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## Erythrocyte Aging: A More than Superficial Resemblance to Apoptosis?

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### Key Words

Aging • Anion transporter • Apoptosis • Asplenia • Cytoskeleton • Erythrocyte • Hemoglobin • Membrane • Vesicles

### Abstract

In physiological circumstances, erythrocyte aging leads to binding of autologous IgG followed by recognition and removal through phagocytosis, mainly by Kupffer cells in the liver. This process is triggered by the appearance of a senescent erythrocyte-specific antigen. The functional and structural characteristics of senescent erythrocytes strongly suggest that this antigen originates on band 3, probably by calcium-induced proteolysis. Generation of vesicles enriched in denatured hemoglobin is an integral part of the erythrocyte aging process. These vesicles are also removed by Kupffer cells, with a major role for exposure of phosphatidylserine. Moreover, senescent erythrocyte-specific antigens are present on vesicles. Thus, vesicles and senescent erythrocytes may be recognized and removed through the same signals. These and other, recent data support the theory that erythrocyte aging is a

form of apoptosis that is concentrated in the cell membrane, and provide the context for future studies on initiation and regulation of the erythrocyte aging process. Insight into the normal aging mechanism is essential for understanding the fate of erythrocytes in pathological circumstances and the survival of donor erythrocytes after transfusion.

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### Introduction

There is no dispute that erythrocytes have a definite lifespan in all animal species investigated so far, and there is little dispute on the length of the species-specific lifespans [1]. These simple statements imply that, as in other cells, life and death are well regulated for erythrocytes, in spite of their lack of capacity for protein synthesis. During the last decade, a general consensus has been established on the mechanisms by which senescent erythrocytes are removed from the circulation. However, the identity of the mechanisms by which erythrocytes become senescent, i.e. by which

erythrocytes arrive at the verge of being removed, is still a subject of discussion. Here we will briefly review this discussion, and introduce a new aspect, namely vesicle formation, as an important and hitherto largely overlooked mechanism of erythrocyte aging and removal. We will incorporate this aspect in the established hypotheses on erythrocyte aging, and discuss the possibility that erythrocyte aging is a form of apoptosis.

## Recognition of senescent erythrocytes

In physiological circumstances, senescent erythrocytes are bound by autologous IgG, which leads to recognition and phagocytosis. This represents one of the first demonstrations of a physiological role for an autoimmune process. In humans the responsible phagocytes are mainly Kupffer cells in the liver, but in other species, most notably rat and mouse, phagocytosis may also occur in other parts of the reticulo-endothelial system, such as the spleen [1, 2]. The IgG that is bound to senescent erythrocytes represents one of the few accessible clues to the identity of the antigen(s) it binds to, and thereby to the aging mechanism itself. Therefore, characterization of this IgG has been given a good deal of attention (for a recent, extensive review see [3]). However, this attention has not led to an unambiguous view, since it has been clouded by the use of research strategies that have depended too heavily on experiments *in vitro*, in combination with plasma as a source of naturally occurring antibodies. Plasma contains erythrocyte-specific antibodies with various affinities, specificities and activities: leading to complement activation or not, binding to intracellular erythrocyte proteins such as actin and spectrin, binding to extra- and intracellular domains of membrane proteins such as band 3, and of low and high affinity. The clearest view, based on analyses in which the materials were as clean and as close to the *in vivo* situation as possible, suggests that low numbers of bound IgG (e.g. 200 molecules/erythrocyte) are sufficient for phagocytosis, and that significant IgG binding does not occur until during the last few weeks [4, 5]. Activation of complement is probably not involved. When erythrocytes of various ages were probed with antibodies against the anti-complement proteins CD55 and CD59, the mean fluorescence intensity decreased with erythrocyte age (CD55,  $89 \pm 1.8\%$ ; CD59  $92 \pm 2.8\%$  in the oldest relative to the youngest erythrocytes). However, the mean surface decreased even more, resulting in a small increase with

aging in the number of CD55 and CD59 per unit of surface area [6]. The low number of specifically erythrocyte-bound IgG found under physiological conditions *in vivo* indicates that removal of senescent erythrocytes is an efficient and well-orchestrated process. However, this also implies that it is difficult to obtain enough IgG for analysis. In those situations in which more bound antibodies can be obtained from 'aged' erythrocyte populations, it must be assumed that either the aging mechanism and/or the removal machinery are disturbed, and any data obtained in such circumstances must be doubted with regard to their validity for the physiological aging process. Various examples may illustrate this important *caveat*: 1, whereas senescent erythrocyte-specific immunoglobulins are all IgG, the immunoglobulins present on sickled erythrocytes consist of various classes, including IgA [7]; 2, antibodies that bind to experimentally aged erythrocytes have a low affinity, or may represent a blood group reaction [3, 8, 9]; 3, the amount of autologous immunoglobulin binding after oxidation *in vitro* mainly correlates with the degree of lysis, and probably results from lysis-induced appearance of intracellular autoantigens on the outside of intact erythrocytes (Bosman GJCGM and Kay MMB, unpublished observations).

