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CD47 in Erythrocyte Ageing and Clearance – the Dutch Point of View

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Keywords
CD47 · Aged erythrocytes · Phagocytosis · SIRPα

Abstract
Recently, an important role for CD47, a well-known 'don't eat me' signal, in the clearance of aged erythrocytes was revealed. Experimental data support the conversion of CD47 from a 'don't eat me' to an 'eat me' signal through a conformational change in CD47. Intriguingly, erythrocyte phagocytosis after this switch seems to be mediated by the same receptor that normally signals inhibition of phagocytosis, SIRPα. In this review, the possible molecular mechanisms leading to this conformational change in CD47 as well as the possible signal transduction events leading to phagocytosis after this switch are discussed. Lastly, the consequences of this newly identified mode of erythrocyte phagocytosis for the clearance of aged erythrocytes during normal turnover and after erythrocyte transfusion are addressed.

Schüsselwörter
CD47 · Gealterte Erythrozyten · Phagozytose · SIRPα

Zusammenfassung
Vor kurzem ist eine wichtige Rolle für CD47, einem bekannten 'Don't-Eat-Me'-Signal, bei der Beseitigung gealterter Erythrozyten aufgedeckt worden. Experimentelle Daten belegen die Umwandlung von CD47 von einem 'Don't-Eat-Me'- zu einem 'Eat-Me'-Signal durch konformative Veränderungen. Interessanterweise scheint die Erythrozytenphagozytose nach der Umwandlung von dem gleichen Rezeptor (SIRPα) vermittelt zu werden, der normalerweise die Hemmung der Phagozytose signalisiert. In dieser Übersichtsarbeit werden sowohl die potentiellen molekularen Mechanismen, die zu dieser Umwandlung von CD47 führen, als auch die möglichen Signaltransduktionsvorgänge, die nach der Umwandlung die Phagozytose einleiten, diskutiert. Des Weiteren werden die Konsequenzen dieses neu identifizierten Modus der Erythrozytenphagozytose für die Beseitigung gealterter Erythrozyten während des normalen Turnovers sowie nach Erythrozytentransfusion beleuchtet.

Introduction

Erythrocyte clearance has been studied for many years in the context of normal turnover [1, 2], enhanced clearance due to defects in erythrocyte metabolism or changes in membrane composition [3–5], and in blood transfusion [6]. Traditionally, erythrocyte phagocytosis has been proposed to be the result of the accumulation of 'eat me' signals on the membrane of the ageing erythrocyte. Several 'eat me' signals have been identified to be important for the clearance of aged erythrocytes by macrophages residing in the red pulp of the spleen, or alternatively, in the liver [1, 7, 8]. Two possible mechanisms that would lead to erythrocyte clearance are i) autoantibody binding to Band 3 after conformational changes in this protein due to ageing (the so-called 'senescent cell antigen') [9, 10], and ii) expression of phosphatidylserine (PS) on the outer

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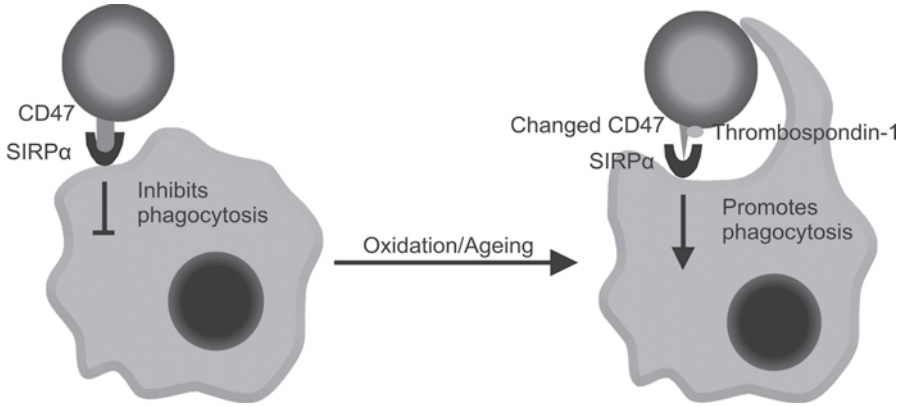

Fig. 1. Oxidation and ageing cause a conformational change in CD47 upon which TSP-1 can bind. Under normal conditions, CD47 on the erythrocyte inhibits phagocytosis by interaction with SIRPα on the phagocyte. After the conformational change in CD47, TSP-1 is able to bind upon which interaction between CD47 and SIRPα leads to phagocytosis.

leaflet of the erythrocyte membrane [11]. Both of these mechanisms are discussed in detail in this issue of Transfusion Medicine and Hemotherapy.

