

## Maturation loss of the vitamin C transporter in erythrocytes

James M. May <sup>a,\*</sup>, Zhi-chao Qu <sup>a</sup>, Huan Qiao <sup>a</sup>, Mark J. Koury <sup>a,b</sup>

<sup>a</sup> Department of Medicine, Vanderbilt University School of Medicine, 715 Preston Research Building, Nashville, TN 37232-6303, USA

<sup>b</sup> VA Tennessee Valley Healthcare System, Nashville, TN 37232-6303, USA

Received 7 June 2007

Available online 18 June 2007

### Abstract

Erythrocytes have the same intracellular concentration of ascorbate as plasma, which is much lower than that of nucleated cells. To determine why erythrocytes are unable to concentrate ascorbate, we tested for the presence of ascorbate transporters in these cells. Human erythrocytes had very low rates of uptake of radiolabeled ascorbate, which was accounted for by the lack of ascorbate transporter SVCT2 in immunoblots. Using a cell culture model of Friend virus-infected mouse erythroblasts, immunoblots showed that the SVCT2 was present in the erythroblast stages, but was lost following extrusion of the nucleus in the formation of the reticulocyte stage. Rates of specific ascorbate transport correlated with the presence of the SVCT2. These results show that mature erythrocytes fail to concentrate ascorbate due to the loss of SVCT2 during maturation in the bone marrow.

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**Keywords:** Ascorbic acid transport; Erythroblasts; Developmental maturation; Erythrocytes; Mouse; Human

Human erythrocytes have the same concentration of ascorbate as that in plasma [1,2]. This contrasts with nucleated blood cells and tissues, in which ascorbate concentrations are many-fold those in plasma [3,4]. For example, at plasma and erythrocyte ascorbate concentrations of 40–80  $\mu$ M, lymphocyte ascorbate concentrations are about 4 mM [4]. The cause of this difference likely relates to the absence of a specific ascorbate transporter in erythrocytes, which have very low rates of ascorbate uptake [5–7]. In contrast, erythrocytes rapidly take up dehydroascorbate (DHA), the two-electron oxidized form of ascorbate, and reduce it to ascorbate [5–7]. Because of its negative charge at physiologic pH and hydrophilic nature, ascorbate crosses biological membranes only very slowly. Thus, ascorbate generated from DHA uptake is trapped in the erythrocyte and can temporarily reach concentrations to as high as 1–2 mM [2]. Nonetheless, the fact that ascorbate concentrations are the same in erythrocytes and plasma

argues that DHA uptake and reduction does not generate the ascorbate concentration gradient seen in other cells.

The concentration gradient of ascorbate across the plasma membrane of nucleated cells is due to the presence of one or more specific ascorbate transporters, two of which have been cloned and termed “SVCT” (sodium-dependent vitamin C transporter) [8]. The SVCT1 and SVCT2 mediate sodium- and energy-dependent ascorbate transport and generate a gradient supported by the plasma membrane sodium–potassium ATPase [8]. *Xenopus* oocytes also lack specific ascorbate uptake, and when injected with mRNA for the SVCT2, they acquire this transport and can concentrate ascorbate [8]. One would presume that erythrocytes lack both the SVCT1 and SVCT2, thus accounting for their inability to take up and accumulate ascorbate against a gradient. However, the absence of the SVCT protein(s) in erythrocytes has not been documented, nor has there been a mechanism proposed to account for this lack. In this work, we confirm the absence of SVCT proteins in mature human and mouse erythrocytes and show in a mouse erythroblast culture system that the SVCT2 is lost with extrusion of the nucleus during reticulocyte formation.

*Abbreviations:* DHA, dehydroascorbic acid; PBS, phosphate-buffered saline; SVCT, sodium-dependent vitamin C transporter.

\* Corresponding author. Fax: +1 615 936 1667.

E-mail address: [james.may@vanderbilt.edu](mailto:james.may@vanderbilt.edu) (J.M. May).