CD47, also known as integrin-associated membrane protein, mediates adhesion-related processes [10]. Recent data have pointed towards CD47 as another molecule that may be involved in recognition of senescent erythrocytes by macrophages [11]. Erythrocytes from CD47 knock-out mice, in which they have a normal lifespan, rapidly disappeared from the circulation when brought into wild-type recipients. So, regulation of CD47 expression might serve as a mechanism to control elimination of senescent RBC. However, erythrocytes from heterozygous animals survived normally in wild-type mice, which argues against a gradual disappearance of CD47 as the sole mechanism that regulates erythrocyte survival. It is more likely that the absence of CD47 triggers other processes leading to erythrocyte removal, that have not yet been identified in mice. In humans, CD47 is part of the Rhesus core complex, and Rhesus<sub>null</sub> individuals with less than 25% CD47 have hemolytic anemia and stomatocytosis, but there is no evidence that Rh<sub>null</sub> erythrocytes are phagocytosed more readily than erythrocytes with normal amounts of CD47 [12].

Exposure of phosphatidylserine at the outside of erythrocytes is widely implicated as a trigger for the removal of senescent erythrocytes (for a recent review

see [13]), notwithstanding the paucity of data showing that exposure of phosphatidylserine is actually increased during physiological aging. In fact, flow cytometry data show the virtual absence of erythrocytes exposing phosphatidylserine, as measured by the capacity to bind annexin V, in all age fractions (the youngest erythrocytes,  $0.31 \pm 0.16$  %; the oldest erythrocytes,  $0.11 \pm 0.06$  % positive cells [14]). The arguments for this theory are mainly based on pathological data, and on results from manipulation *in vitro* and *in vivo*. In erythrocyte pathologies such as sickle cell anemia, thalassemia and spherocytosis, a decrease in erythrocyte survival is associated with an increase in phosphatidylserine exposure [13]. Treatments leading to an increased calcium influx and oxidation, that induce more or less presumed aspects of erythrocyte aging and decreased erythrocyte survival, also lead to inhibition of flipase and/or activation of scramblase, both leading to loss of phospholipid asymmetry [15]. In general, all processes that lead to increased destruction are associated with an increased exposure of phosphatidylserine at the erythrocyte surface. In view of this evidence together with the general role that phosphatidylserine plays in recognition and removal of apoptotic cells [16], we are inclined to give phosphatidylserine the benefit of the doubt regarding its role in normal erythrocyte aging. It can not be excluded that exposure of phosphatidylserine leads to such a fast removal that it is underestimated [13, 14]. A decrease in phospholipid asymmetry may very well be one of the consequences of physiological aging at the level of the membrane, and may result in exposure of phosphatidylserine that, in conjunction with other signals, partakes in the recognition and removal of senescent erythrocytes by phagocytes [16].

Thus, the accumulation of autologous, high-affinity, and specific IgG during the final part of the erythrocyte lifespan triggers, above a relatively low threshold, binding and phagocytosis of senescent erythrocytes. Removal may be promoted by the exposure of phosphatidylserine at the erythrocyte surface.

### **Identity of the senescent erythrocyte-specific antigen**

Together with the consensus on the involvement of the immune system in the recognition and removal of senescent erythrocytes, a consensus also seems to have been reached on the involvement of band 3 in the

