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Enhanced suicidal death of erythrocytes from gene-targeted mice lacking the Cl-/HCO(3)(-) exchanger AE1

Akel, A ; Wagner, C A ; Kovacikova, J ; Kasinathan, R S ; Kiedaisch, V ; Koka, S ; Alper, S L ; Bernhardt, I ; Wieder, T ; Huber, S M ; Lang, F

Abstract: Genetic defects of anion exchanger 1 (AE1) may lead to spherocytic erythrocyte morphology, severe hemolytic anemia, and/or cation leak. In normal erythrocytes, osmotic shock, Cl(-) removal, and energy depletion activate Ca(2+)-permeable cation channels with Ca(2+)-induced suicidal erythrocyte death, i.e., surface exposure of phosphatidylserine, cell shrinkage, and membrane blebbing, all features typical for apoptosis of nucleated cells. The present experiments explored whether AE1 deficiency favors suicidal erythrocyte death. Peripheral blood erythrocyte numbers were significantly smaller in gene-targeted mice lacking AE1 (AE1(-/-) mice) than in their wild-type littermates (AE1(+/+) mice) despite increased percentages of reticulocytes (AE1(-/-): 49%, AE1(+/+): 2%), an indicator of enhanced erythropoiesis. Annexin binding, reflecting phosphatidylserine exposure, was significantly larger in AE1(-/-)erythrocytes/reticulocytes (approximately 10%) than in AE1(+/+) erythrocytes (approximately 1%). Osmotic shock (addition of 400 mM sucrose), Cl(-) removal (replacement with gluconate), or energy depletion (removal of glucose) led to significantly stronger annexin binding in AE1(-/-) erythrocytes/reticulocytes than in AE1(+/+) erythrocytes. The increase of annexin binding following exposure to the Ca(2+) ionophore ionomycin (1 muM) was, however, similar in AE1(-/-) and in AE1(+/+) erythrocytes. Fluo3 fluorescence revealed markedly increased cytosolic Ca(2+) permeability in AE1(-/-) erythrocytes/reticulocytes. Clearance of carboxyfluorescein diacetate succinimidyl ester-labeled erythrocytes/reticulocytes from circulating blood was more rapid in AE1(-/-) mice than in AE1(+/+) mice and was accelerated by ionomycin treatment in both genotypes. In conclusion, lack of AE1 is associated with enhanced Ca(2+) entry and subsequent scrambling of cell membrane phospholipids.

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Enhanced suicidal death of erythrocytes from gene-targeted mice lacking the Cl^-/HCO_3^- exchanger AE1

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Akel A, Wagner CA, Kovacikova J, Kasinathan RS, Kiedaisch V, Koka S, Alper SL, Bernhardt I, Wieder T, Huber SM, Lang F. Enhanced suicidal death of erythrocytes from gene-targeted mice lacking the Cl^-/HCO_3^- exchanger AE1. *Am J Physiol Cell Physiol* 292: C1759–C1767, 2007. First published January 24, 2007; doi:10.1152/ajpcell.00158.2006.—Genetic defects of anion exchanger 1 (AE1) may lead to spherocytic erythrocyte morphology, severe hemolytic anemia, and/or cation leak. In normal erythrocytes, osmotic shock, Cl^- removal, and energy depletion activate Ca^{2+}-permeable cation channels with Ca^{2+}-induced suicidal erythrocyte death, i.e., surface exposure of phosphatidylserine, cell shrinkage, and membrane blebbing, all features typical for apoptosis of nucleated cells. The present experiments explored whether AE1 deficiency favors suicidal erythrocyte death. Peripheral blood erythrocyte numbers were significantly smaller in gene-targeted mice lacking AE1 ($AE1^{-/-}$ mice) than in their wild-type littermates ($AE1^{+/+}$ mice) despite increased percentages of reticulocytes ($AE1^{-/-}$: 49%, $AE1^{+/+}$: 2%), an indicator of enhanced erythropoiesis. Annexin binding, reflecting phosphatidylserine exposure, was significantly larger in $AE1^{-/-}$erythrocytes/reticulocytes (~10%) than in $AE1^{+/+}$ erythrocytes (~1%). Osmotic shock (addition of 400 mM sucrose), Cl^- removal (replacement with gluconate), or energy depletion (removal of glucose) led to significantly stronger annexin binding in $AE1^{-/-}$ erythrocytes/reticulocytes than in $AE1^{+/+}$ erythrocytes. The increase of annexin binding following exposure to the Ca^{2+} ionophore ionomycin (1 μM) was, however, similar in $AE1^{-/-}$ and in $AE1^{+/+}$ erythrocytes. Fluo3 fluorescence revealed markedly increased cytosolic Ca^{2+} permeability in $AE1^{-/-}$ erythrocytes/reticulocytes. Clearance of carboxyfluorescein diacetate succinimidyl ester-labeled erythrocytes/reticulocytes from circulating blood was more rapid in $AE1^{-/-}$ mice than in $AE1^{+/+}$ mice and was accelerated by ionomycin treatment in both genotypes. In conclusion, lack of AE1 is associated with enhanced Ca^{2+} entry and subsequent scrambling of cell membrane phospholipids.

annexin; cell volume; osmolarity; phosphatidylserine; energy depletion

ANION EXCHANGER 1 (AE1), a Cl^-/HCO_3^- exchanger, is the most abundant intrinsic protein of the erythrocyte cell membrane (27). AE1 mediates the rapid exchange of Cl^- with HCO_3^- and thus allows the erythrocyte to release HCO_3^- into the systemic capillaries that perfuse acidic, CO_2-producing peripheral tissues. In pulmonary capillaries, erythrocyte AE1 takes up plasma HCO_3^- for intracellular conversion by carbonic anhydrase to CO_2, which can then diffuse from the cell back into the plasma and across the alveolar epithelium for exhalation (27). Humans with defective AE1 may suffer from hereditary spherocytic anemia, ovalocytosis (1, 25, 33, 47), or stomatocytosis (11, 43). Animals completely lacking AE1 exhibit severe hemolytic anemia, often in the setting of hydrops fetalis (11, 42, 48). Most recently, several distinct missense mutations of AE1 have been associated with enhanced erythroid cation leak (11).

In normal erythrocytes, cation channels are activated by cell injury, such as oxidative stress, osmotic shock, or energy depletion (17, 24), leading to entry of Ca^{2+} and subsequent erythrocyte shrinkage, membrane blebbing, and breakdown of cell membrane phosphatidylserine (PS) asymmetry (28). The channels are also activated and PS exposure is triggered by removal of Cl^- (16). PS exposure, cell shrinkage, and membrane blebbing could be similarly elicited by treatment of erythrocytes with the Ca^{2+} ionophore ionomycin (6, 9, 13, 28). Accounting for the similarities with and differences from apoptosis of nucleated cells (20, 21), the term "eryptosis" has been coined to describe this type of erythrocyte death (29).

To explore whether the machinery leading to suicidal erythrocyte death is modified by lack of AE1, the effects of osmotic shock, Cl^- removal, and energy depletion were studied in erythrocytes from AE1 knockout ($AE1^{-/-}$) mice and their wild-type ($AE1^{+/+}$) littermates. Furthermore, we tested whether $AE1^{-/-}$ and $AE1^{+/+}$ erythrocytes are more rapidly cleared from circulating blood in vivo and whether an increased Ca^{2+} concentration influences the removal of $AE1^{-/-}$ and $AE1^{+/+}$ erythrocytes.

METHODS

Mice. Experiments were performed in erythrocytes from gene-targeted mice lacking AE1 ($AE1^{-/-}$) and their $AE1^{+/+}$ littermates (age: 3–5 mo). The generation and initial characterization of $AE1^{-/-}$ mice were as described by Peters et al. (42). The genotype was determined by PCR. For detection of the wild-type allele, oligonucleotides 5′-AGGTACACGGACAAGGTTTCTTGAT-3′ and 5′-AGGCCAGAGGG TTAGAGGTGAATGTT-3′ were used as forward and reverse primers, respectively, which amplified a 400-bp fragment. The primer pair 5′-CTTGGGTGGAGAGGCTATTC-3′ and 5′-AGGTGAGATGACAGGAGATC-3′ served to detect the mutant allele and amplified a 280-bp fragment. PCR results were detected on a 1.8%

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Table 1. *Blood counts of $AE1^{-/-}$ and $AE1^{+/+}$ mice*

	$AE1^{+/+}$	$AE1^{-/-}$	*P* Value
Red blood cells, 10^6 cells/μl	10.4±0.09	2.42±0.04	$<10^{-6}$
Hematocrit, %	51.2±0.7	15.1±0.3	$<10^{-5}$
Platelets, 10^3 cells/μl	337±8.6	512±68	NS
Hemoglobin, g/100 ml	19.3±0.1	6.7±0.1	$<10^{-7}$
Reticulocytes, %	2.18±0.05	48.7±0.39	$<10^{-10}$

Values are arithmetic means ± SE of red blood cell numbers, packed cell volume (hematocrit), platelets, hemoglobin, and reticulocytes in peripheral blood; $n = 4$ mice/group. NS, not significantly different. *P* values indicate significant differences between anion exchanger 1 (AE1)-deficient ($AE1^{-/-}$) mice and their wild-type ($AE1^{+/+}$) littermates.

agarose gel. Animal experiments were conducted according to the guidelines of the American Physiological Society as well as the German law for the welfare of animals and were approved by local authorities. To obtain erythrocytes, animals were lightly anesthetized with isofluran (Abbott, Wiesbaden, Germany), and ~200 μl of blood were withdrawn into heparinized capillaries by puncturing the retroorbital plexus.