## Materials and methods

**Materials.** Analytical reagents, including, ascorbic acid, ascorbate oxidase, and dehydroascorbic acid, were supplied by Sigma/Aldrich Chemical Co. (St. Louis, MO).

**Preparation and culture of mouse erythroblasts and reticulocytes.** Developmentally synchronized proerythroblasts were purified from spleens of 8- to 10-week-old, female CD2F1 mice in the acute erythroblastic stage of the disease caused by the “anemia-inducing” strain of Friend leukemia virus [9]. Erythroblasts were cultured for times indicated before removal of cells for experiments. At 44 h, some cultures were harvested, separated by unit gravity sedimentation to isolate fractions that were highly enriched in reticulocytes, extruded nuclei, or erythroblasts that had not undergone enucleation [10]. The purified reticulocytes were placed back in culture for another 3 days, and samples of reticulocytes were harvested at various times during their maturation *in vitro*.

**Erythrocyte and erythrocyte membrane preparation.** Human erythrocytes were obtained by venipuncture from normal human donors. Mouse erythrocytes were obtained by exsanguination following euthanization of 10- to 12-week-old C57Bl/6 mice. Erythrocytes were rinsed three times by centrifugation in phosphate-buffered saline (PBS, consisting of 140 mM NaCl and 12.5 mM sodium phosphate, pH 7.4). With each rinse, the “buffy” coat of white cells was removed and discarded. White or “leaky” erythrocyte ghosts were prepared as described by Steck and Kant [11] in 5 mM phosphate buffer, pH 7.4, and stored at  $-70^{\circ}\text{C}$  for subsequent electrophoresis.

**Preparation of mouse brain microvascular endothelial cells.** Endothelial cells were prepared exactly as described by Song et al. [12] and cultured for 7 days before they were scraped from the plate and prepared for gel electrophoresis.

**Assay of erythrocyte ascorbate transport.** Erythrocytes at a 6% packed cell volume or the indicated numbers of erythroid precursors were incubated at  $37^{\circ}\text{C}$  in 0.5 ml of PBS that contained 5 mM D-glucose, 0.66 mM GSH, and 0.05  $\mu\text{Ci}$  of L-[1- $^{14}\text{C}$ ]ascorbic acid. The total ascorbate concentration was 6–9  $\mu\text{M}$ . At times indicated, cells were pelleted by centrifugation, and the supernatant was aspirated. The cells were rinsed three times in 2 ml of ice-cold “stop” solution that consisted of 25  $\mu\text{M}$  cytochalasin B in PBS. The cells were lysed by vigorous mixing with 0.2 ml of 25% metaphosphoric acid and the lysate was partially neutralized with addition of 0.75 ml of phosphate-EDTA. The latter consisted of 100 mM sodium phosphate and 5 mM EDTA, pH 8.0. The lysate was centrifuged for 1 min at 13,600g and 0.5 ml of the supernatant was added with mixing to 4 ml of Ecolume liquid scintillation fluid (ICN, Costa Mesa, CA). The radioactivity of duplicate samples was measured in a Packard CA-2200 liquid scintillation counter, after allowing at least 1 h for decay of chemiluminescence. Results were normalized to the intracellular aqueous volume of the cells for erythrocytes, which was taken to be 70% of the packed cell volume [13] and are shown as means  $\pm$  standard error.

