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Research paper

Tubulin is retained throughout the human hematopoietic/erythroid cell differentiation process and plays a structural role in sedimentable fraction of mature erythrocytes

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ABSTRACT

We investigated the properties of tubulin present in the sedimentable fraction ("Sed-tub") of human erythrocytes, and tracked the location and organization of tubulin in various types of cells during the process of hematopoietic/erythroid differentiation. Sed-tub was sensitive to taxol/nocodazole (drugs that modify microtubule assembly/disassembly), but was organized as part of a protein network rather than in typical microtubule form. This network had a non-uniform "connected-ring" structure, with tubulin localized in the connection areas and associated with other proteins. When tubulin was eliminated from Sed-tub fraction, this connected-ring structure disappeared. Spectrin, a major protein component in Sed-tub fraction, formed a complex with tubulin. During hematopoietic differentiation, tubulin shifts from typical microtubule structure (in pro-erythroblasts) to a disorganized structure (in later stages), and is retained in reticulocytes following enucleation. Thus, tubulin is not completely lost when erythrocytes mature; it continues to play a structural role in the Sed-tub fraction.

1. Introduction

Erythrocytes have been a major focus of biochemical studies during the 20th and 21 st centuries because they are easily obtained, are the simplest of human cells, lack internal organelles, and play an important physiological role (Goodman et al., 2013). Morphological, metabolic, and functional changes in human erythrocytes are associated with altered interactions between the lipid bilayer and cytoskeleton (Ferru et al., 2011; Lewis et al., 2009; Liu et al., 2010; Manno et al., 2010; Rodriguez-Garcia et al., 2015). Our previous studies have shown that rheological changes are associated with altered content and distribution of tubulin in both the membrane fraction (Mem-tub) and sedimentable fraction (Sed-tub) of human erythrocytes (Amaiden et al., 2012; Amaiden et al., 2015; Monesterolo et al., 2012; Nigra et al., 2016). We have also demonstrated that erythrocyte tubulin is able to form complexes with P-ATPases such Na^+,K^+-ATPase (NKA) and plasma membrane Ca^{++}-ATPase (PMCA) and thereby regulate enzyme activities, and that such interaction is related to hemorheological properties of erythrocytes (Amaiden et al., 2012; Monesterolo et al., 2015). However, the composition and structure of tubulin in the Sed-tub fraction remain unknown.

Erythrocyte "ghosts" lose the biconcave shape characteristic of intact erythrocytes, although they retain the membrane and membranous cytoskeleton. It has therefore been suggested that maintenance of erythrocyte shape involves interaction of certain intracellular components with the plasma membrane (Terasawa et al., 2006). In the present study, we analyzed the structure, composition, and hematopoietic origin of tubulin in the Sed-tub fraction we described previously (Amaiden et al., 2011).

Abbreviations: EPO, erythropoietin; IL3, interleukin; NKA, Na^+,K^+-ATPase; PMCA, plasma membrane Ca^{++}-ATPase; SCF, stem cell factor; Sed-tub, tubulin in sedimentable fraction of erythrocytes; TEM, transmission electron microscopy

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2. Materials and methods

2.1. Materials

Anti-mouse IgG conjugated with peroxidase, anti-α-tubulin mouse mAb DM1-A, fluorescein-conjugated anti-mouse IgG, bovine serum albumin (BSA), Sepharose 4B, nitrocellulose membrane, nocodazole, paclitaxel (taxol), and Triton X-100 were from Sigma-Aldrich. Lumigen PS-3 detection kit and high-performance chemiluminescence film were from GE Healthcare. Human progenitor cell enrichment kit (Easy Sep), Stem Spam medium, LTCM medium, interleukin L-3 (IL-3), stem cell factor (SCF), fetal bovine serum (FBS), erythropoietin (EPO), and LR White resin.

2.2. Human subjects and erythrocyte preparation

Male and female patients were recruited for this study from the Hospital Regional de Río Cuarto, Córdoba, Argentina. The informed consent protocol was approved by the hospital's Human Studies Committee. Fresh blood samples were collected from healthy volunteer subjects (age 25–40 years) in Vacutainer tubes (Becton-Dickinson; Plymouth, UK), with EDTA (1 mg/mL) as anticoagulant. Erythrocytes were isolated from these samples by conventional centrifugal separation and used immediately.