Solutions. Experiments were performed at 37°C in Ringer solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM HEPES-NaOH, 5 mM glucose, and 1 mM $CaCl_2$ (pH 7.4). Where indicated, cells were exposed to osmotic shock (700 mosM by the addition of 400 mM sucrose), to Cl^- removal (isosmotic replacement by gluconate), to energy depletion (glucose removal with or without the addition of 2-deoxyglucose), or to 1 μM of the Ca^{2+} ionophore ionomycin (Sigma, Taufkirchen, Germany). In further experiments, the HEPES-NaOH buffer of the Cl^--free solution was replaced by 25 mM $NaHCO_3$, and cells were incubated in a 5% CO_2 atmosphere. Where indicated, DIDS was added at a concentration of 100 μM.

Blood count. Erythrocyte number, packed cell volume (hematocrit), platelet number, and hemoglobin concentration were determined in blood from the mice using an electronic hematology particle counter (type MDM 905, Medical Diagnostics Marx, Butzbach, Germany) equipped with a photometric unit for hemoglobin determination and with volume settings adjusted for use with mouse erythrocytes.

Determination of GSH. Freshly drawn mouse erythrocytes (3% hematocrit) were washed once with glucose-free Ringer solution and incubated for 7 h at 37°C in Ringer solution, in glucose-free Ringer solution, or in glucose-free Ringer supplemented with 10 mM 2-deoxyglucose. After incubation, the supernatant was removed, and the pellet washed once with ice-cold PBS. Samples were then deproteinated by the addition of 200 μl of ice-cold sulfosalicylic acid (1%) and centrifuged for 5 min at 15,000 *g*. Lysates were diluted in distilled water (1:10), and the GSH content was determined by the addition of 100 μl of assay cocktail mix [92.3 μl of assay buffer (0.1 M NaP_i and 1 mM EDTA; pH 7.5), 4 μl of 10 mM NADPH, 3 μl of 10 mM 5,5′-dithio-bis-(2-nitrobenzoic acid), and 0.7 μl of glutathione reductase (2 U/μl)] to 10 μl of the diluted sample. Finally, the absorbance of the samples was measured at 405 nm and compared with known concentrations of standard samples.

PS exposure. PS exposure was determined by annexin binding in FACS analysis as previously described (2, 28). After incubation, cells were washed in annexin binding buffer containing (in mM) 125 NaCl, 10 HEPES-NaOH (pH 7.4), and 5 $CaCl_2$. Erythrocytes were stained with Annexin-Fluos (Roche Diagnostics, Mannheim, Germany) at a 1:100 dilution. After 15 min, samples were diluted 1:5 and measured by flow cytometric analysis on a FACS-Calibur from Becton Dickinson (Heidelberg, Germany). Annexin fluorescence intensity was measured in the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 515–545 nm.

Double staining. Cells were exposed to osmotic shock (700 mosM by the addition of 400 mM sucrose to Ringer solution) for 4 h and to Cl^- removal (isosmotic replacement by gluconate) for 7 h. Thereafter, cells were washed in annexin binding buffer and incubated (15 min) in annexin binding buffer containing annexin V-568 (1:50 dilution, Roche Diagnostics) and the DNA/RNA-specific dye Syto16 (30 nM, Molecular Probes, Leiden, The Netherlands). Samples were diluted 1:5, and Syto16- and annexin V-568-specific fluorescence was analyzed by flow cytometry in fluorescence channel FL-1 and FL-2 (emission wavelength of 564–606 nm), respectively.

Measurements of intracellular Ca^{2+} activity. Intracellular Ca^{2+} measurements were performed as previously described (4). Briefly, erythrocytes were loaded with Fluo-3 AM (Calbiochem, Bad Soden, Germany) by the addition of 2 μl of a Fluo-3 AM stock solution (1 mM in DMSO) to 1 ml of the erythrocyte suspension [0.3% hematocrit in Fluo-3 AM buffer containing 123 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 25 mM HEPES-NaOH (pH 7.4), 10 mM glucose, 10 mM pyruvate, and 2 mM $CaCl_2$ (or 0 mM $CaCl_2$ and 0.5 mM EGTA)]. Cells were incubated at 37°C for 15 min under shaking and with protection from light. An additional 2 μl of Fluo-3 AM (1 mM) were added with incubation carried out for 25 min without shaking. Fluo-3 AM-loaded erythrocytes were centrifuged at 1,800 rpm for 5 min at 22°C and washed with Ringer solution (or Ca^{2+}-free Ringer

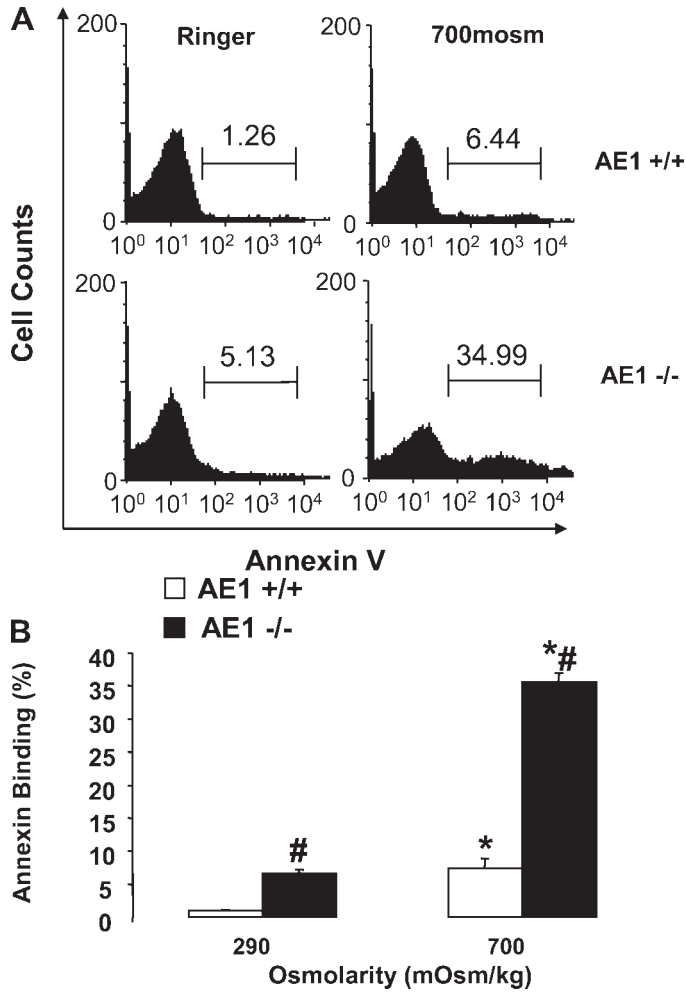

Fig. 1. Annexin binding to normal and anion exchanger 1 (AE1)-deficient erythrocytes: effect of osmotic shock. Annexin V binding was determined by FACS analysis of erythrocytes from AE1-deficient ($AE1^{-/-}$) mice and their wild-type ($AE1^{+/+}$) littermates after 4 h of incubation in isotonic solution (Ringer solution) or in 700 mosM hypertonic solution (addition to an isotonic solution of 400 mM sucrose). *A*: representative histograms illustrating the percentages of annexin V-binding erythrocytes. *B*: arithmetic means ± SE ($n = 5$) of the percentages of annexin V-binding erythrocytes. *Significant difference between control conditions and treatment with 700 mosM; #significant difference between $AE1^{-/-}$ and $AE1^{+/+}$ mice.