generation of the senescent erythrocyte-specific antigen. Band 3 is, at one million copies per cell, quantitatively the major protein of the erythrocyte membrane. The C-terminal half of the protein is an integral membrane domain that catalyzes the electroneutral exchange of chloride and bicarbonate. The N-terminal cytoplasmic domain provides, through the intermediate action of ankyrin, the interaction site by which the spectrin/actin cytoskeleton is anchored to the lipid bilayer [17]. In addition, the membrane domain carries the antigens of the Diego blood group system, and the cytoplasmic domain has high-affinity binding sites for key enzymes of the glycolysis such as glyceraldehyde-3-phosphate dehydrogenase, aldolase and phosphofructokinase [18]. In addition, carbonic anhydrase II and IV may be associated with band 3 at the intra- and extracellular side of the membrane, respectively, forming a 'transport metabolon' [19, 20]. Binding of glyceraldehyde-3-phosphate dehydrogenase to band 3 inhibits enzyme activity, suggesting that these associations, regulated by hitherto little explored signal transduction pathways, have a functional meaning in maintaining erythrocyte homeostasis. This is not restricted to metabolism and anion transport, since the linkage with ankyrin, the association with glycophorin A, and the association with the Rhesus complex through protein 4.2 all place band 3 at the center of a complex that regulates cell shape and transport of other metabolites such as glucose and  $\text{NH}_3$ , as well [5, 21, 22]. Also, deoxy-hemoglobin and especially denatured hemoglobin (hemichromes) have a high affinity for the cytoplasmic domain of band 3 [5]. There may also be a role for band 3 in release of NO during the blood flow [23].

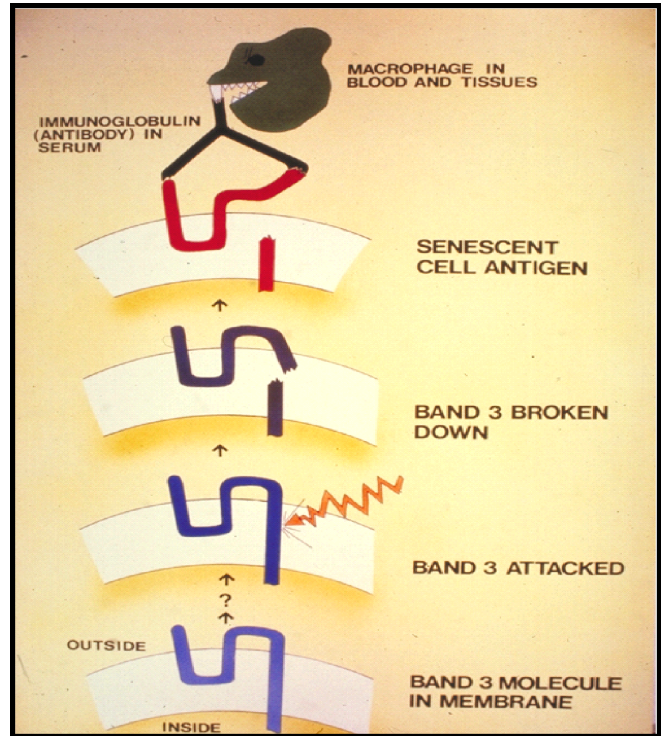
IgG that becomes specifically bound to erythrocytes during the final stages of their lifespan recognizes band 3, so it is obvious that changes in band 3 occurring during the erythrocyte lifespan must precede this binding. However, the molecular identity of the senescent erythrocyte antigen that is recognized by this IgG, and thus the nature of these changes, have not been unambiguously identified. The discussion focuses on aggregation versus breakdown of band 3 as the ultimate steps in neoantigen formation. In the first hypothesis, binding of denatured hemoglobin to the cytoplasmic domains of band 3 causes oligomerization of band 3, resulting in the formation of a neoantigen that is recognized by senescent erythrocyte-specific IgG. This hypothesis is supported by many data showing an association between increased IgG binding and band 3 oligomerization *in vitro* and *in vivo*. The concomitant

increase in membrane-bound globin chains supports the involvement of hemoglobin in this process [3, 4]. In the second hypothesis, breakdown of band 3 within the membrane domain is the central event that leads to conformational changes resulting in the generation of a senescent erythrocyte-specific neoantigen, senescent cell antigen (Figure 1, [5]). This hypothesis is supported by immunoblot data showing aging-related breakdown, and by functional data such as an aging-related decrease in anion transport capacity [5]. Both theories explain the loss of interaction between membrane and cytoskeleton, as indicated by the increased mobility of band 3, and the decreased numbers of high-affinity binding sites for ankyrin [4, 24]. In both scenarios there is a central role for oxidation as a causative event, be it through hemichrome generation in the oligomerization hypothesis or through inducing an increased sensitivity of band 3 to proteolysis in the breakdown hypothesis. Various data indicate that age-related breakdown of band 3 is caused by activation of calcium-sensitive proteases [25].