2.3. Hematopoietic cells and cell culture

Human umbilical cord blood samples were obtained from patients at the National Medical Center, Mexican Social Security Institute, Mexico City, in compliance with guidelines of the Ethics Committee. Hematopoietic progenitor cells were purified as described previously (Flores-Guzman et al., 2006). In brief, cord blood was fractionated on Ficoll density gradients (Lymphoprep; Stem Cell Technologies) to obtain mononuclear cells. A cell fraction enriched in CD34+ lineage-negative cells was obtained using a human progenitor cell enrichment kit (Easy Sep; Stem Cell Technologies) and maintained in Stem Span medium. Cells were cultured for 7 days (37 °C, 5% CO_2 atmosphere) in MyeloCult medium (H5100; Stem Cell Technologies) with 20% FBS, 50 ng/mL IL-3, 50 ng/mL SCF, and 2 IU/mL EPO. All cells were recovered at the end of 7 days, reseeded in Stem Span supplemented with 20% FBS and 2 IU/mL EPO, at a density of 30,000 cells/mL, and culture was continued for 12 days (Ubukawa et al., 2012). To promote the enucleation process of erythroid cells, cells were transferred to a poly-D-lysine Lab-Tek chamber (Nunc; Denmark) and cultured for 24 h.

2.4. Evaluation of the erythroid maturation process

Maturation of erythroid cells in culture was evaluated both by morphology and by immunophenotype. For morphology analysis, cells were spun onto slides using a Cytospin and were stained with Wright-Giemsa. On the indicated days, 500 cells per slide were scored, based on standard morphology criteria. For Flow Cytometry analysis, the following antibodies were used: antiCD34-PECy7, antiCD38-FITC, antiCD45RA-APC, antiCD71-PE, antiCD33-APC, and antiCD235a-PE. All the antibodies were purchased from BD Bioscience, except for antiCD235a, which was purchased from R & D Systems Inc. All of them were selected based on the antibodies approved by EuroFlow Guide for Flow Cytometry. Progenitor and precursor cells were identified based on the expression (or lack of expression) of CD34 and CD38. CD71 and CD235a were used as early and late erythroid markers, respectively, whereas CD45RA and CD33 were used as early and late myeloid markers, respectively. Erythroid progenitors were defined as CD34+ CD38+ CD71+ CD45RA- cells, whereas erythroid precursors were defined as CD34- CD38- CD235a+ CD33-. Cell immunophenotype was analyzed using the FlowJo X v10 Software (FlowJo® LLC).

2.5. Isolation of sed-tub fraction

Erythrocytes isolated from 2 mL human blood were resuspended in 3 mL lysis buffer (7.5 mM sodium phosphate buffer, pH 7.5, containing 0.01 mM taxol and 0.5% Triton X-100) and incubated for 3 min at RT. Lysate was centrifuged (20,000 x *g*) for 20 min at 30 °C. Supernatant was immediately centrifuged at 100,000 x g for 30 min at 37 °C. The pellet (Sed-tub fraction) was resuspended in 200 μL TBS buffer and stored at −20 °C until use (Amaiden et al., 2011).

2.6. Rat brain tubulin preparation

Tubulin was obtained as described previously (Casale et al., 2001). In brief, brains from 30- to 60-day-old Wistar rats were homogenized at 4 °C in 1 vol MEM buffer (0.1 M Mes, pH 6.7, containing 1 mM EGTA and 1 mM $MgCl_2$). The homogenate was centrifuged at 100,000 x *g* for 45 min, and the pellet was discarded. Tubulin was purified by two cycles of assembly/disassembly followed by phosphocellulose chromatography.