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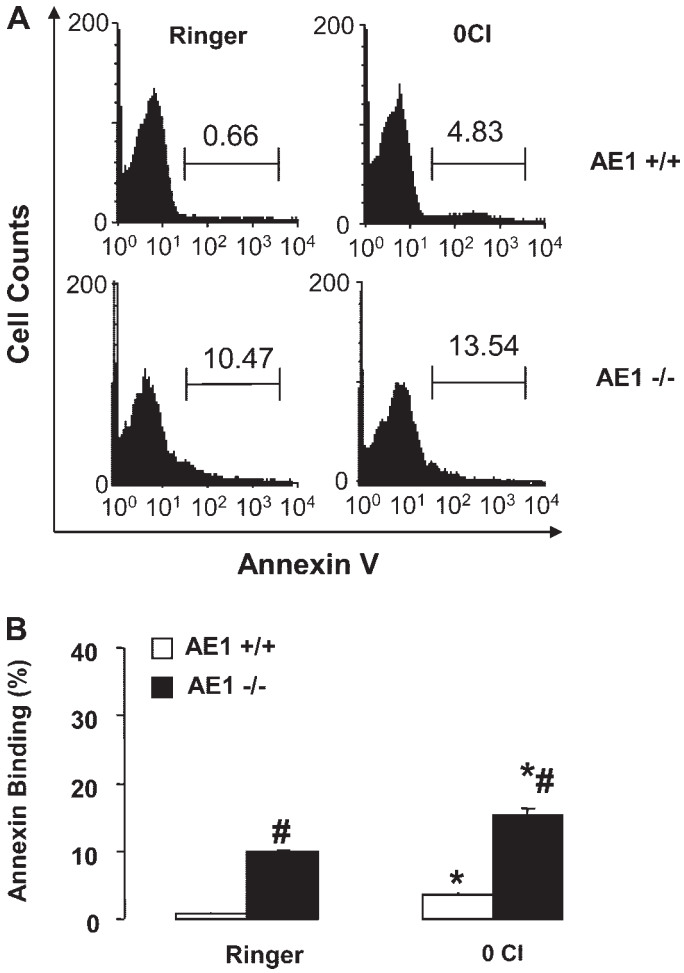

Fig. 2. Effect of Cl^- removal on erythrocyte annexin binding. Annexin V binding was determined by FACS analysis of erythrocytes from *AE1*$^{-/-}$ and *AE1*$^{+/+}$ mice after 7 h of incubation in control Ringer solution or in Cl^--free Ringer solution (0 Cl^-), i.e., Cl^- replaced with gluconate. *A*: representative histograms illustrating the percentages of annexin V-binding erythrocytes. *B*: arithmetic means ± SE (n = 5 each) of the percentages of annexin V-binding erythrocytes. *Significant difference between control conditions and Cl^- replacement; #significant difference between *AE1*$^{-/-}$ and *AE1*$^{+/+}$ mice.

solution additionally containing 0.5 mM EGTA). For flow cytometry, Fluo-3 AM-loaded erythrocytes were resuspended in 1 ml Ringer solution (0.3% hematocrit) containing the appropriate experimental solution with the Ca^{2+} ionophore ionomycin (1 μM) or vehicle alone (0.1% DMSO) and incubated for different time periods at 37°C. Ca^{2+}-dependent fluorescence intensity was then measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of the half-life of fluorescence-labeled erythrocytes. Fluorescence-labeled erythrocytes were obtained by staining the cells with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes). The labeling solution was prepared by the addition of adequate amounts of a CFSE stock solution (10 mM in DMSO) to PBS to yield a final concentration of 5 μM. Cells were then incubated with labeling solution for 30 min at 37°C under light protection. Cells were pelleted at 2,000 rpm for 5 min, washed twice in PBS containing 1% FCS, and pelleted at 2,000 rpm for 5 min. The pellet was then resuspended in fresh, prewarmed Ringer solution. Fluorescence-labeled erythrocytes were injected in a volume of 100 μl into the tail veins of healthy C57BL/6 mice (female, 4 mo old). After the respective time periods, blood was taken from the injected mice, and the CFSE-dependent fluorescence intensity of the erythrocytes was measured in the fluorescence channel FL-1 as described above. Percentages of CFSE-positive erythrocytes were calculated as percentages of the total erythrocyte number. Control experiments revealed that the labeling procedure induced <10% lysis of *AE1*$^{-/-}$ or *AE1*$^{+/+}$ erythrocytes (data not shown).

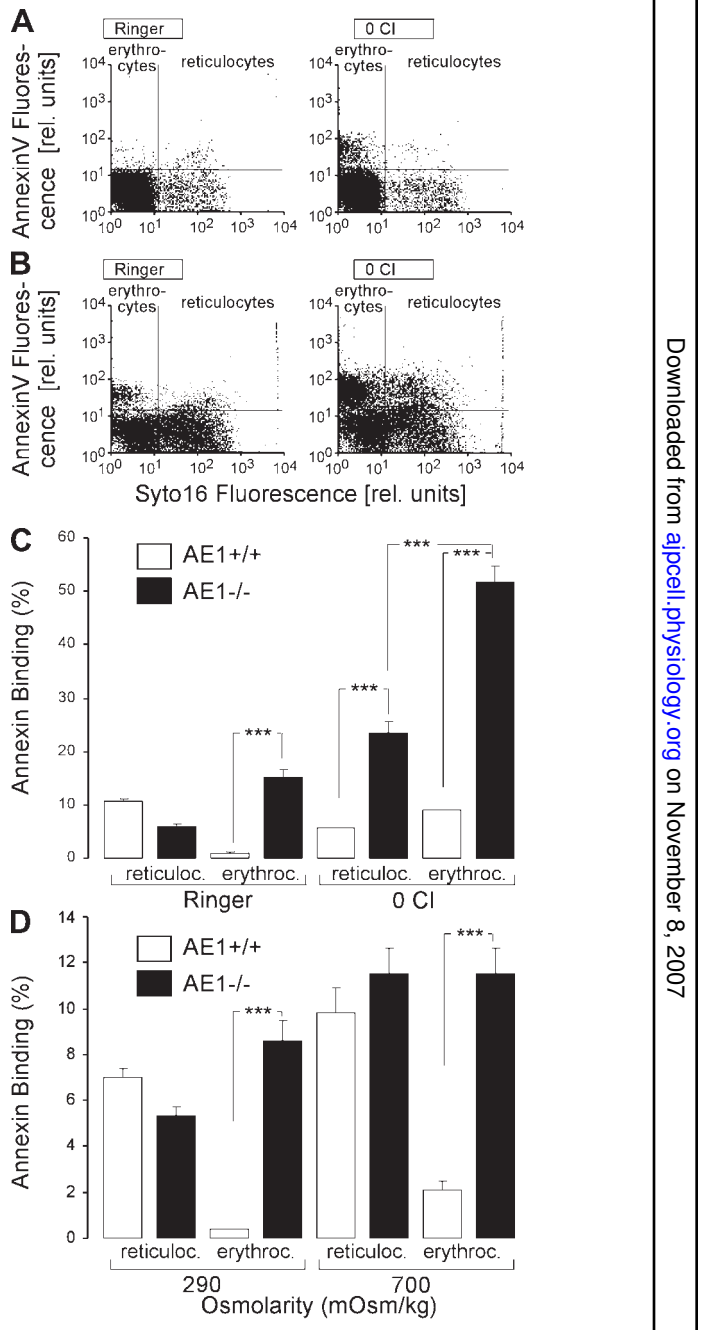

Fig. 3. Phosphatidylserine (PS) exposure following removal of extracellular Cl^- or osmotic shock in reticulocytes and erythrocytes. *A* and *B*: dot blots of cells double stained with fluorescence-labeled annexin and the DNA/RNA-specific dye Syto16 depicting the annexin binding of erythrocytes (i.e., low Syto16 fluorescence) and reticulocytes (i.e., high Syto16 fluorescence) from *AE1*$^{+/+}$ (*A*) and *AE1*$^{-/-}$ (*B*) mice. *C* and *D*: percentages of annexin-binding cells in erythrocytes and reticulocytes from *AE1*$^{+/+}$ and *AE1*$^{-/-}$ mice after 7 h (*C*) or 4 h (*D*) of incubation in Ringer solution (Ringer solution and 290 mosm/kg, respectively) and in Cl^--free Ringer solution (*C*) or Ringer solution containing an additional 400 mM sucrose (700 mosm/kg; *D*), respectively. Data are arithmetic means ± SE (n = 3–4). ***$P \leq 0.001$ (by ANOVA).