However, in 2000, Oldenborg et al. [12, 13] identified the membrane protein CD47 to be an important additional factor regulating phagocytosis of erythrocytes. In contrast to the 'eat me' signals as mentioned above, CD47 was shown to inhibit phagocytosis of erythrocytes by macrophages [12–16] (fig. 1). Moreover, the expression of CD47 by erythrocytes was found to be essential to prevent their uptake in the spleen by macrophages; erythrocytes from CD47-deficient mice were rapidly cleared when transfused into wild-type animals [13, 17]. Thus, clearance of erythrocytes was now believed to be determined by the balance between the accumulation of 'eat me' signals and the expression of the 'don't eat me' signal CD47. Loss of CD47 expression during erythrocyte ageing was proposed as an alternative mechanism for erythrocyte clearance in the normal ageing process [18, 19]. CD47 exerts its inhibitory effect through binding to SIRPα on the macrophage, which induces inhibitory signaling by the immunoreceptor tyrosine-based inhibition motifs (ITIMs) residing in the cytoplasmic tail of SIRPα [20, 21]. Upon ligation of SIRPα by CD47, the tyrosine phosphatases SHP-1 and SHP-2 are recruited to the ITIMs and activated, which in turn regulates, generally in a negative fashion, downstream signaling pathways and effector functions.

In a recent study, in which we attempted to identify the mechanism(s) that underlie the well-described clearance of a significant percentage of donor erythrocytes in the first 24 h after transfusion, CD47 was identified to be able to function as an 'eat me' signal after undergoing a conformational change (fig. 1) [22]. Strikingly, SIRPα was found to be the counter-receptor that recognized and bound this form of CD47 as well, revealing another layer of complexity of this ligand/receptor pair. Thus, next to its well-known role as inhibitor of phagocytosis, we propose that CD47 can switch its conformation and thereby its function to an 'eat me' signal on aged erythrocytes. In this review, we discuss this finding and the possible molecular mechanisms that are responsible for it.

Evidence for a Role of CD47 in the Phagocytosis of Aged Erythrocytes

Intriguingly, evidence exists in the literature that the CD47-SIRPα interaction, besides its inhibitory function on phagocytosis, can also promote cell-cell interactions [23] and even mediate phagocytosis of apoptotic cells [24–26]. First, SIRPα can mediate trans-endocytosis of CD47-containing membranes of adjacent cells, as reported by Kusakari et al. [23]. This work revealed that CD47-SIRPα signaling can, inversely to its known inhibitory effect on phagocytosis, actually lead to internalization of parts of the membrane of adjacent cells through SIRPα signaling. Furthermore, another report indicated that CD47-SIRPα interaction is very important for proper recognition and phagocytosis of apoptotic cells. Expression of CD47 in a lymphoma cell line, which did not express CD47 endogenously, was essential to allow phagocytosis of this cell line after the induction of apoptosis [25]. The parental cell line, i.e. without expression of CD47, was not phagocytosed after induction of apoptosis. Furthermore, the effect of expression of CD47 was abrogated when the lymphoma cells were treated with an antibody recognizing SIRPα, thereby blocking the interaction with CD47. These data were the first indication that CD47 and SIRPα are more than just negative regulators of phagocytosis, but might be involved in the efficient removal of apoptotic cells as well. These findings led us to hypothesize that the CD47-SIRPα interaction might play a role in the removal of aged erythrocytes. Using SIRPα-transfected cell lines, we observed that aged erythrocytes, either isolated from fresh blood or experimentally aged by oxidative treatment, were bound and phagocytosed through CD47-SIRPα interaction [22]. In addition, experimentally aged erythrocytes were found to be phagocytosed by human red pulp macrophages. Strikingly, human red pulp macrophages were unable to phagocytose anti-D-opsonized or PS-exposing erythrocytes (Burger et al., data unpublished), which is an indication that these mechanisms do not play a dominant role in the phagocytosis of aged erythrocytes by this particular cell type.