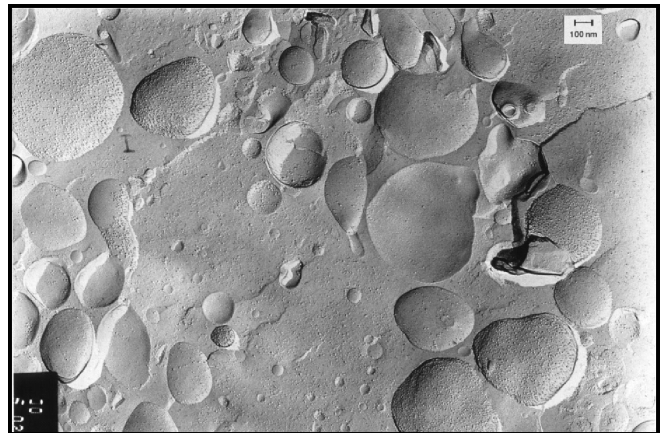
At the moment both hypotheses remain unfalsified, probably for a number of reasons: 1, changes in the cytoplasmic domain of band 3 affect structure and function of the membrane domain and *vice versa*, which makes it difficult to pinpoint the original site of disturbance; 2, because of the central role of band 3 in erythrocyte homeostasis, many processes induce changes in band 3, including erythrocyte-specific pathology but possibly also other conditions such as old age, pregnancy, cardiovascular pathology, and diabetes [26-28]. In addition, the presence of denatured hemoglobin per se, such as in unstable hemoglobinopathies, is not clearly associated with an accelerated erythrocyte aging phenotype [29]. This makes it difficult to establish a chain of cause and effect; 3, in view of the above, it is also difficult to establish the value of artificial and pathological model systems for all possibly relevant aspects of the physiological aging process; 4, structural changes in especially the membrane domain of band 3 that are present *in situ* may easily be lost upon membrane isolation and further analytical procedures; 5, obtaining erythrocyte populations of well-defined cellular age is a laborious procedure.

### The role of vesiculation during erythrocyte aging

Using a combination of counterflow centrifugation and subsequent density centrifugation it was possible to determine unequivocally that during aging the erythrocyte

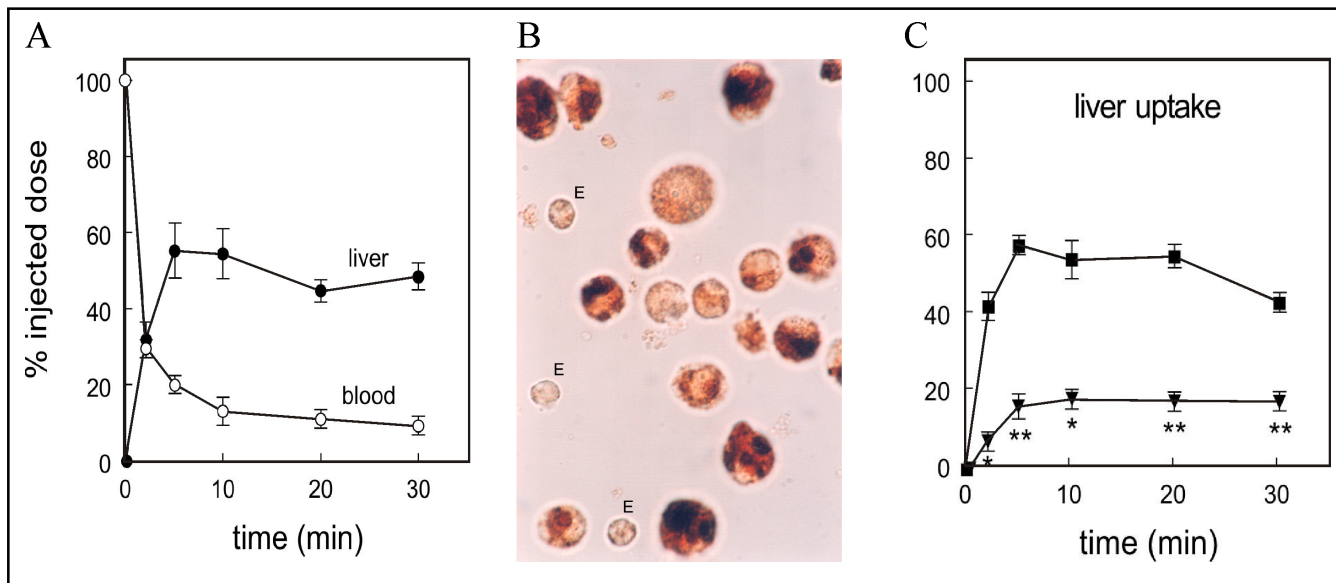


**Fig. 1.** Breakdown of band 3 within the membrane domain leads to a change in protein conformation, and generates senescent cell antigen [5].