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Statistics. Data are expressed as arithmetic means ± SE, and statistical analysis was made by paired or unpaired *t*-test or ANOVA using Dunnett's, Bonferroni, or Tukey's test as post hoc tests where appropriate.

RESULTS

Blood counts revealed severe anemia in $AE1^{-/-}$ mice. As shown in Table 1, erythrocyte number, packed cell volume, and plasma hemoglobin concentration in $AE1^{-/-}$ mice were only a fraction of the respective values in $AE1^{+/+}$ mice. In contrast, $AE1^{-/-}$ mice had nearly 50% reticulocytosis compared with ~2% reticulocytes in $AE1^{+/+}$ mice. Thus, as shown previously by Peters et al. (42) and Southgate et al. (48), the severe anemia was not due to impaired erythropoiesis but rather to hemolysis and/or accelerated clearance of circulating erythrocytes. Although slightly elevated, platelet counts in $AE1^{-/-}$ mice were not statistically different from those of $AE1^{+/+}$ mice ($P < 0.07$).

The loss of mature erythrocytes could have been due to cell membrane phospholipid scrambling leading to PS exposure at the cell membrane (28). Annexin binding, reflecting breakdown of PS asymmetry with exposure of PS at the cell surface, was indeed significantly larger in erythrocytes from $AE1^{-/-}$ mice than in erythrocytes from $AE1^{+/+}$ mice (Fig. 1), a finding in accordance with earlier studies (22, 23). These data suggest a less stable PS asymmetry of the $AE1^{-/-}$ erythrocyte cell membrane.

Osmotic shock is known to stimulate PS exposure (28) in the absence of substantial hemolysis (30). As illustrated in Fig. 1, osmotic shock indeed led to breakdown of PS asymmetry of the cell membrane in erythrocytes from both genotypes. However, the increase in annexin binding following osmotic shock was significantly larger in $AE1^{-/-}$ erythrocytes than in $AE1^{+/+}$ erythrocytes (Fig. 1).

Similar to osmotic shock, Cl^- removal (isosmotic replacement with gluconate) from the extracellular fluid triggered breakdown of PS asymmetry in erythrocytes from both $AE1^{-/-}$ and $AE1^{+/+}$ mice (Fig. 2). Exposure to Cl^--free extracellular solution significantly increased the number of annexin binding cells in both $AE1^{+/+}$ and $AE1^{-/-}$ erythrocytes. Again, percentages of annexin-binding erythrocytes were significantly higher in $AE1^{-/-}$ blood than in $AE1^{+/+}$ blood (Fig. 2). However, the absolute increment in the proportion of annexin-binding erythrocytes in Cl^--free medium was the same for both genotypes.

To explore whether the accelerated suicidal erythrocyte death in $AE1^{-/-}$ mice was due to high reticulocyte numbers in the $AE1^{-/-}$ blood, annexin binding was determined separately for erythrocytes and reticulocytes. Cells from $AE1^{-/-}$ and $AE1^{+/+}$ mice were exposed for 7 h to Cl^--free extracellular solution or for 4 h to hyperosmotic shock and subsequently double stained with the DNA/RNA-specific dye Syto16 and fluorescence-labeled annexin (Fig. 3, *A* and *B*). As a result, under control conditions (Ringer solution) and following both removal of extracellular Cl^- (Fig. 3*C*) and hyperosmotic shock (Fig. 3*D*), mature $AE1^{-/-}$ erythrocytes (i.e., cells with low Syto16 fluorescence) bound significantly more annexin than $AE1^{+/+}$ erythrocytes. Annexin binding of reticulocytes (i.e., cells with high Syto16 fluorescence) did not differ between $AE1^{-/-}$ and $AE1^{+/+}$ mice under control conditions (Fig. 3, *C* and *D*) and was significantly higher in $AE1^{-/-}$ than $AE1^{+/+}$ mice following Cl^- removal (Fig. 3*C*) but similar in $AE1^{-/-}$ and $AE1^{+/+}$ mice following hyperosmotic shock (Fig. 3*D*). Taken together, these experiments indicated that the observed higher susceptibility of $AE1^{-/-}$ blood cells to suicidal death stimuli does not simply reflect the higher number of reticulocytes in $AE1^{-/-}$ blood.

In an additional series of experiments, Cl^- removal experiments were repeated in 25 mM HCO_3^-/5% CO_2-buffered saline. Furthermore, annexin binding following Cl^- removal was studied in standard HEPES-buffered solutions in the presence of the AE1 inhibitor DIDS (100 μM). As shown in Fig. 4, *A* and *B*, respectively, neither the use of the HCO_3^-/CO_2 buffer nor of DIDS blunted the increase in annexin binding elicited by extracellular Cl^- removal. Thus, the enhanced sensitivity of $AE1^{-/-}$ cells to removal of extracellular Cl^- was not due to the missing anion exchanger function of AE1.

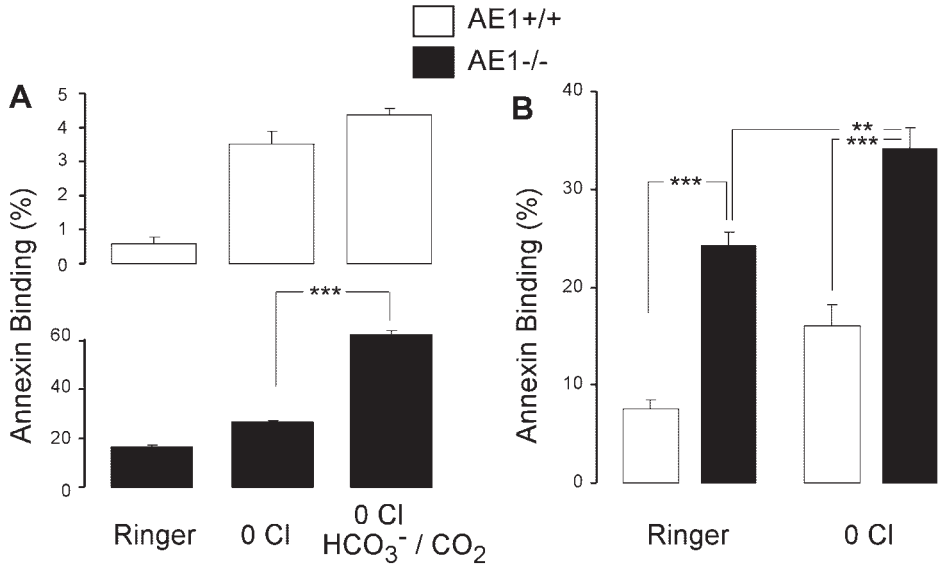

Fig. 4. Effect of AE1 activity on PS exposure of $AE1^{+/+}$ and $AE1^{-/-}$ erythrocytes in Cl^--free medium. *A*: mean percentages of annexin-binding cells (±SE; $n = 4$) in $AE1^{+/+}$ and $AE1^{-/-}$ erythrocytes incubated for 7 h in Ringer solution, in Cl^--free Ringer solution, or in Cl^--free solution buffered with 25 mM HCO_3^-/5% CO_2. ***$P \leq 0.001$ (by ANOVA). *B*: mean percentages of annexin-binding cells (±SE; $n = 4$) in $AE1^{+/+}$ and $AE1^{-/-}$ erythrocytes incubated in the presence of the AE1 inhibitor DIDS (100 μM) for 7 h in Ringer solution or in Cl^--free Ringer solution. **$P \leq 0.01$ and ***$P \leq 0.001$ (by ANOVA).

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Energy depletion, i.e., removal of glucose from the extracellular fluid, a maneuver that reduced the cellular ATP content by ~45% during 7 h of incubation (26), similarly led to sustained breakdown of PS asymmetry in erythrocytes from both $AE1^{-/-}$ and $AE1^{+/+}$ mice (Fig. 5). Exposure to glucose-free extracellular fluid significantly increased the number of annexin binding cells in both genotypes, but the increment in annexin binding associated with energy depletion was again significantly larger in erythrocytes from $AE1^{-/-}$ mice (Fig. 5). Glucose depletion decreased the concentration of GSH in $AE1^{-/-}$ and $AE1^{+/+}$ cells similarly (Fig. 6*A*), suggesting similar oxidation of $AE1^{-/-}$ and $AE1^{+/+}$ cells upon glucose depletion. Even 2 h of glucose depletion or incubation with 2-deoxyglucose (10 mM) elicited annexin binding of >20% of $AE1^{-/-}$ cells while having no effect on $AE1^{+/+}$ erythrocytes (Fig. 6*B*). Since 2-deoxyglucose reportedly fuels the pentose-phosphate cycle leading to partial NADPH replenishment and glutathione regeneration (49), the difference in glucose depletion-stimulated annexin binding between $AE1^{-/-}$ and $AE1^{+/+}$ cells was probably not due to a difference in redox state.