Conformational Change in CD47 Forms the Basis for Recognition by SIRPa as an 'Eat Me' Signal

It has been known for some time that CD47 can exist in different conformations on erythrocytes. Evidence for this was provided by Brittain et al. [27] who had used a set of antibodies directed against CD47 to study the conformational status of CD47 on sickle erythrocytes. CD47 expression levels did not vary between erythrocytes of normal controls and sickle patients, but the conformation of this protein, as determined by binding of antibodies against particular conformation-dependent epitopes, seemed to differ between the 2 groups. In addition, thrombospondin-1 (TSP-1), a well-known ligand for CD47 [28], was able to bind to CD47 on sickle erythrocytes but not to CD47 on erythrocytes of healthy controls, suggesting that the conformation of CD47 also determines whether or not it binds to TSP-1. Intriguingly, binding of TSP-1 to apoptotic cells has been reported to enhance phagocytosis of the apoptotic cell without inducing the secretion of pro-inflammatory cytokines [29]. Furthermore, TSP-1, which is a large homotrimeric glycoprotein, can mediate several cell-cell and cell-matrix interactions and can bind PS on erythrocytes [30]. Previous work by Head et al. [31] had indicated that inducing CD47 signaling on erythrocytes via antibodies but also through prolonged incubation with the TSP-1-derived peptide 4N1K leads to erythrocyte death. Together, these data indicated that the recognition of aged erythrocytes through CD47-SIRPα, which was found to be serum dependent, could be explained by a conformational change in CD47 resulting in TSP-1 binding and subsequent SIRPα recognition. In line with the experiments conducted by Brittain et al. [27], we observed that the conformation of CD47 on experimentally aged erythrocytes was found to have undergone a conformational change similar to that seen in sickle erythrocytes. Also, the TSP-1-derived peptide 4N1K was found to bind only to experimentally aged erythrocytes, i.e. to CD47 that had undergone a change in conformation. After testing binding and phagocytosis by both SIRPα-transfected cell lines as well as red pulp macrophages, we concluded that the change in conformation of CD47, followed by TSP-1 binding, marks erythrocytes for phagocytosis through SIRPα recognition.

It is presently unknown what molecular mechanism(s) induce(s) the change in conformation in CD47 leading to TSP-1 binding. Several possible mechanisms can be envisioned. First, since CD47 is part of the Band 3 complex in erythrocytes [32], changes in this membrane complex could induce conformational changes in CD47 that lead to the 'eat me' configuration. As discussed elsewhere in this issue of Transfusion Medicine and Hemotherapy, Band 3 itself is subject to many modifications, including clustering [33], phosphorylation [34], and cleavage [35], which lead to the generation of the senescent cell antigen. These modifications in Band 3 are well documented to occur during ageing [36–38], and this would certainly be a very elegant way to couple intracellular ageing events resulting in Band 3 modifications to the conformational change in CD47 and thus create an extracellular 'eat me' signal at the expense of the 'don't eat me' signal that CD47 normally fulfills in erythrocytes. Another interesting phenomenon that occurs on apoptotic erythrocytes is the clustering of CD47 [32]. This clustering is supposed to be the result of increased mobility of CD47, and might lead to a change in conformation and subsequent 4N1K binding which would then be recognized by SIRPα as an 'eat me' signal [22]. The contribution of this phenomenon to the inhibition or facilitation of clearance through SIRPα is still unexplored. Finally, it is also possible that CD47 is directly modified during ageing, i.e. through oxidation, which would directly alter the conformation of CD47 and the recognition by SIRPα. Although the latter option appears somewhat unlikely, evidence was obtained that this indeed occurs. Firstly, the conformational change in CD47 can be induced by oxidative treatment. Secondly, beads coated with the extracellular domain of CD47 were found to bind the TSP-1-derived peptide 4N1K only after oxidation and binding of oxidized CD47 to SIRPα proved to be dependent on the binding of 4N1K. Of course, the above proposed changes in CD47 leading to a conformational switch are certainly not mutually exclusive and might also occur simultaneously.

Signal Transduction by SIRPa Leading to Phagocytosis of Aged Erythrocytes

SIRPα signaling leading to the inhibition of phagocytosis is based on signaling through the 4 ITIMs residing in the cytoplasmic tail [20]. The ITIMs become phosphorylated upon CD47 binding after which the tyrosine phosphatases SHP-1 and SHP-2 are recruited and activated through another phosphorylation event. SHP-1 and SHP-2 then dephosphorylate specific protein substrates that are important for phagocytosis, and thereby exert a negative effect on this process. At present it is unknown how SIRPα signaling induced by the CD47/TSP-1 complex on aged erythrocytes induces phagocytosis. Inspired by the report that the membrane proximal region of SIRPα regulates endocytosis independent of the ITIMs [23], we explored the phagocytic capacity of different SIRPα mutants of aged erythrocytes. Phagocytosis of aged erythrocytes through CD47/SIRPα interaction proved to be dependent on the presence of the cytoplasmic tail, but did not require functional ITIMs [22]. The latter could be concluded from the fact that a SIRPα mutant, in which all tyrosines in the ITIMs were replaced with phenylalanines, was as effective as the wild-type protein. This essentially rules out a role for SHP-1 and SHP-2, but raises the question which signaling proteins are involved in this process. Interestingly, SIRPα was postulated to be a scaffold for the recruitment of signaling complexes related to integrin activity [24]. 2 different complexes were identified; one containing the tyrosine kinase

