells at various phases of maturation. The myelopoiesis exhibited no differences between *ob/ob*-mice and control mice. In both types of mice the cell sizes diminished with maturation.

In erythropoiesis no major difference was detected at the early proerythroblastic/early basophilic stage. At the next stage of maturation, however, the basophilic erythroblasts appeared greater in *ob/ob*-mice than in the control mice. An analysis of variance for these cells revealed no significant difference between individual mice within either group of animals ($F = 2.36$, $p > 0.05$). On average, the variance between individual mice within a group was only 5.5% of that between cells, which justifies pooling of the cells. When the pooled late stage basophilic erythroblasts in *ob/ob*-mice and control mice were compared, the difference proved significant with $p < 0.01$ (Table 2).

Reticulocyte and erythrocyte morphology

Both the reticulocytes and the mature erythrocytes had a significantly larger diameter in the *ob/ob*-mice than in control mice (Table 2). There was no significant correlation between the size of reticulocytes and their relative number in whole blood (data not shown).

Blood cell indices

EVF and MCV were increased in *ob/ob*-mice as compared with lean control mice, whereas the MCHC was decreased (Table 3). The concentration of leucocytes or ery-

throcytes did not differ significantly between the two types of mice and there was no difference in haemoglobin or MCH. Carbon-monoxide haemoglobin was measured as an indicator of haemolysis and showed no difference between *ob/ob*-mice and control mice (Table 3).

Osmotic resistance

ob/ob-Mouse erythrocytes were less resistant to osmotic manipulation than control erythrocytes (Fig. 1). The NaCl concentration at which an interpolated 50% of the erythrocytes had lysed was 77.3 ± 1.0 mmol/l in *ob/ob*-mice and 73.0 ± 1.1 mmol/l in control mice (Fig. 1).

Discussion

Previous reports have shown that the circulating erythrocytes in *ob/ob*-mice are enlarged and of a somewhat different shape than those in lean normoglycaemic control mice [9, 10]. Those results are confirmed by the present microscopic data on diameter, as well as by Coulter measurements of MCV. In addition, consistent with the decreased area-to-volume ratio of *ob/ob*-mouse erythrocytes, these cells lysed more readily than control cells in hypo-osmolar buffer solutions. The impaired deformability, as reflected both in the subnormal osmotic resistance and in the previously reported decrease in filtrability through polycarbonate sieves [5, 6], probably reflects the altered morphology of the *ob/ob*-mouse erythrocytes, even though other mechanisms may contribute.

It has been natural to speculate that the altered erythrocyte morphology, and hence the disturbed rheological properties, in *ob/ob*-mice are due to aberrations in erythropoiesis, perhaps related to the marked hyperinsulinaemia in these animals [9]. The present results support this hypothesis. Indicating an enhancement of erythropoiesis there were an augmented number of reticulocytes in the

Table 3. Properties of circulating blood cells in *ob/ob* and control mice

		Control	<i>ob/ob</i>
LC	($10^9/l$)	5.54 ± 0.82	5.41 ± 0.40
EC	($10^{12}/l$)	7.02 ± 0.19	7.45 ± 0.17
Hb	(g/l)	147 ± 4.3	150 ± 4.1
EVF	(%)	33.5 ± 0.92	37.5 ± 0.76^a
MCV	(fl)	47.4 ± 0.31	50.4 ± 0.53^b
MCH	(pg)	20.9 ± 0.30	20.1 ± 0.28
MCHC	(g/l)	441 ± 7.8	400 ± 8.8^a
Hb-CO	(%)	0.78 ± 0.13	0.70 ± 0.08

Leucocyte concentration (LC), erythrocyte concentration (EC), haemoglobin (Hb), erythrocyte volume fraction (EVF), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) in ten *ob/ob*-mice and ten control mice, and carbon-monoxide haemoglobin (Hb-CO) in five *ob/ob*-mice and five control mice. Mean values $\pm$ SEM; ^a $p < 0.005$; ^b $p < 0.001$ for comparison between the two types of mice (two-tailed Student's *t*-test with correction for unequal variance)

circulation, slightly elevated EVF, increased size of erythroblasts and mature erythrocytes, and an increased iron content of the bone marrow.

Reticulocytosis has previously been found to correlate with a diminished erythrocyte filtrability [21], a relationship confirmed by the present findings. Young erythrocytes are known to be larger than old ones, although both are of the same membrane area-to-volume ratio [22, 23]. Therefore, the enhanced number of reticulocytes may contribute to the increase in average erythrocyte size in *ob/ob*-mice and also the observed decrease in MCHC. However, other explanations must also be assumed, as the area-to-volume ratio was clearly decreased in the *ob/ob*-mice as judged from the decrease in osmotic resistance and from previous investigations on individual *ob/ob*-mouse erythrocytes [9, 10].

Since the concentration of erythrocytes in whole blood was slightly, though not significantly, elevated in *ob/ob*-mice it is rather unlikely that erythropoiesis was indirectly stimulated by any haemolytic disorder. Haemolysis was also ruled out by the fact that the concentration of Hb-co did not differ between the two types of mice. Growth-factors, and in particular the marked hyperinsulinaemia in the *ob/ob*-mice are rather more likely stimulators of the erythroid cells in the *ob/ob*-mice. In addition to its classic metabolic effects, insulin has been shown to be mitogenic for a wide spectrum of cells in culture [24]. Insulin in elevated doses is thought to directly stimulate erythroid colony forming cells, plausibly on their insulin-like growth factor (IGF-I or IGF-II) receptors [12]. IGF and insulin appear to act differently from other erythropoietic growth factors [13].

Interestingly, the administration of growth hormone to hypophysectomized rats has recently been shown to stimulate erythropoiesis and increase the volume of the erythrocytes. As a consequence the area-to-volume ratio diminished and the erythrocytes offered an enhanced resistance against filtration through polycarbonate sieves (Engström, Ohlsson, and Oscarsson, unpublished results). Furthermore, our hypothesis that insulin causes bone marrow alterations, and hence changes in circulating erythrocytes, is strongly supported by a recent publication [25], which reported that insulin administration to Type 1 (insulin-dependent) diabetic subjects, stimulates the bone marrow to produce erythrocytes containing fetal-Hb.

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