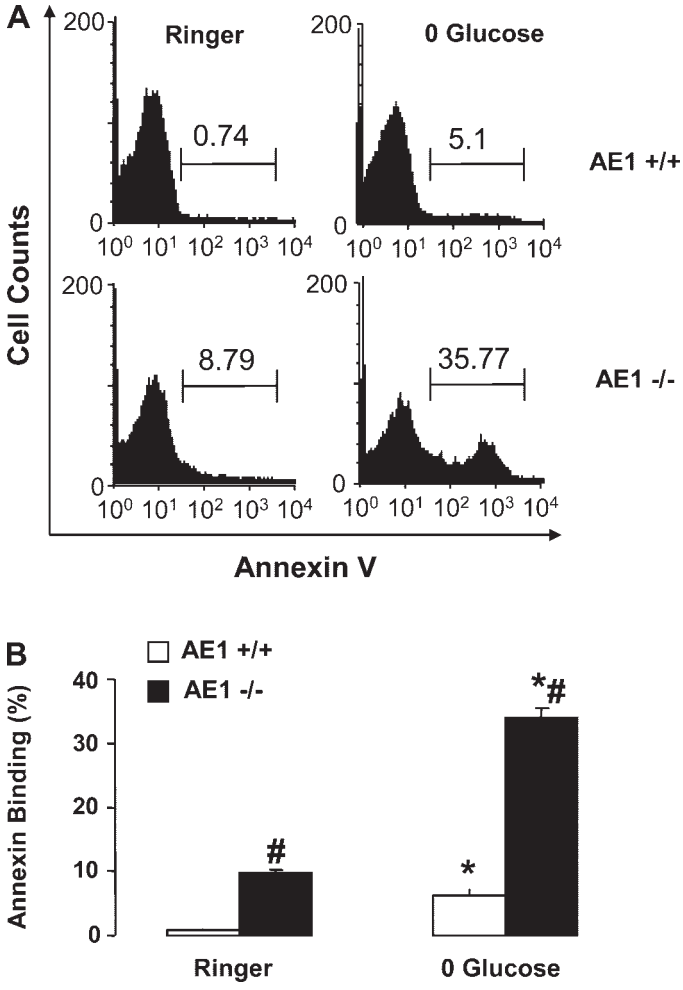

Fig. 5. Effect of glucose depletion on erythrocyte annexin binding. Annexin binding was determined by FACS analysis of erythrocytes from $AE1^{-/-}$ and $AE1^{+/+}$ mice following 7 h of incubation in control Ringer solution or glucose-free Ringer solution [0 glucose]. *A*: representative histograms illustrating the percentages of annexin V-binding erythrocytes. *B*: arithmetic means ± SE (n = 5) of the percentages of annexin V-binding erythrocytes. *Significant difference between control conditions and glucose removal; #significant difference between $AE1^{-/-}$ and $AE1^{+/+}$ mice.

The Ca^{2+} ionophore ionomycin (1 μM), which increases cytosolic Ca^{2+} activity, triggered breakdown of PS asymmetry in erythrocytes from both $AE1^{-/-}$ and $AE1^{+/+}$ mice (Fig. 7). In the presence of ionomycin, elevated percentages of annexin binding erythrocytes did not differ between $AE1^{+/+}$ and $AE1^{-/-}$ mice. Hence, the sensitivity of erythrocytes to elevated intracellular Ca^{2+} and subsequent suicidal erythrocyte death did not depend on the presence of AE1.

From the experiments described above, we concluded that the enhanced basal level of PS exposure in $AE1^{-/-}$ erythrocytes could be due to enhanced Ca^{2+} entry (28). The increase of Fluo 3-dependent fluorescence during osmotic shock, as a measure of cytosolic free Ca^{2+} concentration, was indeed significantly steeper in $AE1^{-/-}$ mice than in $AE1^{+/+}$ mice (Fig. 8, *A–C*). Moreover, the difference in annexin binding between $AE1^{+/+}$ and $AE1^{-/-}$ cells following exposure to Cl^--free extracellular solution between $AE1^{+/+}$ and $AE1^{-/-}$ cells was significantly blunted when experiments were performed in Ca^{2+}-free solution (Fig. 8*D*). The observations are consistent with enhanced Ca^{2+} permeability of $AE1^{-/-}$ cells during cellular stress.

To explore the physiological significance of PS exposure, the clearance of erythrocytes from circulating blood was determined in vivo. To this end, erythrocytes were labeled with CFSE, leading to nearly complete labeling of the cell population (not shown). Additionally, a portion of erythrocytes was treated ex vivo with 1 μM ionomycin before being labeled, which leads to PS exposure in the majority of erythrocytes of both genotypes (~65%; see also Fig. 7). Given a lifetime of ~40 days, 10% of wild-type mouse erythrocytes are expected to be cleared within 4 days. In the present study, ~15% of CFSE-labeled wild-type erythrocytes were cleared during this time period (Fig. 9, open circles), indicating that CFSE labeling per se is not a strong trigger of erythrocyte clearance. The comparison of the results shown in Fig. 9 indicated that nontreated, CFSE-labeled erythrocytes from $AE1^{-/-}$ mice (closed circles) disappeared more rapidly from circulating blood than those from $AE1^{+/+}$ mice (open circles) during the first 10 h of the experiment. In contrast to the nontreated cells, the vast majority of ionomycin-treated, CFSE-labeled erythrocytes from either genotype disappeared at a rapid clearance rate (Fig. 9, closed and open triangles). After 20 min, ~85% of the cells were already cleared. This rapid clearance was not due to cellular lysis during the labeling procedure, which was below 10% (data not shown).

DISCUSSION

The present results clearly demonstrate that the stress-stimulated Ca^{2+} permeability is higher in erythrocytes from $AE1^{-/-}$ mice than in erythrocytes from $AE1^{+/+}$ littermates. Human and murine erythrocytes express Ca^{2+}-permeable cation channels, which are opened by osmotic shock, Cl^- removal, or energy depletion (16, 17, 24). An increased cytosolic Ca^{2+} concentration triggers Ca^{2+}-sensitive scrambling of cell membrane lipids (15, 54) with subsequent PS exposure at the cell surface (6, 9, 28, 31, 32).

PS in the external leaflet of the plasma membrane of apoptotic cells reportedly is an "eat me signal" contributing to the

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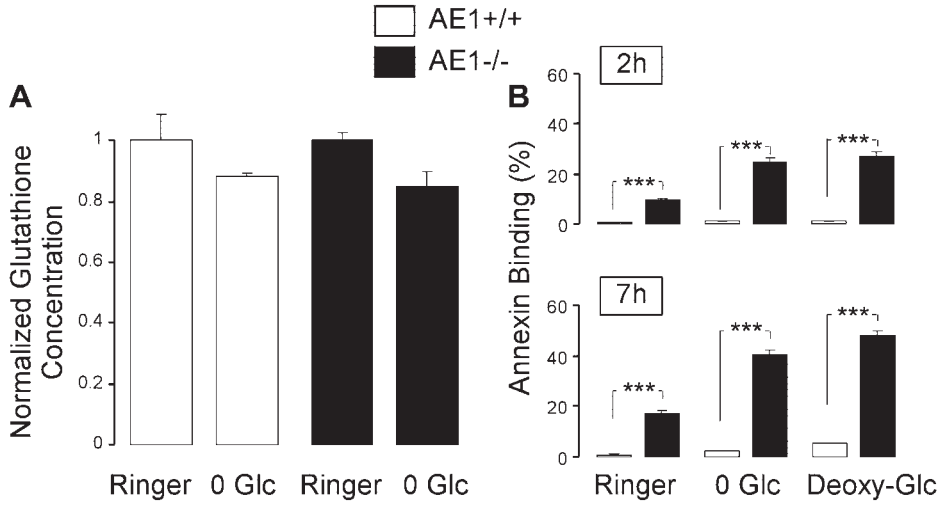

Fig. 6. Energy depletion-induced changes in redox state and PS exposure of $AE1^{+/+}$ and $AE1^{-/-}$ erythrocytes. *A*: normalized concentration (means ± SE; $n = 3$–4) of GSH in $AE1^{+/+}$ and $AE1^{-/-}$ erythrocytes incubated for 7 h in Ringer solution and in glucose-free Ringer solution. *B*: mean percentage of annexin-binding cells (±SE; $n = 4$) in $AE1^{+/+}$ and $AE1^{-/-}$ erythrocytes incubated for 2 h (*top*) and 7 h (*bottom*) in Ringer solution, in glucose-free Ringer solution, or in glucose-free Ringer solution supplemented with 10 mM 2-deoxyglucose (Deoxy-Glc). ***$P \leq 0.001$ (by ANOVA).

