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The sorting of blood group active proteins during enucleation

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Abstract

Enucleation represents the critical stage during red blood cell development when the nucleus is extruded from an orthochromatic erythroblast in order to generate a nascent immature reticulocyte. Extrusion of the nucleus results in loss of a proportion of the erythroblast plasma membrane, which surrounds the nucleus, the bulk of the endoplasmic reticulum and a small region of cytoplasm. For this reason enucleation provides an important point in erythroblast differentiation at which proteins not required for the function of the erythrocyte can be lost, whilst those that are important for the structure-function properties of the mature erythrocyte must be efficiently retained in the reticulocyte plasma membrane. Disturbances in protein distribution during enucleation are envisaged to occur during human diseases such as Hereditary Spherocytosis. This article will discuss the current knowledge of erythroblast enucleation in the context of retention and loss of proteins that display antigenic blood group sites and that exist within multiprotein complexes within the erythrocyte membrane.

Introduction

The term erythropoiesis describes the complex process by which an immature erythroid progenitor cell (erythroblast) undergoes differentiation in order to generate a biconcave erythrocyte or 'red blood cell'. As an erythroblast differentiates, it commits to a series of events that will see it transit through a variety of classically described cellular morphological stages (proerythroblast, basophilic, polychromatic and orthochromatic). These transitions are accompanied by the induction of rapid and large scale expression of a host of erythroid specific proteins including haemoglobin, a reduction in cell volume, condensation of the nucleus and culminate in the complete extrusion of the condensed nucleus in order to generate an anucleate nascent reticulocyte. This process of nuclear extrusion from an orthochromatic erythroblast is known as enucleation and involves the removal or pinching off of a plasma membrane enclosed intact nucleus accompanied by endoplasmic reticulum (ER) membrane and a small region of cytoplasm. The resultant nascent reticulocyte must express at its surface all of the antigenic erythrocyte proteins that provide the basis of the complex erythrocyte blood group systems. Since enucleation is accompanied by loss of a significant proportion of membrane material, mechanisms are required for the sorting or partitioning of specific proteins between the plasma membrane surrounding the extruded

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nucleus and that defining the incipient reticulocyte. This article will discuss the current knowledge of erythroblast enucleation in the context of retention and loss of erythrocyte membrane proteins that display antigenic blood group sites and exist within multiprotein complexes that maintain the structural integrity of the red blood cell.

The specialised red blood cell membrane and multicomponent protein complexes

The plasma membrane of the mature erythrocyte is comprised of an asymmetric lipid membrane that is attached to an underlying spectrin-actin based cytoskeletal meshwork via the interaction of cytoskeletal adaptor proteins (ankyrin, protein 4.1 and adducin) with integral membrane proteins. Many of these membrane proteins comprise major blood group proteins and known to be localised in multicomponent membrane complexes in the red blood cell membrane [1, 2]. These include proteins of the ankyrin/band 3 tetramer based multiprotein complex such as band 3 (Diego), glycophorin A/glycophorin B (MNS), RhD/CE (RH), RhAG (RHAG) and ICAM4 (LW) and proteins within the protein 4.1/adducin anchored junctional complex including glycophorin C (Gerbich), Kell (Kell), XK (Kx) and band 3 (Diego)[3-5].

Genetic mutations that impact on cytoskeletal proteins, adaptor proteins or integral membrane proteins can lead to haemolytic disorders such as Hereditary Spherocytosis and Hereditary Elliptocytosis [6]. Detailed characterisation of these naturally occurring human genetic mutations carried out over many years has formed the basis of our current understanding of the structure of the red cell membrane [7]. For example, studies of Rh null individuals (both amorph and regulator type) provided the principal evidence that identified the components of the Rh sub-complex [8, 9], whilst the study of erythrocytes from a band 3 null patient (homozygous band 3 Coimbra)[1] demonstrated for the first time that band 3 comprises the core of a large ankyrin associated macrocomplex that includes protein 4.2 and the Rh sub-complex components. An observation that was later corroborated in a separate band 3 partially deficient individual (homozygous band 3 Courcouronnes)[10]. This model describing the existence of a major membrane complex containing band 3 associated proteins accounts for an earlier observation in which multiple genetically unrelated blood group antigens were found to be depressed on erythrocytes from patients possessing the band 3 Southeast Asian Ovalocytosis N-terminal deletion mutation [11]. More recent studies have shown that a proportion of band 3 associates with the junctional complex via interactions with adducin [2] and potentially protein 4.1 [12] whilst a further population is thought to exist in a mobile state without direct cytoskeletal constraint [13].