**Electrophoresis and immunoblotting of SVCT proteins.** Erythrocyte ghost membranes in 5 mM phosphate buffer were solubilized in a lysis buffer consisting of 150 mM NaCl, 1% Nonidet P40 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% sodium lauryl sulfate (w/v), 0.1 mg/ml phenylmethylsulfonyl fluoride, and leupeptin, pepstatin, and aprotinin, each at 0.01 mg/ml. The lysate was mixed and stored on ice for 30 min. To this was added an equal volume of sample buffer, which consisted of 125 mM Tris-HCl, 20% (v/v) glycerol, 4% (w/v) sodium lauryl sulfate, 10% (v/v) mercaptoethanol, and 0.0025% bromophenol blue (w/v), pH 6.8. Samples were centrifuged for 10 s at 13,000g, and the solubilized material was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli [14]. Electrophoresis and transfer to poly(vinylidene difluoride) membrane was carried out as previously described [15]. The blot was probed with an affinity-purified rabbit polyclonal antibodies specific for the human or mouse SVCT2 or SVCT1 (#9926 and #9921, respectively, Santa Cruz Biochemicals, Santa Cruz, CA) at 1:400 dilutions. The secondary antibody was anti-rabbit IgG conjugated to horseradish peroxidase (#A0545, Sigma-Aldrich, Inc., St. Louis, MO) and used at a 1:5000 dilution. Bands were stained using ECL

Plus Western blotting reagents (RPN 2132 from Amersham Biosciences, Piscataway, NJ). Locations of the bands were determined using pre-stained molecular weight markers.

## Results

Human erythrocytes took up radiolabeled ascorbate very slowly (Fig. 1, triangles) compared to erythrocytes also incubated with ascorbate oxidase (Fig. 1, circles). This enzyme oxidizes ascorbate to its free radical, which then dismutates and generates radiolabeled DHA that is rapidly taken up by the cells on the glucose transporter. Uptake of L-[1- $^{14}\text{C}$ ]DHA generated by ascorbate oxidase was so rapid that it was complete by about 10 min. When erythrocytes were treated just before the transport assay with 25  $\mu\text{M}$  cytochalasin B, a specific glucose transport inhibitor, the apparent uptake of L-[1- $^{14}\text{C}$ ]ascorbate was decreased to very low levels. This indicates that most of the observed uptake of radiolabeled ascorbate was actually due to uptake of L-[1- $^{14}\text{C}$ ]DHA on the glucose transporter.

This lack of ascorbate transport in human erythrocytes was explained by the finding that the SVCT2 was not detected in immunoblots of human (Fig. 2, lane A) and mouse (Fig. 2, lane B) erythrocyte membranes. In contrast, there was strong immunostaining of a 74–76 kDa band in primary cultures of endothelial cells from mouse brain capillaries (Fig. 2, lane C). The amount of protein loaded was comparable or slightly greater in the erythrocyte membranes, as indicated by actin immunostaining on the membrane prepared from the same gel.

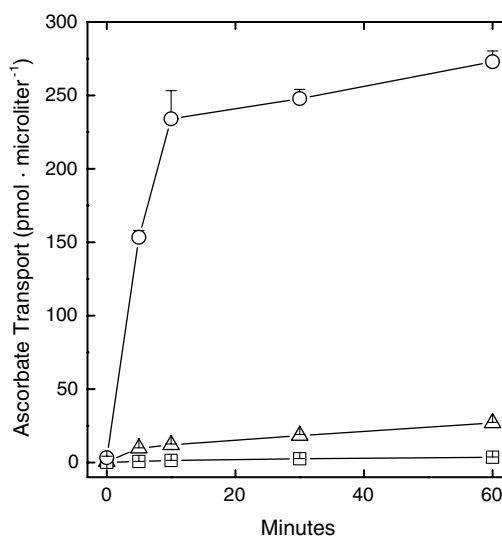


Fig. 1. Ascorbate and DHA transport by human erythrocytes. Erythrocytes at a 6% packed cell volume were incubated at  $37^{\circ}\text{C}$  in PBS that contained 5 mM D-glucose, 0.66 mM GSH, 0.05  $\mu\text{Ci}$  of L-[1- $^{14}\text{C}$ ]ascorbate, and either no further additions (triangles), 0.1 U/ml ascorbate oxidase (circles), or 25  $\mu\text{M}$  cytochalasin B (squares). At the times indicated the cells were pelleted by centrifugation, rinsed three times in ice-cold “stop” solution, and taken for measurement of intracellular radioactivity as described under Methods. Results are shown from four experiments.