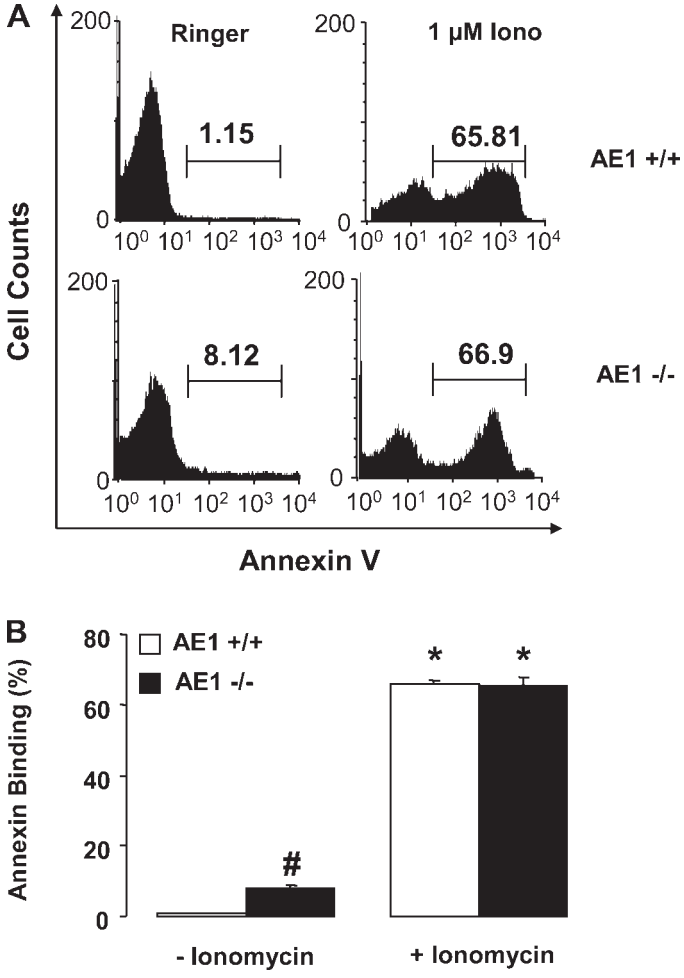

Fig. 7. Effect of the Ca^{2+} ionophore ionomycin (Iono) on erythrocyte annexin binding. Annexin binding was determined by FACS analysis of erythrocytes from $AE1^{-/-}$ and $AE1^{+/+}$ mice incubated for 30 min in control Ringer solution with (+) or without (−) the Ca^{2+} ionophore Iono (1 μM). *A*: representative histograms illustrating the percentages of annexin V-binding erythrocytes. *B*: arithmetic means ± SE ($n = 3$) of the percentages of annexin V-binding erythrocytes. *Significant difference between control conditions and treatment with ionomycin; #significant difference between $AE1^{-/-}$ and $AE1^{+/+}$ mice.

recognition and phagocytosis of apoptotic cells by macrophages that are equipped with PS receptors (18, 19). PS exposure might also contribute to the clearance of erythrocytes. Accordingly, PS exposure parallels the time-dependent clearance of biotin-labeled rabbit erythrocytes in vivo (7). Moreover, erythrocytes are engulfed rapidly by macrophages in vitro (51) and cleared in vivo from peripheral blood after the insertion of a synthetic fluorescent PS analog into the erythrocyte membrane (45). Thus, the observed increase in Ca^{2+} permeability and the subsequent PS exposure are instrumental in the clearance of affected erythrocytes.

The present observations thus provide an explanation for the severe anemia of AE1 knockout mice (42, 48) and, potentially, also for the anemia of humans with AE1 mutations (1, 25, 33, 47). As noted previously by Peters et al. (42) and Southgate et al. (48), the dramatic increase of reticulocyte number in the $AE1^{-/-}$ mouse reflects profound stimulation of erythropoiesis that, however, is unable to prevent the severe anemia. No attempts have been made to discriminate between reticulocytes and mature erythrocytes during the determination of erythrocyte clearance. Thus, the relative large number of reticulocytes in $AE1^{-/-}$ mice may have influenced the magnitude of rapidity of erythrocyte clearance. Nevertheless, accelerated erythrocyte death accounts for, but does not result from, increased reticulocyte numbers.

Despite the absence of AE1, which normally constitutes 50% of intrinsic membrane protein, along with the secondary absence of other membrane proteins (10), $AE1^{-/-}$ erythrocytes contain a morphologically intact cytoskeleton (42). Therefore, the cytoskeletal consequences of loss of AE1 might not be expected directly to result in destabilization and randomization and, possibly, reorganization of the membrane lipids.

The enhanced PS exposure of AE1-deficient erythrocytes is reminiscent of the enhanced PS exposure of thrombocytes leading to a hypercoagulable state (22, 23). PS-exposing erythrocytes may contribute to vasoocclusion by binding to receptors in the vascular wall, thereby leading to obstruction of the microcirculation (3, 12). The correlation between PS exposure and thrombosis may, however, not hold in hereditary spherocytosis. All tested recessive hereditary spherocytosis diseases of the mouse (53) have been characterized by erythrocytes with elevated annexin V binding, whereas dominant hereditary

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spherocytosis in humans did not share this property (14). Moreover, the severity of thrombosis in spherocytic mice deficient in β-spectrin or ankyrin (15–22% incidence of systemic thrombosis) or deficient in α-spectrin (85–100% incidence of thrombosis) did not correlate with the proportion or magnitude of erythrocyte annexin V binding (53).

The premature death of erythrocytes from *AE1*$^{-/-}$ mice is in seeming contrast to the putative role of AE1 in the machinery leading to physiological erythrocyte senescence (37–39, 46). Senescent erythrocytes are thought to increasingly bind naturally occurring anti-band 3 antibodies with subsequent activation of the classical complement pathway eventually leading to opsonization (36–40). Accordingly, the clearance of transfused red blood cells (RBCs) is delayed when blood to be banked undergoes the removal of complement (50). Oxidative stress damages hemoglobin and leads to the formation of hemichromes, which associate with the cytoplasmic domain of AE1 (34, 41, 52, 55), resulting in binding of endogenous circulating anti-band 3 antibody, activation of the complement system, and deposition of complement C3 molecules on the RBC surface (for reviews, see Refs. 35, 36, and 46). Although the survival of erythrocytes from *AE1*$^{-/-}$ mice was reduced despite the absence of the above AE1-dependent mechanism of physiological senescence, the present observations do not necessarily challenge the view that AE1-dependent complement activation leads to senescence and subsequent clearance of normal erythrocytes. Instead, AE1-dependent senescence and (stress-induced) suicidal erythrocyte death may be two distinct mechanisms limiting erythrocyte survival. While AE1-dependent senescence selects aged erythrocytes for removal from the circulation, suicidal erythrocyte death affects injured or stressed erythrocytes of all ages. In addition to senescence and suicidal erythrocyte death as mechanisms of erythroid demise, neocytolysis preferentially affects young erythrocytes, at least in humans (44). Several distinct pathways (5, 8, 29, 44, 46) may thus lead to premature or timely death of erythrocytes.