**Fig. 2.** Plasma contains small (< 200 nm) vesicles with right-side-out and inside-out-oriented membranes, and larger (200 – 800 nm) vesicles with predominantly right-side-out membranes [33].

volume decreases with time, with an increase in density especially during the first, and a decrease in hemoglobin content especially during the second part of the lifespan [30]. These changes are associated with a loss of



**Fig. 3.** A, within 5 minutes,  $^{51}\text{Cr}$ -chromate-labeled vesicles are removed from the circulation with a concomitant uptake by the liver; B, nearly all liver activity is found in Kupffer cells, with a

concomitant uptake of hemoglobin; C, this uptake is inhibited by preinjection of phosphatidylserine liposomes ( $\blacktriangle$ ), but not phosphatidylcholine liposomes ( $\blacksquare$ ) [32].

cholesterol and phospholipid and a linear decrease in the mean surface area of 20 per cent, which all pointed towards a loss of membrane during aging. This loss could be readily explained through the formation of microvesicles by erythrocytes of all ages (Figure 2), with vesicles derived from older erythrocytes containing more hemoglobin. Immunological analysis of these vesicles by flow cytometry and immunoblotting revealed that a portion, but not all of these vesicles contain IgG at their surface, expose phosphatidylserine, and may contain breakdown products of band 3 that are also found on the oldest erythrocytes (Willekens et al, in preparation). HPLC analysis showed that the hemoglobin composition of these vesicles resembles that of the oldest erythrocytes, including HbA1c and what are probably more advanced glycation and carbamylation products [31]. Vesicles disappear rapidly from the circulation: in a rat model, Kupffer cells in the liver remove naturally occurring, hemoglobin-containing, erythrocyte-derived vesicles from the circulation within minutes, mainly by scavenger receptors and with phosphatidylserine as the principal ligand (Figure 3, [32]). Taken together, these data represent the discovery of a new and hitherto obscure pathway of erythrocyte removal in which 20 per cent of the hemoglobin and the cell membrane are involved. They also place a considerable fraction of the premortal substrate at our disposal. It should be noted that the spleen plays an

important, although poorly understood role in the expulsion and possibly also the removal of vesicles. After splenectomy, vacuoles - the presumed precursors of vesicles - start to accumulate in erythrocytes of all ages. In older erythrocytes absolute increases of HbA1c and HbA1e2 are measured. Also, an increase in total hemoglobin content and an increase in surface area relative to those of control old cells could be demonstrated [33, 34]. Taken together, these observations point towards the retention of vesicles especially in erythrocytes containing older hemoglobin. Therefore, it can be stated that the spleen facilitates the erythrocyte vesiculation process. It is plausible that Heinz bodies and other inclusion bodies leave the erythrocyte the same way. It can also be speculated that the beneficial effect of splenectomy in some patients with hemolytic anemia caused by inborn errors in erythrocyte membrane proteins [35], may be caused by the prevention of inappropriate vesiculation which, in these patients, would result in more robust erythrocytes.

A full proteomic and immunologic comparative analysis of the vesicles produced in physiological circumstances *in vivo* and erythrocyte fractions of various ages is on the way (Bosman et al, in preparation). However, the available data already allow the combination of aging-associated vesiculation with the current hypotheses described above, into a new hypothesis on