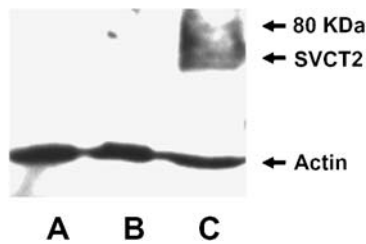


Fig. 2. Immunoblot of the SVCT2 in erythrocyte and endothelial cell membranes. Purified membranes from human (A) and mouse (B) erythrocytes as well as total endothelial cell membranes were loaded in each of three gel lanes, electrophoresed and immunoblotted for SVCT2 and actin, as indicated in the figure. The migration of the 80 kDa molecular weight marker is indicated.

Membranes prepared from cultures of mouse erythroblasts, on the other hand, showed a doublet at the expected molecular weight range for the SVCT2 at 0–48 h of culture (Fig. 3). However, after the erythroblasts enucleated to form reticulocytes, they showed only very faint SVCT2 staining that gradually disappeared as culture was continued to 60 h. No band of a similar molecular weight was detected when an antibody against the SVCT1 was used (results not shown). These results show that the SVCT2 protein is present in the erythroblast stages, but is lost with extrusion of the nucleus.

When cells in culture for 44 h were separated by sedimentation velocity, the fractions enriched for nuclei showed greater staining for SVCT2, although the bands were present in all fractions tested (Fig. 4A). In this separation, Fractions 1–7 and 8–10 contain largely nucleated erythroblasts, Fractions 11–12 and 13–18 contain mostly extruded nuclei, and Fractions 17–21 contain nuclei and reticulocytes [10]. Ascorbate transport was also measured in cells derived from the experiment shown in Fig. 4A, all in the presence of 25  $\mu$ M cytochalasin B. As noted earlier, the latter will prevent uptake of any L-[1- $^{14}$ C]DHA on the glucose transporter. As can be seen in Fig. 4B, specific ascorbate transport remained high in the first four fractions, but was much lower in the last fraction containing

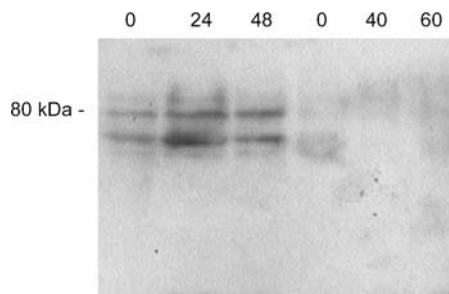


Fig. 3. Immunoblotting of cultured erythroid cell membranes for the SVCT2. The cells were rinsed once in PBS and were extracted with lysis buffer. Protein from approximately  $5 \times 10^7$  cells was applied to each lane of the gel. Electrophoresis and staining for SVCT2 was carried out as described under Methods. The location of the relevant molecular weight marker is shown to the left, and each lane is identified by the hours in culture before extrusion of the nucleus at 48 h and after extrusion to 60 h.