Pyk2, the other SKAP55hom/R and FYB/SLAP-130 [39]. Moreover, it was shown that tyrosine phosphorylation of the ITIMs in SIRPα was not a prerequisite for the formation of these different complexes. The possibility that SIRPα cooperates with integrins to mediate phagocytosis of apoptotic cells is appealing, as many reports have already pointed out a role for integrins in the non-inflammatory removal of apoptotic cells [40, 41].

CD47/TSP-1 as an 'Eat Me' Signal for Normal Erythrocyte and Donor Erythrocyte Removal

Based on our previous data in which a small proportion of erythrocytes from full blood – residing in the fraction of erythrocytes that contain the oldest erythrocytes – was found to be bound and phagocytosed by SIRPα-expressing cells, we propose that CD47/TSP-1 functions as a removal signal in the normal turnover of erythrocytes (fig. 1). In line with this, human macrophages isolated from the red pulp of the spleen were found to be able to recognize and phagocytose erythrocytes expressing this removal signal. Human red pulp macrophages express high levels of SIRPα, in contrast to for instance Fc receptors which are expressed only at low levels (Burger et al., data unpublished). Human red pulp spleen macrophages were found to phagocytose anti-D-opsonized erythrocytes at very low levels, in contrast to monocyte-derived macrophages which phagocytosed erythrocytes treated in this way at high levels (Burger et al., data unpublished). Even PS-expressing erythrocytes were not phagocytosed at a level above background by red pulp macrophages which are the most relevant cell type for the removal of aged erythrocytes (Burger et al, data unpublished). In short, these results favor CD47 as a removal signal for aged erythrocytes in vivo over other potential removal signals, such as immunoglobulin binding or PS exposure. In addition, we speculate that this mechanism may also be relevant for other blood cell types, since CD47 is ubiquitously expressed on hematopoietic cells, and evidence exist in the literature that CD47 is also necessary for efficient phagocytosis of apoptotic cells of other origin [24, 25]. It is well known that transfusion of erythrocytes leads to a rapid clearance of 10–25% of the transfused cells, depending on storage time [42, 43]. The data obtained may also explain this high percentage of removal of erythrocytes after transfusion. A significant proportion of donor erythrocytes, stored for several weeks, show the conformational change in CD47 favoring TSP-1 binding when incubated in fresh blood. Short-stored erythrocytes, which are also cleared at a much lower rate after transfusion, do not show this increase. In addition, long-stored erythrocytes bind TSP-1 when diluted into whole blood, whereas short-stored cells do not. Importantly, the change in conformation and the subsequent TSP-1 binding can only be observed after dilution in whole blood and subsequent incubation at 37 °C. This finding provides a possible explanation for the high rate of clearance of long-stored erythrocytes after transfusion. Although it has been suggested that the expression of CD47 decreases during erythrocyte storage, hinting at an enhanced clearance due to diminished inhibition through SIRPα signaling, we and others have found that CD47 levels are not diminished after prolonged storage [44, 45]. Thus, a conformational change in CD47 resulting in the expression of the 'eat me' configuration of CD47 in long-stored erythrocytes seems to be an attractive mechanism for the high percentage of removal of donor erythrocytes.

Conclusion

CD47 undergoes a conformational change during ageing, which causes TSP-1 binding and recognition of CD47 as an 'eat me' signal by SIRPα. This newly identified mode of erythrocyte clearance provides a very elegant, possibly non-inflammatory, way of clearing aged erythrocytes. The conformational status of CD47 can be changed through oxidative stress or, possibly, after changes in the Band 3 complex, and is thus a potential extracellular indicator for intracellular damage and ageing. From our point of view, CD47 controls erythrocyte lifespan both positively through inhibition of phagocytosis via SIRPα, and negatively by triggering phagocytosis through SIRPα. The fact that aged erythrocytes, which can be phagocytosed through a SIRPα-mediated mechanism, can be isolated from normal blood, and primary human red pulp macrophages can phagocytose aged erythrocytes through this mechanism, strongly suggests that this mechanism is operational in vivo, but further studies are required to establish this.

Disclosure Statement

The authors declare no competing financial interests.

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