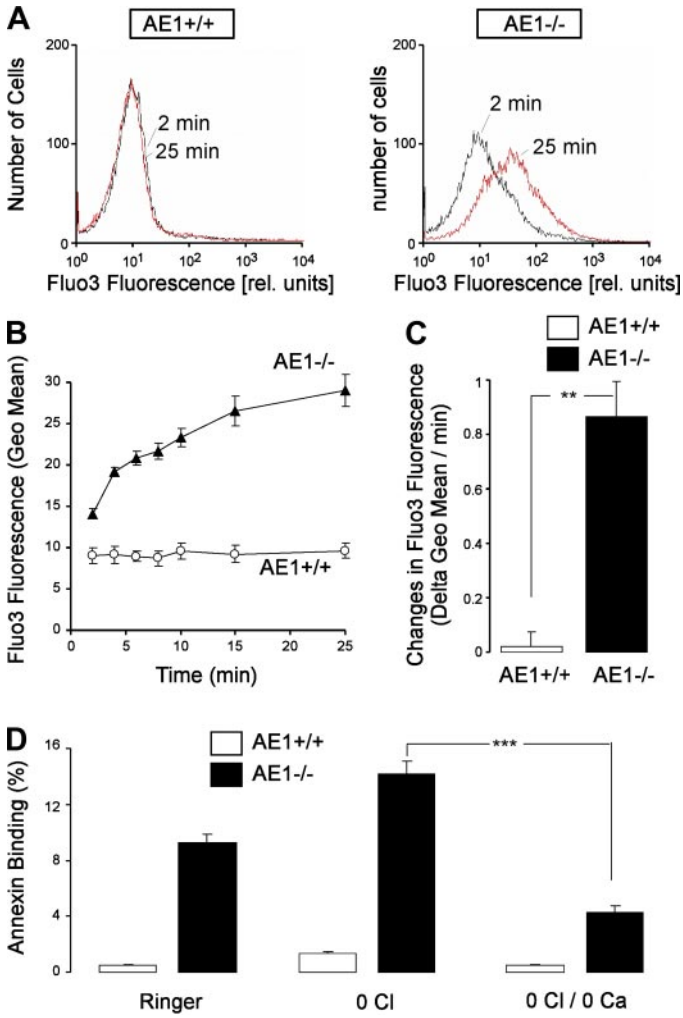

Fig. 8. Increased Ca^{2+} entry into erythrocytes from *AE1*$^{-/-}$ mice following hyperosmotic or isosmotic cell shrinkage. *A*: overlay histograms showing Fluo 3 fluorescence of erythrocytes from *AE1*$^{+/+}$ and *AE1*$^{-/-}$ mice at 2 and 25 min after the addition of Ca^{2+} and 400 mM sucrose to the medium. *B*: time course of mean Fluo 3 fluorescence changes (±SE; $n = 4$) in *AE1*$^{+/+}$ and *AE1*$^{-/-}$ erythrocytes during hypertonic shock (addition of Ca^{2+} and 400 mM sucrose to the medium at time = 0 min). *C*: mean slope of the Fluo 3 fluorescence increase (±SE; $n = 4$) in *AE1*$^{+/+}$ and *AE1*$^{-/-}$ erythrocytes as calculated from *B* between 2 and 10 min of recording. $^{**}P \leq 0.01$ (by a two-tailed Welch-corrected *t*-test). *D*: annexin binding of *AE1*$^{+/+}$ and *AE1*$^{-/-}$ erythrocytes following 7 h of incubation in Ringer solution, in Cl^--free Ringer solution (0 Cl), and in Cl^--free/Ca^{2+}-free Ringer solution (0 Cl/0 Ca). Data are arithmetic means ± SE ($n = 5$). $^{***}P \leq 0.001$ (by ANOVA).

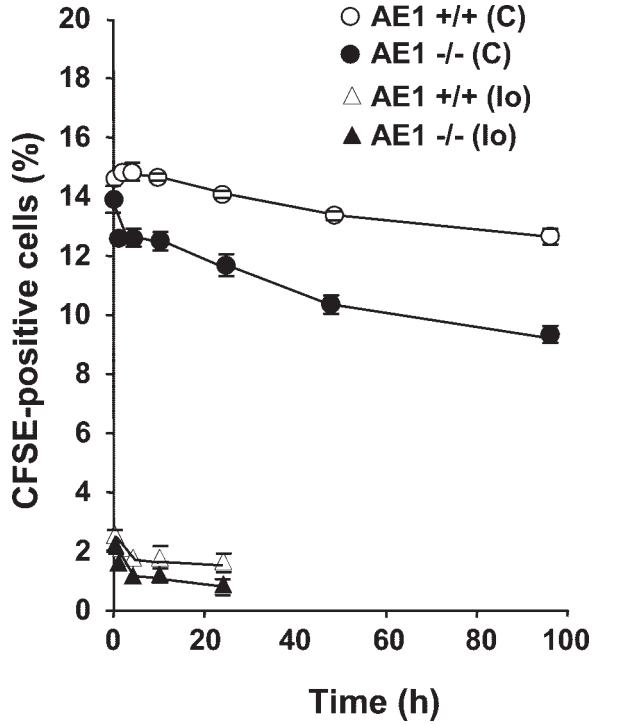

Fig. 9. Clearance of normal and AE1-deficient erythrocytes from circulating blood in C57BL/6 mice. Shown is the time-dependent decay of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled circulating erythrocytes originally taken from *AE1*$^{-/-}$ or *AE1*$^{+/+}$ mice, labeled with CFSE, and reinjected into C57BL/6 mice either without [control (C)] or with prior treatment with ionomycin (Io; 1 μM, 30 min). The percentage of CFSE-labeled cells is plotted against time after injection. Values are arithmetic means ± SE ($n = 4$). Some SEs are smaller than the respective symbols.