2.7. Preparation of antibody linked to sepharose

Anti-α and −β spectrin mAbs and purified tubulin were covalently bound to cyanogen bromide-activated Sepharose 4 B beads as described previously (Hubbert et al., 2002), with slight modification. Beads were washed with 100 vol 0.001 M HCl at 21 °C. Packed beads (1 mL) were mixed with antibody (2.5 mg protein) in 1 mL coupling buffer (0.5 M NaCl containing 0.2 M $NaHCO_3$, pH 8.2). The mixture was agitated on a rocking platform overnight at 4 °C and loaded into a small chromatographic column. Unbound antibodies or protein were removed by washing with 5 mL coupling buffer. Antibody-coated beads were transferred to a beaker and suspended in 1 mL coupling buffer containing 0.2 M glycine to block unreacted sepharose sites. The mixture was agitated overnight at 4 °C, and unbound glycine was removed by washing the beads with 10 mL coupling buffer. Resulting antibody-coated beads were washed with 1.5 mL 0.01 mM Tris-HCl, pH 8, containing 0.14 M NaCl and 0.025% NaN_3, and stored at 4 °C until use (2 days or less).

2.8. Immunoprecipitation

Erythrocyte homogenate was mixed with packed antibody-sepharose beads and incubated overnight at 4 °C with gentle agitation. Samples were centrifuged, and precipitated material was washed five times with NaCl/Tris-Triton. Fractions (50 μL) of packed beads were resuspended in 50 μL Laemmli sample buffer, heated at 100 °C for 6 min, and centrifuged. Aliquots (20 μL) were subjected to SDS-PAGE.

2.9. Electrophoresis and immunoblotting

Proteins were separated by SDS-PAGE on 10% polyacrylamide slab gels (Laemmli, 1970), transferred to nitrocellulose sheet, and reacted with mouse mAb DM1-A (dilution 1:1000) to determine α-tubulin content, or with mouse anti-spectrin mAb (dilution 1:1000) to determine α- and β-spectrin content. The nitrocellulose sheet was reacted with peroxidase-conjugated anti-mouse IgG. Intensities of tubulin bands were quantified by the Scion imaging software program.

2.10. Immunofluorescence

Erythrocytes or progenitor cells were fixed with methanol at −20 °C. Samples were rehydrated, blocked with 2% BSA, and stained by indirect immunofluorescence using mAb DM1-A (dilution 1:1000) in NaCl containing 2% BSA. Fluorescein-conjugated anti-mouse IgG (dilution 1:100) was used as secondary antibody. Coverslips were mounted on Fluor-Save, and epifluorescence was detected by confocal

microscopy (Amaiden et al., 2012).

2.11. Transmission electron microscopy (TEM)

Erythrocyte Sed-tub fraction obtained from 10 mL blood was fixed with 2% glutaraldehyde/4% formaldehyde. Samples were dehydrated by ethanol concentration gradient (50%, 70%, 90%) and embedded in LR White resin at 4 °C (Skepper and Powell, 2008). Capsules were incubated at 50 °C for 48 h. Sections were blocked with 5% BSA, washed with Milli-Q water for 2 min, incubated with primary mAb DM1A (1:200 in PBS/1% BSA), washed with PBS, incubated with secondary anti-mouse IgG conjugated with 5-nm colloidal gold (1:30 in PBS/1% BSA) for 45 min at 37 °C, washed, stained with aqueous uranyl acetate for 1 min, and observed by TEM.

2.12. Two-dimensional (2-D) electrophoresis

Samples (50 µL) of Sed-tub fractions from erythrocytes (50 µg) were concentrated using 2D Clean-Up kit (GE Healthcare), and resuspended in 125 µL rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG 3–11 NL buffer, 0.002% bromophenol blue) with 17 mM DTT. For the first dimension of 2-D electrophoresis, 50 µg protein was loaded to isoelectric focusing strips (DryStrip 4–7, 7 cm; GE Healthcare) by passive rehydration overnight at RT. Samples were equilibrated with balancing buffer (6 M urea, 75 mM Tris pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue) with DTT (10 mg/mL) for 15 min at RT with stirring, and then with iodoacetamide (25 mg/mL) for 15 min at RT with stirring. For the second dimension, SDS-PAGE (12% gel; 10 × 10 × 0.1 cm) was performed using a low-MW marker from a Molecular Weight Calibration Kit (Amersham), and the gel was stained with Coomassie Blue. Proteins in spots were analyzed by matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF-MS).