the erythrocyte aging mechanism. We propose that protein modification by oxidation, glycation, carbamylation, nitrosylation, or ubiquitinylation occurs already early in the life of the erythrocyte. The occurrence of all these processes in the erythrocyte has been documented, in some cases also modifying band 3 [36]. If hemoglobin is the main victim, this may result in the binding of denatured hemoglobin to the cytoplasmic domain of band 3 and subsequent breakdown within the band 3 membrane domain. When band 3 is the main victim, this may result in the binding of hemoglobin to band 3 breakdown products that are already present. Activation of proteases by calcium is likely to be involved in band 3 breakdown, with calcium entering the erythrocyte possibly through activation of cell volume-sensitive cation channels [37, 38]. Either theory explains the presence of band 3 breakdown products in all but the youngest fraction at much higher concentrations than seem to be necessary for binding of the few hundred IgG molecules that are sufficient for phagocytosis of the senescent fraction [5]. The presence of band 3 breakdown products may also explain the loss of water during the first part of the lifespan as an osmotic consequence of a decrease in band 3-catalyzed anion transport capacity [5, 30]. Lateral diffusion of the band 3 fragments, driven either by aggregation of the associated hemichromes, or by exclusion from the functional units consisting of band 3 di- and tetramers, may lead to their aggregation which would increase membrane curvature and thereby induce vesiculation [39]. Alternatively, breakdown of band 3 within its membrane domain is likely to disturb the lipid organization by loss of lipid anchorage, and affect the mobility of lipids in successive layers. It remains to be established if this is reflected by or related to raft formation [40, 41]. This could lead to vesicle formation where the cytoskeleton remains intact, such as occurs in the erythrocytes of band 3 knock-out animals [42]. Thus, vesicles containing denatured hemoglobin also display the signal for their removal in the form of senescent erythrocyte-antigen on band 3 fragments. Through this process, non-functional and/or toxic products are continuously removed from the erythrocyte for the better part of its lifespan. At the end, however, the cellular defenses wear down and the vesicle formation system becomes overburdened, resulting in removal of the whole erythrocyte. Although there are similarities between the mechanisms and receptors involved in recognition and phagocytosis of vesicles and senescent erythrocytes, it is not clear whether they are completely identical.

## **Erythrocyte aging represents the execution phase of the apoptosis process**

Cell death is considered as apoptosis when it processes through an ordered chain of events, that are triggered by an extra- or intracellular stress signal (the activation phase), and when: 1, the protein components of the cell death process are constitutively expressed; 2, the execution phase involves a calcium-dependent proteolytic cascade (the execution phase); 3, activation is controlled by dedicated regulatory proteins; 4, the remains are removed by phagocytosis, without inflammation (burial phase). In addition, destabilization of the connection between the cytoskeleton and the cell membrane, resulting in blebbing and fragmentation, may be a regulated event, and phagocytosis of cell fragments is triggered by 'distinguishing features' such as the presence of phosphatidylserine at their surface [16, 43]. A compilation of the keywords of the processes that lead to the removal of senescent erythrocytes from the circulation described above – aging, autoimmunity, calcium-dependent proteolysis, vesiculation, oxidation, phagocytosis, phosphatidylserine exposure – shows an almost complete overlap with the terms that characterize the apoptotic process of nucleated cells. This has already been put forward by others [37, 38, 44], and is supported by data suggesting the involvement of calcium, showing oxidation and aging-related activation of caspases [45, 46], possibly mediated by membrane-associated Bcl-X(L) [47], and leading to phosphatidylserine exposure, breakdown of band 3 and phagocytosis [45, 48]. In this context it should be emphasized that the data obtained during the last decade show that the erythrocyte contains a complex, functional set of regulatory systems. ATP content, concentrations of anions and cations, cell volume and cell shape are not the immutable consequences of the erythrocyte's surroundings but, as in all cells, are regulated as a response to and meeting the demands of neighbouring cells and organs [49-51]. Thus, various signal transduction pathways are present and active, putting life and death of the erythrocyte once more at a central position in the study of organismal homeostasis.

The current knowledge of the erythrocyte aging process reviewed here, together with the view of the erythrocyte as a specialized cell with its own homeostasis and signal transduction machinery, lead inevitably to the thesis that the processes leading to removal of erythrocytes from the circulation are those of the execution phase of a apoptosis program. This conclusion

may accelerate the slowly progressing research on erythrocyte aging by inspiring the search for the pieces that are still missing from a complete apoptosis picture. Perhaps more accessible problems than the long-time elusive molecular identity of the senescent erythrocyte-specific antigen can now be addressed, such as the identity of the activation signal, the mechanism of calcium increase, the identity of any regulatory proteins, etc. In a reciprocal view, the erythrocyte may once more constitute

a valuable model system, e.g. for the study of the final stages of apoptosis such as membrane blebbing, vesicle formation, and immune recognition and phagocytosis. Finally, an unequivocal description of the physiological erythrocyte aging pathway will be invaluable in understanding the fate of erythrocytes in pathological circumstances and the survival of donor erythrocytes after blood transfusion, and thereby open the road towards rational intervention procedures.

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