In addition to the study of erythrocytes from humans with naturally occurring protein mutations or deficiencies, the study of erythrocytes from artificially generated knockout mouse models has provided a valuable tool for elucidation of the composition of the erythrocyte membrane and its complexes. Whilst there are many similarities between the plasma membrane complexes in humans and mice, it is important to note that a number of significant species specific differences exist [14]. Mice erythrocytes do not have glycophorin B, the glucose transporter Glut1 [15, 16] and possess only a single version of Rh protein, and there are also variations in the assemblies of multiprotein complexes as

illustrated by the reduced expression of Rh in erythrocytes from the protein 4.1 null mouse [17] but not from humans completely deficient in this protein (unpublished data referred to in [18]). Recently published global RNA expression comparison studies of erythropoietic differentiation in mouse and humans are increasingly highlighting the relevance of these species differences not only in erythrocytes but throughout the process of erythropoiesis [19, 20]. These studies serve to further emphasise that caution should be taken when extrapolating data derived from mouse studies to a human setting and also highlight the need for further studies to understand the biogenesis of the erythrocyte membrane during human erythropoiesis.

Membrane protein assembly and sorting during terminal differentiation

In the prelude to enucleation, transcriptional activity of the uncondensed nucleus is high as the differentiating erythroblast rapidly synthesises, processes and delivers the erythrocyte specific components required to assemble the unique erythrocyte membrane architecture. Over the past two decades a number of studies employing a variety of *in vitro* culture models have provided important information regarding the timing of protein expression of key erythroid and blood group proteins during erythropoiesis [21-29]. Cytoskeletal proteins α -spectrin, β -spectrin and the adaptor proteins protein 4.1 and ankyrin are all present in proerythroblasts [23, 27, 30] along with some integral membrane proteins that are also found in erythrocytes such as CD44, CD47 and glycophorin C. Whether these proteins exist within proerythroblast multiprotein complexes and how these may differ from the well characterised complexes in which they reside in the erythrocyte membrane is currently unknown. Several of these proteins, including CD44 [31], CD47 [32] and protein 4.1 [33-35] are expressed as multiple isoforms. Protein 4.1 undergoes a differential splicing driven shift in the prevalence of specific isoforms during erythropoiesis, with the alternative isoforms exhibiting differential affinity for particular membrane proteins e.g. CD44 and GPC [35], that may contribute to the development of the erythrocyte junctional complex. Differentiation specific isoform expression has also previously been reported for CD44 during the early phase of haematopoiesis [36]. The relative expression level of CD47 isoforms at distinct stages of erythropoiesis and how this relates to the multiprotein complex residence of CD47 is unknown and is a source of current investigation within our laboratory.

Following induction of human terminal erythroid differentiation erythrocyte specific proteins such as band 3, along with other major membrane proteins including protein 4.2, Rh, RhAG and Glut1 are rapidly expressed [16, 21, 25, 26]. This wave of new protein expression and delivery to the plasma membrane floods the intracellular compartments of erythroblasts during the early stages of differentiation and is largely absent by the orthochromatic stage with mostly only plasma membrane localisation of membrane proteins such as band 3 and RhAG detectable [22].

This window of transcription, synthesis and delivery to the plasma membrane within the first 72 hours of erythropoiesis [22] is consistent with the loss of many secretory pathway proteins within this timeframe [37] and the continued condensation of the nucleus and polarisation prior to extrusion.