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GRANTS

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REFERENCES

1. **Alloisio N, Texier P, Vallier A, Ribeiro ML, Morle L, Bozon M, Bursaux E, Maillet P, Goncalves P, Tanner MJ, Tamagnini G, Delaunay J.** Modulation of clinical expression and band 3 deficiency in hereditary spherocytosis. *Blood* 90: 414–420, 1997.
2. **Andree HA, Reutelingsperger CP, Hauptmann R, Hemker HC, Hermens WT, Willems GM.** Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. *J Biol Chem* 265: 4923–4928, 1990.
3. **Andrews DA, Low PS.** Role of red blood cells in thrombosis. *Curr Opin Hematol* 6: 76–82, 1999.
4. **Andrews DA, Yang L, Low PS.** Phorbol ester stimulates a protein kinase C-mediated agatoxin-TK-sensitive calcium permeability pathway in human red blood cells. *Blood* 100: 3392–3399, 2002.
5. **Barvitenko NN, Adragna NC, Weber RE.** Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance. *Cell Physiol Biochem* 15: 1–18, 2005.
6. **Berg CP, Engels IH, Rothbart A, Lauber K, Renz A, Schlosser SF, Schulze-Osthoff K, Wesselborg S.** Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. *Cell Death Differ* 8: 1197–1206, 2001.
7. **Boas FE, Forman L, Beutler E.** Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. *Proc Natl Acad Sci USA* 95: 3077–81, 1998.
8. **Bosman GJ, Willekens FL, Werre JM.** Erythrocyte aging: a more than superficial resemblance to apoptosis? *Cell Physiol Biochem* 16: 1–8, 2005.
9. **Bratosin D, Estaquier J, Petit F, Arnoult D, Quatannens B, Tissier JP, Slomianny C, Sartiaux C, Alonso C, Huart JJ, Montreuil J, Ameisen JC.** Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. *Cell Death Differ* 8: 1143–1156, 2001.
10. **Bruce LJ, Beckmann R, Ribeiro ML, Peters LL, Chasis JA, Delaunay J, Mohandas N, Anstee DJ, Tanner MJ.** A band 3-based macrocomplex of integral and peripheral proteins in the RBC membrane. *Blood* 101: 4180–4188, 2003.
11. **Bruce LJ, Robinson C, Guizouarn H, Borgese F, Harrison P, King MJ, Goede JS, Coles SE, Gore DM, Lutz HU, Ficarella R, Layton DM, Iolascon A, Ellory JC, Stewart GW.** Monovalent cation leaks in human red cells caused by single amino-acid substitutions in the transport domain of the band 3 chloride-bicarbonate exchanger, AE1. *Nat Genet* 37: 1258–1263, 2005.
12. **Closse C, Dachary-Prigent J, Boisseau MR.** Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. *Br J Haematol* 107: 300–302, 1999.
13. **Daugas E, Cande C, Kroemer G.** Erythrocytes: death of a mummy. *Cell Death Differ* 8: 1131–1133, 2001.
14. **de Jong K, Larkin SK, Eber S, Franck PF, Roelofsen B, Kuypers FA.** Hereditary spherocytosis and elliptocytosis erythrocytes show a normal transbilayer phospholipid distribution. *Blood* 94: 319–325, 1999.
15. **Dekkers DW, Comfurius P, Bevers EM, Zwaal RF.** Comparison between Ca^{2+}-induced scrambling of various fluorescently labelled lipid analogues in red blood cells. *Biochem J* 362: 741–747, 2002.
16. **Duranton C, Huber S, Tanneur V, Lang K, Brand V, Sandu C, Lang F.** Electrophysiological properties of the *Plasmodium falciparum*-induced cation conductance of human erythrocytes. *Cell Physiol Biochem* 13: 189–198, 2003.
17. **Duranton C, Huber SM, Lang F.** Oxidation induces a Cl^--dependent cation conductance in human red blood cells. *J Physiol* 539: 847–855, 2002.
18. **Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM.** A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* 405: 85–90, 2000.
19. **Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL.** Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J Biol Chem* 276: 1071–1077, 2001.
20. **Green DR, Reed JC.** Mitochondria and apoptosis. *Science* 281: 1309–1312, 1998.
21. **Gulbins E, Jekle A, Ferlinz K, Grassme H, Lang F.** Physiology of apoptosis. *Am J Physiol Renal Physiol* 279: F605–F615, 2000.
22. **Hassoun H, Hanada T, Lutchman M, Sahr KE, Palek J, Hanspal M, Chishti AH.** Complete deficiency of glycophorin A in red blood cells from mice with targeted inactivation of the band 3 (AE1) gene. *Blood* 91: 2146–2151, 1998.
23. **Hassoun H, Wang Y, Vassiliadis J, Lutchman M, Palek J, Aish L, Aish IS, Liu SC, Chishti AH.** Targeted inactivation of murine band 3 (AE1) gene produces a hypercoagulable state causing widespread thrombosis in vivo. *Blood* 92: 1785–1792, 1998.
24. **Huber SM, Gamper N, Lang F.** Chloride conductance and volume-regulatory nonselective cation conductance in human red blood cell ghosts. *Pflügers Arch* 441: 551–558, 2001.
25. **Jarolim P, Rubin HL, Liu SC, Cho MR, Brabec V, Derick LH, Yi SJ, Saad ST, Alper S, Brugnara C.** Duplication of 10 nucleotides in the erythroid band 3 (AE1) gene in a kindred with hereditary spherocytosis and band 3 protein deficiency (band 3PRAGUE). *J Clin Invest* 93: 121–130, 1994.
26. **Klarl BA, Lang PA, Kempe DS, Niemoeller OM, Akel A, Sobiesiak M, Eisele K, Podolski M, Huber SM, Wieder T, Lang F.** Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion. *Am J Physiol Cell Physiol* 290: C244–C253, 2006.
27. **Knauf P, Pal P.** Mediated transport. In: *Red Cell Membrane Transport in Health and Disease*, edited by Bernhardt I, Llory JC. Berlin: Springer Verlang, 2003, p. 257–302.
28. **Lang KS, Duranton C, Poehlmann H, Myssina S, Bauer C, Lang F, Wieder T, Huber SM.** Cation channels trigger apoptotic death of erythrocytes. *Cell Death Differ* 10: 249–256, 2003.
29. **Lang KS, Lang PA, Bauer C, Duranton C, Wieder T, Huber SM, Lang F.** Mechanisms of suicidal erythrocyte death. *Cell Physiol Biochem* 15: 195–202, 2005.
30. **Lang KS, Myssina S, Brand V, Sandu C, Lang PA, Berchtold S, Huber SM, Lang F, Wieder T.** Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes. *Cell Death Differ* 11: 231–243, 2004.
31. **Lang KS, Roll B, Myssina S, Schittenhelm M, Scheel-Walter HG, Kanz L, Fritz J, Lang F, Huber SM, Wieder T.** Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. *Cell Physiol Biochem* 12: 365–372, 2002.
32. **Lang PA, Warskulat U, Heller-Stilb B, Huang DY, Grenz A, Myssina S, Duszenko M, Lang F, Haussinger D, Vallon V, Wieder T.** Blunted apoptosis of erythrocytes from taurine transporter deficient mice. *Cell Physiol Biochem* 13: 337–346, 2003.
33. **Lima PR, Gontijo JA, Lopes de Faria JB, Costa FF, Saad ST.** Band 3 campinas: a novel splicing mutation in the band 3 gene (AE1) associated with hereditary spherocytosis, hyperactivity of Na^+/Li^+ countertransport and an abnormal renal bicarbonate handling. *Blood* 90: 2810–2818, 1997.
34. **Low PS.** Structure and function of the cytoplasmic domain of band 3: center of erythrocyte membrane-peripheral protein interactions. *Biochim Biophys Acta* 864: 145–167, 1986.
35. **Low PS, Waugh SM, Zinke K, Drenckhahn D.** The role of hemoglobin denaturation and band 3 clustering in red blood cell aging. *Science* 227: 531–533, 1985.
36. **Lutz HU.** Erythrocyte clearance. In: *Blood Cell Biochemistry*, edited by Harris JR. New York: Plenum, 1990, p. 81–120.
37. **Lutz HU, Bussolino F, Flepp R, Fasler S, Stammler P, Kazatchkine MD, Arese P.** Naturally occurring anti-band-3 antibodies and complement together mediate phagocytosis of oxidatively stressed human erythrocytes. *Proc Natl Acad Sci USA* 84: 7368–7372, 1987.
38. **Lutz HU, Fasler S, Stammler P, Bussolino F, Arese P.** Naturally occurring anti-band 3 antibodies and complement in phagocytosis of oxidatively-stressed and in clearance of senescent red cells. *Blood Cells* 14: 175–203, 1988.
39. **Lutz HU, Jelezarova E.** Complement amplification revisited. *Mol Immunol* 43: 2–12, 2006.
40. **Lutz HU, Nater M, Stammler P.** Naturally occurring anti-band 3 antibodies have a unique affinity for C3. *Immunology* 80: 191–196, 1993.
41. **Matarrese P, Straface E, Pietraforte D, Gambardella L, Vona R, Maccaglia A, Minetti M, Malorni W.** Peroxynitrite induces senescence

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and apoptosis of red blood cells through the activation of aspartyl and cysteinyl proteases. *FASEB J* 19: 416–418, 2005.
42. **Peters LL, Shivdasani RA, Liu SC, Hanspal M, John KM, Gonzalez JM, Brugnara C, Gwynn B, Mohandas N, Alper SL, Orkin SH, Lux SE.** Anion exchanger 1 (band 3) is required to prevent erythrocyte membrane surface loss but not to form the membrane skeleton. *Cell* 86: 917–927, 1996.
43. **Reid ME, Mohandas N.** Red blood cell blood group antigens: structure and function. *Semin Hematol* 41: 93–117, 2004.
44. **Rice L, Alfrey CP.** The negative regulation of red cell mass by neocytolysis: physiologic and pathophysiologic manifestations. *Cell Physiol Biochem* 15: 245–250, 2005.
45. **Schroit AJ, Madsen JW, Tanaka Y.** In vivo recognition and clearance of red blood cells containing phosphatidylserine in their plasma membranes. *J Biol Chem* 260: 5131–5138, 1985.
46. **Schwarzer E, Kühn H, Valente E, Arese P.** Band 3/complement-mediated recognition and removal of normally senescent and pathological human erythrocytes. *Cell Physiol Biochem* 16: 133–146, 2005.
47. **Shayakul C, Alper SL.** Defects in processing and trafficking of the AE1 Cl^-/HCO_3^- exchanger associated with inherited distal renal tubular acidosis. *Clin Exp Nephrol* 8: 1–11, 2004.
48. **Southgate CD, Chishti AH, Mitchell B, Yi SJ, Palek J.** Targeted disruption of the murine erythroid band 3 gene results in spherocytosis and severe haemolytic anaemia despite a normal membrane skeleton. *Nat Genet* 14: 227–230, 1996.
49. **Suzuki M, O'Dea JD, Suzuki T, Agar NS.** 2-Deoxyglucose as a substrate for glutathione regeneration in human and ruminant red blood cells. *Comp Biochem Physiol B* 75: 195–197, 1983.
50. **Szymanski IO, Odgren PR, Valeri CR.** Relationship between the third component of human complement (C3) bound to stored preserved erythrocytes and their viability in vivo. *Vox Sang* 49: 34–41, 1985.
51. **Tanaka Y, Schroit AJ.** Insertion of fluorescent phosphatidylserine into the plasma membrane of red blood cells. Recognition by autologous macrophages. *J Biol Chem* 258: 11335–11343, 1983.
52. **Teti D, Crupi M, Busa M, Valenti A, Loddo S, Mondello M, Romano L.** Chemical and pathological oxidative influences on band 3 protein anion-exchanger. *Cell Physiol Biochem* 16: 77–86, 2005.
53. **Wandersee NJ, Tait JF, Barker JE.** Erythroid phosphatidyl serine exposure is not predictive of thrombotic risk in mice with hemolytic anemia. *Blood Cells Mol Dis* 26: 75–83, 2000.
54. **Woon LA, Holland JW, Kable EP, Roufogalis BD.** Ca^{2+} sensitivity of phospholipid scrambling in human red cell ghosts. *Cell Calcium* 25: 313–320, 1999.
55. **Zhang D, Kiyatkin A, Bolin JT, Low PS.** Crystallographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3. *Blood* 96: 2925–2933, 2000.

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