Enhanced susceptibility to suicidal death of erythrocytes from transgenic mice overexpressing erythropoietin

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WT erythrocytes. Fluorescence reflecting cytosolic Ca2+ activity was significantly larger and forward scatter significantly decreased in tg6 than in WT erythrocytes. The KCl loss and cell shrinkage. The cation channels and thus eryptosis are stimulated by K+ entry and increased K+ channel activity. The term "eryptosis" has been coined to describe the suicidal death of erythrocytes characterized by decrease of forward scatter and phosphatidylserine exposure (43). Similar to erythrocyte senescence (6, 13, 43, 70), eryptosis leads to accelerated clearance of circulating erythrocytes (40).

Ca2+ may enter erythrocytes through Ca2+-permeable cation channels (27, 38), which are activated following osmotic shock by increase of extracellular osmolarity, oxidative stress by addition of t-butylhydroperoxide, and energy depletion by removal of extracellular glucose (42). The channels are inhibited by Cl− and are disinhibited by removal of Cl− (26, 38). Activation of those channels triggers breakdown of phosphatidylserine asymmetry and subsequent erythrocyte death (42). The entry of Ca2+ further leads to stimulation of Ca2+-activated, charybdotoxin- and clotrimazole-sensitive K+ channels with subsequent erythrocyte hyperpolarization driving Cl− out of the cell (58). The loss of KCl with osmotically obliged water then leads to the observed cell shrinkage (48). Increase of extracellular K+ concentration limits the hyperpolarization by decreasing the equilibrium potential for K+. The cation channels are inhibited by erythropoietin (57), which thus counteracts eryptosis and extends the life span of circulating erythrocytes.

The present study has been performed to elucidate whether eryptosis is altered in erythrocytes from transgenic mice overexpressing erythropoietin, thereby reaching dramatically increased erythropoietin plasma levels (75). Despite hematocrit levels of 0.8 and higher, these animals are able to cope with this excessive erythropoietin but show low exercise performance and reduced life expectancy due to multiple organ failure (36).

METHODS

Mice. Erythrocytes were drawn from the tail vein of adult female transgenic mice overexpressing erythropoietin (tg6) and their female wild-type littermates (WT). The blood was collected in phosphate-buffered saline (PBS) containing 2 mM EDTA. The generation and

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properties of tg6 mice have been described previously (64). Comparison was made between mice hemizygous for the human erythropoietin transgene and wild-type animals.

**Determination of the relative distribution width.** The relative distribution width (RDW) was determined by measuring the area of single red cells on Giemsa-stained blood smears using an image analysis system (MCID, St. Catharines, ON, Canada). At least 1,200 erythrocytes per genotype were analyzed (n = 3 per genotype). This procedure was necessary, since the use of a Coulter counter for tg6 blood showed no separation of the thrombocyte and erythrocyte peak.

**Buffers.** Erythrocytes were washed twice in Ringer solution containing 125 mM NaCl, 5 mM KCl, 1 mM MgSO4, 32 mM HEPES, 5 mM glucose, and 1 mM CaCl2. pH 7.4. Erythrocytes were then incubated in Ringer solution at 37°C for different periods of time as indicated. Where indicated, the cells were exposed to Cl- removal (isosmotic replacement by gluconate), 1 μM Ca2+ ionophore ionomycin (Sigma, Taufkirchen, Germany), 10 mM K+ ionophore valinomycin (Calbiochem, Bad Soden, Germany), or 2 U/ml human recombinant erythropoietin (Biomol, Hamburg, Germany).