In addition to their synthesis and delivery to the plasma membrane, some erythroid membrane proteins must be correctly assembled into membrane protein complexes and then attached to the cytoskeleton. We have previously shown that two proteins at the centre of the ankyrin associated multiprotein complex, band 3 and protein 4.2, associate with each other at an early stage of erythropoiesis in basophilic erythroblasts within an intracellular compartment prior to delivery to the plasma membrane[22]. Dependency of Rh on RhAG, proteins that together form the core of the Rh sub-complex, was found to establish at a similar stage. However these two proteins could only be co-immunoprecipitated from the cell surface suggesting that the Rh subcomplex may assemble in the plasma membrane (although a weaker intracellular interaction between these two proteins cannot be excluded) [22]. The stage and cellular location at which the subcomplexes come together and associate with the underlying cytoskeleton remains to be established in humans.

Concomitant with the assembly of erythrocyte multiprotein complexes, erythroblast proteins that are not required for erythrocyte function (e.g. integrins) are removed from the cell surface during terminal differentiation. This is achieved by an altered gene expression program [20] in concert with normal cellular protein turnover. It is also possible that membrane protein recycling pathways are altered during erythropoiesis in order to control membrane protein surface stability. Inefficiencies in surface protein removal could account for the presence of some minor blood group active proteins that appear unessential for normal erythrocyte function.

Enucleation; a question of polarity

Once assembled in the orthochromatic erythroblast plasma membrane, the next protein sorting challenge faced by the enucleating cell is effectively one of polarity. How does the cell selectively retain or enrich the cohort of erythroid membrane proteins and cytoskeletal proteins required by the nascent reticulocyte, rather than lose them with the plasma membrane surrounding the nucleus?

Several proteins and processes have been shown to be important for enucleation (reviewed by [38], however a detailed comprehensive mechanism has yet to be elucidated. Nuclear condensation and polarisation to one side of the cell must first occur before extrusion can take place. This occurs progressively throughout erythropoiesis and has been shown to be dependent on histone deacetylation [39], microtubule rearrangements and phosphoinositide 3-kinase activity [40]. Once initiated, enucleation proceeds via the pinching off of a plasma membrane enclosed nucleus in a process mediated by constriction of an actomyosin ring [41] and has been reported to occur within 8 minutes, as measured by direct observation of *in vitro* cultured erythroblasts [42].

We have recently published a study in which we exploit the combination of *in vitro* human erythroblast culture and high speed sensitive mass spectrometry to characterise the identities and relative abundance of proteins present in purified populations of reticulocytes compared to extruded nuclei [43]. Importantly this work confirmed that many erythroid membrane and cytoskeletal proteins partition predominantly or exclusively to the reticulocyte during erythroblast enucleation alongside the expected cytosolic, mitochondrial, endosomal and

lysosomal proteins. In contrast, nuclear proteins, ER proteins, and a contingent of cytosolic and plasma membrane proteins distribute with the extruded nucleus. Although isolated ER remnants remain in the nascent reticulocyte that are further degraded during reticulocyte maturation, enucleation is thus identified as the stage at which the bulk of endoplasmic reticulum is lost during red cell development.

Membrane protein sorting and the role of the cytoskeleton

A variety of model systems have been used to study the mechanism of enucleation, but until recently these have mostly concentrated on studying this process in mouse erythroblasts. In particular, mouse studies have proposed that the spectrin cytoskeleton plays an essential role in regulating membrane protein partitioning in the reticulocyte. Spectrin is restricted to the reticulocyte in mice and humans [44-46] and many important membrane proteins are attached to the cytoskeletal network via associated adaptor proteins and indirectly via membrane protein complexes. Importantly, disruption of cytoskeletal network interactions resulted in mislocalisation of specific membrane proteins to the nucleus in mice [47], suggesting that mislocalisation during enucleation could contribute to the membrane alterations observed in red blood cell membrane disorders such as Hereditary Spherocytosis and Hereditary Elliptocytosis in humans.

To date several membrane and cytoskeletal proteins are known to differentially sort to the reticulocyte in humans and mice. The membrane proteins band 3 [47, 48], GPA [47], GPC [47] and RhAG [47] are reported to be restricted to the reticulocyte in murine cells. Alongside the spectrin cytoskeleton [45, 46], microtubules [40, 46], myosin [40] and actin also were restricted to the reticulocyte in mouse cells. The majority of actin is concentrated at the base of the extruding nucleus where it is important for actomyosin driven enucleation [40, 46]. Some proteins appear to be selectively lost, such as the beta 1 integrin which is lost with the nucleus during both mouse [49] and human [50] enucleation. The vitamin C transporter SVCT2 also localises to the plasma membrane surrounding the extruded nucleus in mouse erythroblasts [51], as does the erythroblast macrophage protein (EMP) [49, 52]. A significant proportion of erythroblast CD147 (Basigin) is also lost with the nucleus during human erythroblast enucleation, possibly due to its association with beta 1 integrin [50]. .