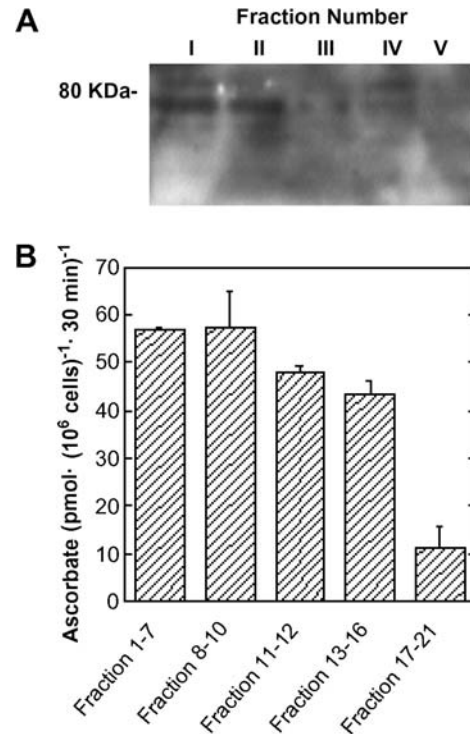


Fig. 4. SVCT2 content and ascorbate transport in various erythroid fractions. Sedimentation velocity was used to separate cells cultured for 44 h into five fractions as described in the text. The cells in each fraction were rinsed in PBS and extracted with lysis buffer. (A) Membranes from approximately  $5 \times 10^7$  cells were loaded into each gel lane. Electrophoresis and immunoblotting were carried out as described under Methods. The location of the relevant molecular weight marker is noted on the left. (B) Ascorbate transport was measured in duplicate in cells after fractionation. Results are shown from one of two such experiments performed.

mostly reticulocytes. Together, the results show that the SVCT2 protein and function is present in fractions containing erythroblasts, but not in fractions containing mostly reticulocytes.

## Discussion

The study shows that mature erythrocytes lack the SVCT2 and that this accounts for their inability to take up ascorbate against a concentration gradient. Although the SVCT2 is present in erythroblasts derived from the bone marrow in mice, it is lost at the time the nucleus is extruded from the cells, which is about 42–48 h in culture and during maturation in the bone marrow *in vivo*. The SVCT2 is an intrinsic membrane protein that spans the plasma membrane 12 times, according to hydropathy analysis of its amino acid sequence [8]. Enucleating erythroblasts are known to shed specific plasma membrane proteins along with residual portions of organelles at this time. In addition to the SVCT2, shed proteins segregated to the plasma membrane surrounding the extruded nucleus include the receptors for concanavalin A [16] and wheat germ agglutinin [17].

Why some membrane proteins are lost during erythrocyte maturation and others, such as the anion and glucose

transporters are not, remains unknown. Loss of the SVCT2 results in intracellular ascorbate concentrations in erythrocytes that are much lower than those in nucleated cells that have either isoform of the SVCT2. Whether erythrocytes might benefit from higher intracellular ascorbate is uncertain, since they do not synthesize collagen or have dioxygenase enzymes for which ascorbate serves as a co-factor [18]. Ascorbate is an excellent antioxidant and it can protect erythrocytes from extracellular oxidant stresses [19,20]. However, in a cell that contains high concentrations of iron, ascorbate could act as a pro-oxidant due to redox cycling of any free  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , which then can react with molecular oxygen to generate superoxide and eventually the highly reactive hydroxyl radical [21]. Nitrite that is generated from oxidation of nitric oxide is taken up by erythrocytes and can form methemoglobin [22]. Ascorbate can reduce this nitrite back to nitric oxide and thus recycle it [23]. We have shown that ascorbate modestly protects erythrocytes from methemoglobin formation due to nitrite, but only at non-physiologic nitrite concentrations above 0.5 mM [22]. Although ascorbate in erythrocytes will certainly act as a primary antioxidant, it does not appear essential that high intracellular erythrocyte concentrations are necessary for cell function or protection.

On the other hand, erythrocytes have a very high capacity to reduce DHA to ascorbate, which then will be sequestered inside the cells. Since erythrocytes are likely to encounter extremes of oxidant stress at sites of vascular inflammation in diseases such as atherosclerosis, substantial local amounts of DHA are likely to be generated from plasma ascorbate. Rapid uptake of DHA on the GLUT1 glucose transporter and reduction to ascorbate within erythrocytes will preserve the vitamin, which can then slowly leak out of erythrocytes back into plasma. Erythrocytes have also been shown to preserve and probably recycle ascorbate through reduction of extracellular free radical [24]. Erythrocytes thus may have a unique function to preserve ascorbate in blood: rather than simply sequestering ascorbate via the SVCT, they recycle it from its oxidized forms and slowly release it with time.

## Acknowledgments

The authors thank Prapaporn Kopsombut and Melissa M. Rhodes for technical assistance. This work was supported by NIH Grant DK050435 (J.M.M.) and a Merit Review Award from the Department of Veterans Affairs (M.J.K.).

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