**FACS analysis.** FACS analysis was performed essentially as described previously (42). After incubation, cells were washed in annexin-binding buffer containing (in mM) 125 NaCl, 10 HEPES, pH 7.4, and 5 CaCl2. Erythrocytes were stained with annexin V-Fluos (Böhringer Mannheim, Mannheim, Germany) at a 1:500 dilution. After 15 min, samples were measured using flow cytometric analysis (FACSCalibur; Becton Dickinson, Heidelberg, Germany). Cells were analyzed using forward scatter, and annexin fluorescence intensity was measured in the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. For determination of relative reticulocyte numbers, 5 μl of whole blood were added to 1 ml of Retic-COUNT (thiazole orange) reagent (Becton Dickinson). Samples were stained for 30 min at room temperature in the dark. FACS analysis of stained samples was then performed according to the manufacturer’s instructions. Forward scatter, side scatter, and thiazole orange fluorescence intensity (in the fluorescence channel FL-1) of the blood cells were measured with a FACSCalibur (Becton Dickinson). The numbers of Retic-COUNT-positive reticulocytes were determined, and relative reticulocyte numbers were expressed as percentages of the total, gated erythrocyte population. Gating of erythrocytes was achieved by analysis of forward vs. side scatter dot plots using CellQuest software. For the determination of annexin V binding to reticulocytes, 2.5 μl of whole blood were added to a solution composed of 200 μl of Retic-COUNT reagent, 5 mM CaCl2, and annexin-V-APC (Becton Dickinson; 1:25 dilution). After incubation for 30 min at 37°C, annexin V binding was assessed in FL-4 (emission wavelength >670 nm). Mature erythrocytes and reticulocytes were distinguished in FL-1 as described above.

**Measurement of intracellular Ca2+.** Intracellular Ca2+ measurements were performed as described. Briefly, erythrocytes were washed in Ringer solution and then loaded with fluo-3 AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 2 mM CaCl2 and 2 μM fluo-3 AM. The cells were incubated at 37°C for 20 min under shaking and washed twice in Ringer solution containing 2 mM CaCl2. The fluo-3 AM-loaded erythrocytes were resuspended in 200 μl Ringer. Ca2+-dependent fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. To test whether measurement of intracellular Ca2+ was affected by different mean corpuscular hemoglobin concentrations (MCHC), erythrocytes were preincubated in PBS solutions of 300, 285, 270, and 240 mosM containing 2 mM CaCl2 and 2 μM fluo-3 AM. Ca2+-dependent fluorescence intensity was then measured as described above. As a result, forward scatter, which reflects erythrocyte volume, approached (all values geo mean ± SE) 294.8 ± 4.7 (300 mosM), 350.5 ± 2.3 (285 mosM), 367.09 ± 3.6 (270 mosM), and 370.6 ± 1.5 (240 mosM), respectively. However, alterations of the bath osmolarity did not significantly affect Ca2+-dependent fluo-3 AM fluorescence, which approached (all values geo means ± SE of FL-1 fluorescence, n = 5) 33.2 ± 0.5 (300 mosM), 34.2 ± 0.5 (285 mosM), 33.1 ± 0.3 (270 mosM), and 33.3 ± 0.3 (240 mosM). Accordingly, changes in erythrocyte volume and thus MCHC did not influence Ca2+-dependent fluo-3 AM fluorescence.

**Measurement of osmotic resistance.** In a 96-well plate, 3 μl of erythrocyte pellets were exposed to varying solutions of decreasing NaCl concentrations for 2 min. After centrifugation for 5 min at 500 g, the supernatant was transferred to a new 96-well plate. In an ELISA reader, absorption at 405 nm was determined as a measure of hemolysis. Absorption in distilled water was defined as 100% hemolysis.

**Statistics.** Data are means ± SE, and statistical analysis was made by unpaired t-test or by ANOVA using Bonferroni’s test as post hoc test, where appropriate.

**RESULTS**

Erythrocyte parameters were determined in mice overexpressing erythropoietin (tg6) and their WT littermates. As shown in Table 1 and published previously (74), the mean corpuscular volume (MCV) was increased in tg6 mice in vivo, a finding similarly reflected by the increased cell size measured with the image analysis system. The relative difference between both mouse lines of the MCV measured previously and cell size determined with the image analysis system was similar. In addition, Fig. 1A shows smears of freshly retrieved tg6 and WT erythrocytes indicating that tg6 erythrocytes were larger in vivo. The RDW (Table 1) of the red cell size, shown as a coefficient of variation, was about three times higher in tg6 mice compared with WT mice, suggesting an anisocytosis in tg6 mice.