2.13. Protein concentration

Protein concentration was determined by the method of Bradford (1976).

2.14. Statistical analysis

Results were expressed as mean ± SD. Student's *t*-test was used for comparison of two populations. Differences between means with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of taxol and nocodazole on tubulin in sed-tub fraction of erythrocytes

We recently described the composition of various tubulin isotypes in the Sed-tub fraction of erythrocytes (Nigra et al., 2016), but the structure of tubulin in this fraction (hereafter also referred to as "Sed-tub" for convenience) remained unclear. It seemed unlikely to be structured as microtubules, since previous reports suggested that microtubules are not present in erythrocytes. On the other hand, Sed-tub was sensitive to the drugs taxol and nocodazole, which modify the assembly/disassembly state of microtubules. In the present study, we incubated human erythrocytes with taxol or nocodazole, isolated Sed-tub, and determined tubulin content by western blot (Fig. 1A). Incubation with 5 µM nocodazole caused a > 50% decrease of Sed-tub content. In contrast, incubation with 10 µM taxol caused a > 2-fold increase of Sed-tub content. Due to tubulin of Sed-tub fraction is part of a macrostructure that sediment to 100.000 x g (Amaiden et al., 2011) and taxol and nocodazole are able to change the content of tubulin in Sed-tub fraction (Fig. 1A), then, this drugs would affect structure of this fraction. To corroborate this hypothesis structure of Sed-tub fraction was analyzed by immunofluorescence. To do that, Sed-tub was isolated from cells pretreated or not with nocodazole or taxol, embedded in LR White resin, and total tubulin was labeled with specific fluorescent antibodies. A visual analysis of the images (Fig. 1B) shows that tubulin in Sed-tub fraction is observed in circles of intense fluorescence. Number of these circles is higher when cells were pre-treated with taxol respect to erythrocytes pre-treated with nocodazole (~6 times). No microtubules were observed under these conditions. Results suggest that there is a correlation between content of tubulin (Fig. 1A) and structure of Seb-tub fraction (Fig. 1B), in fact, when content of tubulin increases in the fraction a high number of fluorescent circles are seen, and when content of tubulin decrease number of circles are low. These findings indicate that although Sed-tub did not form typical microtubule structure through polymerization, Sed-tub content in macrostructure was reduced by nocodazole and increased by taxol.

To determine the structure of Sed-tub, we examined it by TEM (Fig. 2). Within pellets from taxol-treated erythrocytes, two distinct zones were observed. One zone contained gold particles, indicating the presence of tubulin (Fig. 2A). This zone was characterized by a repetitive structure of connected rings, with gold particles aggregated in the connection areas. The second zone did not contain gold particles, indicating the absence of tubulin (Fig. 2A).

Connected-ring structure as above was also absent, suggesting that tubulin plays a role in such structure. Within pellets from nocodazole-treated erythrocytes, no gold particles and no repetitive connected-ring structure were observed (Fig. 2B). Typical microtubules were not observed in either taxol- or nocodazole-treated cells. Tubulin present in Sed-tub fraction evidently plays an important role in structural organization of this fraction, since absence of tubulin results in loss of organization. However, the low proportion of tubulin in the fraction suggests that it interacts with other proteins.

Taking together, the results demonstrated that in Sed-tub fraction tubulin is part of a macrostructure that takes the form of connected rings. This structure respond like microtubules to assembly and disassembly drugs (taxol and nocodazol) and tubulin is essential to maintain its integrity, due to when tubulin is displaced of Sed-tub fraction the connected rings structure becomes less organized.

3.2. Tubulin and Spectrin are partners of a complex in the sed-tub fraction of erythrocytes

For partial proteomic analysis of Sed-tub fraction from human erythrocytes, the fraction was isolated and subjected to 2-D electrophoresis. Proteins of various molecular weights and isoelectric points were detected in the 2-D gel (Fig. 3A). Proteins present in spots with MW (55 kDa) or isoelectric point (4.75) similar to that of tubulin were analyzed by MALDI-TOF-MS to determine presence of tubulin. Tubulin was not observed by this method, but since different isoforms of tubulin was detected by immunological techniques (Amiaden et al., 2011 and the present work), this result suggest that content of tubulin in Sed-tub is very low.