In our study, enrichment to the reticulocyte plasma membrane in the late stages of human enucleation was observed for all major erythrocyte proteins investigated including band 3, GPA, RhAG, Rh, GPC and CD47, similar to the distribution observed in mouse bone marrow erythroblasts for GPA, band 3 and GPC [47]. These proteins are located in the band 3 macrocomplex and the 4.1-GPC junctional complex, which represent important connection points between the membrane and the cytoskeleton. Retention of these proteins within the reticulocyte plasma membrane is likely to be heavily dependent on their successful incorporation into multiprotein membrane protein complexes during terminal differentiation prior to enucleation. Disruption or loss of key membrane and adaptor proteins in disease may render these proteins vulnerable to loss during enucleation and this is work we are currently exploring further.

Surprisingly, several proteins highly expressed in erythrocytes were found to be present in the plasma membrane surrounding the nucleus. By confocal immunofluorescence microscopy band 3 was distributed evenly around the whole plasma membrane throughout the later stages of enucleation and was also detected on the separate extruded nuclei. Similar localisation of band 3 was also observed in human bone marrow samples (AJB and AMT unpublished results). The exact mechanism or mechanisms that determine why some proteins are retained in the reticulocyte whilst others are lost remains incompletely understood. The evidence suggests that partitioning is at least partially regulated by degree of association with the cytoskeleton. However some proteins known not to be constrained by cytoskeletal attachment are retained in the reticulocyte membrane such as the lipid raft protein flotillin, whilst the related protein stomatin partitions equally [43]. Some proteins (e.g. integrins) preferentially sort to the membrane surrounding the nucleus. Furthermore, seemingly significant proportions of proteins with well established cytoskeletal linkage such as band 3 and GPC are lost during enucleation. One explanation that may go some way towards explaining this apparent contradiction is to consider enucleation as representing a stage at which protein synthesised in excess of that required for appropriate membrane/cytoskeletal multiprotein assembly is removed from the cell. However, some proteins may specifically be targeted to the plasma membrane surrounding the nucleus in a currently unknown way. Overall, it is likely that a combination of factors govern loss or retention of membrane proteins during erythroblast enucleation. We anticipate that future work exploiting advances in the ability to manipulate protein expression in erythroblasts will lead to a more thorough understanding, both of the factors that regulate protein partitioning and of the mechanisms that drive the process of enucleation itself.

Summary

To summarise, enucleation is an essential step in the developmental process required for the production of the mature red blood cell. This review has highlighted a significant body of recent work that illustrates the concept of differential protein partitioning between the plasma membrane of the reticulocyte and that surrounding the extruded nucleus respectively. What is evident from these studies is that our current model describing the enucleation process must now be updated to incorporate the observation that sorting of proteins during enucleation is not an all or nothing process but rather an enrichment of proteins in specific membrane compartments according to associations with the cytoskeleton.

Despite recent advances in understanding the process of enucleation, there are numerous interesting aspects that merit further investigation. This includes including determining how reticulocyte retention of specific lipid microdomain localised proteins without reported cytoskeletal association such as flotillin [43] is achieved. This observation suggests that additional mechanisms such as selective retention of membrane lipids including cholesterol within the reticulocyte membrane during enucleation may also play a role in retention of associated membrane proteins. More work is also needed to determine the precise mechanisms of protein sorting during enucleation. Whether this is an entirely passive process that relies on direct or indirect associations with the cytoskeleton or an active process with specific delivery and exchange of membrane proteins between the reticulocyte

and nuclei plasma membrane domains or a combination of the two mechanisms remains to be established. We anticipate that further studies in this area will aid in our understanding of loss of proteins that occur secondary to mutations in core cytoskeletal and membrane proteins in diseases such as Hereditary Spherocytosis and Hereditary Elliptocytosis.

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