Erythropoietin stimulated the maturation of erythrocytes. Enhanced formation of erythrocytes should be reflected by an increased percentage of reticulocytes. As shown in Fig. 1, B and C, the percentage of reticulocytes is indeed larger in tg6 than in WT mice. Osmotic resistance, as estimated from erythrocyte lysis following a decrease of extracellular NaCl concentration, was significantly enhanced in tg6 erythrocytes. The half-maximal hemolysis (IC50) of tg6 erythrocytes (n = 12) was at 0.46 ± 0.02% (wt/vol) NaCl solution, whereas the IC50 of WT erythrocytes (n = 12) was at 0.56% ± 0.03% (wt/vol) NaCl solution.

According to forward scatter, erythrocytes from tg6 mice incubated in Ringer solution for 24 h were significantly smaller than erythrocytes from WT mice (Fig. 2). The decrease of cell volume could have been due to erythropoietin deprivation. Thus additional experiments were performed in the presence of erythropoietin. The addition of erythropoietin (2 U/ml)
had, however, little effect on forward scatter of tg6 erythrocytes (Fig. 2).

Surprisingly, the percentage of annexin-binding erythrocytes, which reflects phosphatidylserine exposure, was enhanced in tg6 mice (Fig. 3, A and B). Again, additional experiments were performed with addition of erythropoietin. Erythropoietin slightly decreased the percentage of annexin-binding tg6 erythrocytes but did not reduce the percentage of annexin-binding erythrocytes to the values obtained in WT blood. Thus the enhanced phosphatidylserine exposure was only partially reversed by addition of erythropoietin (Fig. 3, A and B). Upon acute deprivation of erythropoietin, the level of phosphatidylserine exposure was more pronounced in reticulocytes than in mature erythrocytes (Fig. 3C).

The percentage of annexin binding erythrocytes could be enhanced by Cl− depletion (substitution of Cl− for gluconate) in both genotypes (Fig. 4). However, the percentage of annexin-binding erythrocytes following Cl− removal was again significantly larger in tg6 than in WT erythrocytes (Fig. 4). Since gluconate binds Ca2+, thus lowering the free Ca2+ concentration, it could have blunted the effect of Cl− depletion on phosphatidylserine exposure. To explore this possibility, we assessed annexin V binding of WT and tg6 erythrocytes after incubation in Cl−-free Ringer solution with or without addition of 5 mM CaCl2. The percentage of annexin V-positive erythrocytes in WT erythrocytes approached 8.2 ± 2.1% (Cl−-free Ringer) and 7.9 ± 1.4% (Cl−-free Ringer plus 5 mM CaCl2) and in tg6 erythrocytes, 24.2 ± 2.0% (Cl−-free Ringer) and 26.0 ± 3.3% (Cl−-free Ringer plus 5 mM CaCl2) (all values n = 5).

Fig. 1. Reticulocyte number in erythrocytes. Reticulocyte number determined by fluorescence-activated cell sorting (FACS) analysis of blood from transgenic mice overexpressing erythropoietin (tg6) and their wild type littermates (WT). A: smears of tg6 and WT erythrocytes. B: original histogram. C: arithmetic means ± SE (n = 8). ***P < 0.001, significant difference between tg6 and WT mice.

Fig. 2. Forward scatter of tg6 and WT erythrocytes: effect of erythropoietin. Forward scatter was determined by FACS analysis of tg6 and WT erythrocytes incubated for 24 h in Ringer with or without erythropoietin (EPO; 2 U/ml). A: original histograms. B: arithmetic means ± SE (n = 8). ***P < 0.001, significant difference between tg6 and WT mice.

Fig. 3. Annexin binding of tg6 and WT erythrocytes: effect of erythropoietin. Annexin V binding was determined by FACS analysis of tg6 and WT erythrocytes incubated for 24 h in Ringer with or without EPO (2 U/ml). A: original histograms. B: arithmetic means ± SE (n = 8). ***P < 0.001, significant difference between tg6 and WT mice. C: original dot plot of Retic-count (x-axis) and annexin V fluorescence (y-axis) of tg6 erythrocytes.
The absence or presence of Cl\textsuperscript{-} was determined by FACS analysis of tg6 and WT erythrocytes incubated for 24 h in Ringer or in Cl\textsuperscript{-}-depleted Ringer (Cl\textsuperscript{-} replaced by gluconate). A: original histograms. B: arithmetic means ± SE (n = 4). ***P < 0.001, significant difference between tg6 and WT mice.

Additional experiments were performed to test whether the enhanced phosphatidylserine exposure and decreased forward scatter were the result of increased cytosolic Ca\textsuperscript{2+} activity. As illustrated in Fig. 5, Cl\textsuperscript{-} removal increased cytosolic Ca\textsuperscript{2+} activity in erythrocytes from both genotypes. In the absence of Cl\textsuperscript{-}, cytosolic Ca\textsuperscript{2+} concentration was indeed higher in tg6 erythrocytes than in WT erythrocytes. Thus increased Ca\textsuperscript{2+} entry into tg6 erythrocytes could contribute to the eryptotic effect of Cl\textsuperscript{-} removal in tg6 erythrocytes.

Hyperosmotic shrinkage by addition of 300 mosM sucrose led within 90 min to a reduction of forward scatter from 352.3 ± 0.9 to 333.1 ± 8.7 in WT erythrocytes and from 269.5 ± 6.9 to 260.5 ± 12.2 in tg6 erythrocytes. The shrinkage triggered an increase of cytosolic Ca\textsuperscript{2+} activity in erythrocytes from both genotypes to a similar extent as evident from an increase of fluo-3 fluorescence from 36.1 ± 0.4 to 40.4 ± 2.4 (WT) and from 44.7 ± 0.4 to 51.8 ± 3.1 (tg6) (all values n = 4).

Additional experiments were performed to test whether tg6 erythrocytes are more sensitive to the eryptotic effect of Ca\textsuperscript{2+}. As shown in Fig. 6, increase of cytosolic Ca\textsuperscript{2+} activity by exposure to the Ca\textsuperscript{2+}-ionophore ionomycin stimulated annexin binding of erythrocytes from both genotypes, an effect that was somewhat more pronounced in tg6 than in WT erythrocytes.

To test whether differences in cellular K\textsuperscript{+} loss contribute to the differences between tg6 and WT erythrocytes, we performed experiments in Ringer solution containing the K\textsuperscript{+} ionophore valinomycin. As shown in Fig. 7, treatment with the K\textsuperscript{+} ionophore valinomycin indeed stimulated erythrocyte annexin binding to values similar to those in tg6 and WT erythrocytes. Thus valinomycin abrogated the differences between tg6 and WT erythrocytes.

If cellular K\textsuperscript{+} loss accounts for the valinomycin-induced annexin binding and for the enhanced eryptosis in tg6 erythrocytes, then dissipation of the K\textsuperscript{+} gradient across the cell membrane by increasing the extracellular K\textsuperscript{+} concentration should abrogate the effect of valinomycin and the differences between tg6 and WT erythrocytes. Indeed, in both genotypes, an increase of extracellular K\textsuperscript{+} concentration to 125 mM blunted the increase of annexin binding following treatment with valinomycin (Fig. 7). Thus, valinomycin triggered phosphatidylserine scrambling, at least partially, by stimulating cellular K\textsuperscript{+} loss. In the presence of 125 mM KCl, no significant differences were observed between WT and tg6 cells in the absence or presence of valinomycin. Accordingly, K\textsuperscript{+} efflux is required for the differences in annexin binding between tg6 and WT erythrocytes.

DISCUSSION

The present observations clearly demonstrate the enhanced sensitivity of erythrocytes from transgenic animals overexpressing erythropoietin (tg6) to undergo eryptosis, the suicidal death of erythrocytes. Eryptosis is increased in both control extracellular fluid and Cl\textsuperscript{-}-depleted extracellular fluid. Removal of Cl\textsuperscript{-} opens Ca\textsuperscript{2+}-permeable cation channels, leading to Ca\textsuperscript{2+} entry (4, 26, 27, 38). Ca\textsuperscript{2+} activates (Gardos) K\textsuperscript{+} channels (11, 15), which are dependent on the free Ca\textsuperscript{2+} concentration at the cytoplasmic membrane face (f_{\text{free}}[\text{Ca}^{2+}]) (33). The channel activity is increased between 10^{-2} and 10^{-3} M_{\text{free}}[\text{Ca}^{2+}] (24). Thus, at resting f_{\text{free}}[\text{Ca}^{2+}] (below 100 nM), Gardos channels are inactive and the erythrocyte anion channels build up the principal fraction of the whole cell conductance (52). Upon Gardos channel activation, however, the K\textsuperscript{+} conductance of the erythrocyte membrane exceeds the
Cl⁻ conductance by up to several orders of magnitude (47), strongly hyperpolarizing the membrane potential toward the electrochemical equilibrium and thus enhancing the electrical driving force for Cl⁻ exit. In most species, with the notable exception of adult sheep erythrocytes (28), Ca²⁺ entry thus leads to KCl exit and cell shrinkage (48).

Following in vitro exposure, the forward scatter is smaller in tg6 than in WT erythrocytes. Determination of MCV and RDW, however, revealed that erythrocytes are larger in tg6 than in WT mice. The reason for the difference between those two values remained elusive. Replacement of erythropoietin slightly increased forward scatter but did not abrogate or even reverse the difference between tg6 and WT erythrocytes. Possibly, incubation in Ringer solution deprived the erythrocytes from further plasma components influencing cell volume. Alternatively, MCV and forward scatter might not be reflecting identical erythrocyte properties. There is little doubt, however, that the gross decreases of forward scatter paralleling Ca²⁺ entry or eryptosis indeed do reflect cell shrinkage (48).

In addition to its effect on cell volume, the entry of Ca²⁺ stimulates Ca²⁺-sensitive cell membrane scrambling (23, 78) with subsequent phosphatidyserine exposure at the cell surface (42). Since macrophages are equipped with receptors specific for phosphatidylserine (31, 37), erythrocytes exposing phosphatidylserine at their surface will be recognized, engulfed, and degraded (10). Thus eryptosis triggers clearance of circulating erythrocytes.

Since erythropoietin has previously been shown to inhibit the erythrocyte cation channels and eryptosis (57), the accelerated death of erythrocytes from tg6 mice may appear surprising. In vivo, the high erythropoietin concentrations may protect the erythrocytes, at least in part, from early phosphatidylserine scrambling and thus suicidal death. In Ringer solution devoid of erythropoietin, however, the difference becomes apparent. Since during exposure of erythrocytes to Ringer solution the extracellular fluid is the same for tg6 and WT erythrocytes, the difference must be a property of the erythro-

![Fig. 6. Effect of Ca²⁺ ionophore ionomycin on erythrocyte annexin binding and forward scatter. Annexin binding and forward scatter were determined by FACS analysis of tg6 and WT erythrocytes in Ringer with or without addition of the Ca²⁺ ionophore ionomycin (1 μM). A: original histogram. B: arithmetic means ± SE (n = 4–7) of the percentage of annexin-binding cells. C: arithmetic means ± SE (n = 4–12) of forward scatter. **P < 0.01, significant difference between tg6 and WT mice.

![Fig. 7. Effect of K⁺ ionophore valinomycin on erythrocyte annexin binding at 5 and 125 mM extracellular K⁺ concentration. Annexin binding was determined by FACS analysis of tg6 and WT erythrocytes incubated for 24 h in Ringer with or without addition of the K⁺ ionophore valinomycin (10 nM) at 5 and 125 mM K⁺ concentration. A: original histograms. B: arithmetic means ± SE (n = 10–12) of annexin binding of cells incubated in physiological (5 mM) and high-K⁺ (125 mM) concentration. C: arithmetic means ± SE (n = 3–12) of annexin binding of valinomycin-treated cells incubated in physiological (5 mM) and high-K⁺ (125 mM) concentration. *P < 0.05, significant difference between tg6 and WT mice.
cytes. It is tempting to speculate that erythropoietin exerts at least three actions on erythrocytes: 1) Erythropoietin inhibits death of erythrocyte progenitor cells, thus leading to enhanced formation of mature circulating erythrocytes. This effect is reflected by the enhanced reticulocyte number in tg6 mice compared with WT mice (16, 19, 25, 55, 65, 82). 2) Erythropoietin inhibits the cation channel and thus renders circulating erythrocytes more resistant to activators of the cation channel, Ca\(^{2+}\) entry, and subsequent eryptosis (57). Thus erythropoietin may extend the life span of circulating erythrocytes. 3) Erythropoietin stimulates the expression of genes in progenitor cells, which render the erythrocytes more sensitive to eryptosis, and leads to enhanced erythrocyte death as soon as the erythropoietin concentrations decline. In keeping with this, we speculate that the upregulation of proeryptotic effectors in erythrocytes under the influence of high erythropoietin concentrations allows more rapid removal of excessive erythrocytes if enhanced erythrocyte concentration is no longer needed and the plasma erythropoietin concentrations fall. Along those lines, necroptosis (66), the accelerated death of young erythrocytes following a limited exposure to high altitude or space flight, may reflect the death of those erythrocytes that have been generated under high erythropoietin concentrations and are thus more vulnerable to eryptosis. Clearly, additional experiments are needed to prove or dispute this speculation.

Lack of erythropoietin formation, e.g., in renal insufficiency, may not only decrease formation of new erythrocytes but also accelerate suicidal death of circulating erythrocytes. As a matter of fact, the percentage of phosphatidylserine-exposing erythrocytes is slightly enhanced in renal insufficiency (57). On the other hand, erythrocytes of erythropoietin-deficient patients may in the absence of erythropoietin be less vulnerable to eryptosis, a property, however, never tested thus far.

The difference between tg6 and WT erythrocytes could be blunted by increasing K\(^{+}\) conductance of the cell membrane or by dissipation of the K\(^{+}\) gradient across the cell membrane. Those observations point to a permissive or active role of K\(^{+}\) fluxes in the altered sensitivity of tg6 erythrocytes to eryptosis.

Cellular K\(^{+}\) loss has previously been shown to be one determinant of eryptosis (48). Similarly, cellular K\(^{+}\) loss has been implicated in the apoptosis of nucleated cells under a variety of conditions (12, 20, 54, 61, 76, 79), including stimulation of TNF receptors (32), CD95 activation (34, 71), glucocorticoid (7, 80, 81), GABA (30) or dopamine (60) treatment, growth factor depletion (72), thyroid status (3), sulfonlureas (5), etoposide treatment (69), transformation (77), choline deficiency (2), glutamine depletion (68), oxidative stress (8, 62), hypoxia (53), radiation (67), sphenosine treatment (21), amyloid treatment (18), staurosporine (63), urea (56), Cl\(^{-}\) channel blockers (73), and K\(^{+}\) channel blockers (17). However, the evidence for a causal role of K\(^{+}\) channels in the enhanced susceptibility of tg6 erythrocytes to eryptosis is at present circumstantial. Thus additional experimental effort is needed to explore the potential role of K\(^{+}\) channel activity in enhanced suicidal cell death of tg6 erythrocytes.

In conclusion, tg6 erythrocytes display enhanced rates of eryptosis in Ringer solution and under Cl\(^{-}\) deprivation. The difference of annexin binding between tg6 and WT erythrocytes is blunted or even disappears following treatment with the K\(^{+}\) ionophore valinomycin or in the absence of a K\(^{+}\) gradient across the cell membrane. Altered sensitivity to eryptosis of erythrocytes exposed to excessive erythropoietin concentrations may participate in the regulation of life span and number of circulating erythrocytes.

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