On another hand, to determine possible interaction between tubulin and some proteins presents in Sed-tub fraction, the most abundant proteins having MW in the 30–100 kDa range was analyzed by MALDI-TOF-MS. MS results revealed the presence in Sed-tub fraction of actin, spectrin, Band-3 cytoplasmic domain, and albumin (Fig. 3B). All of the above proteins have been reported in proteomic analysis of the membranous cytoskeleton of erythrocytes (Chan et al., 2013; Lux, 2016; Machnicka et al., 2012; Salomao et al., 2008), suggesting that the macrostructure of Sed-tub should be similar. If tubulin is part of the macrostructure, it presumably interacts with some of the proteins detected by MS, and forms complexes with them. To evaluate possible physical interaction of tubulin with proteins present in Sed-tub fraction, we performed immunoprecipitation experiments on tubulin and spectrin (a predominant protein in the membranous cytoskeleton), using

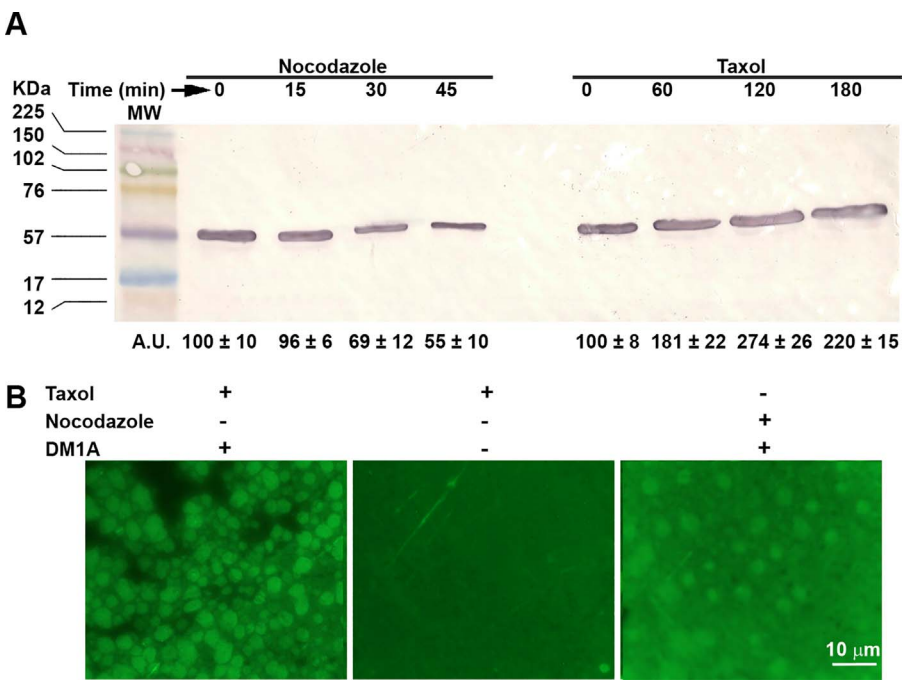


Fig. 1. Effects of taxol and nocodazole on tubulin content in Sed-tub fraction of human erythrocytes. **(A)** Stability of Sed-tub fraction. 6 mL human blood was incubated for the indicated times with 5 μM nocodazole or 10 μM taxol. Sed-tub fraction was then extracted as described in M & M. α-tubulin in each sample was quantified by immunoblotting with mAb DM1A. Bands shown are from a representative experiment from three or more independent replicates. Tubulin bands were quantified using Scion Image software program, and values expressed as arbitrary units (mean ± S.D.). **(B)** Fluorescence microscopy of Sed-tub fraction. Sed-tub fraction was isolated from blood from healthy subjects, pretreated for 1 h with 5 μM nocodazole or 10 μM taxol. Aliquots were fixed and embedded in LR White resin matrix, cut out, and analyzed by indirect immunofluorescence microscopy for detection of α-tubulin (green: mAb DM1A and Fit-c). A control was treated with taxol and incubated with secondary antibody only.

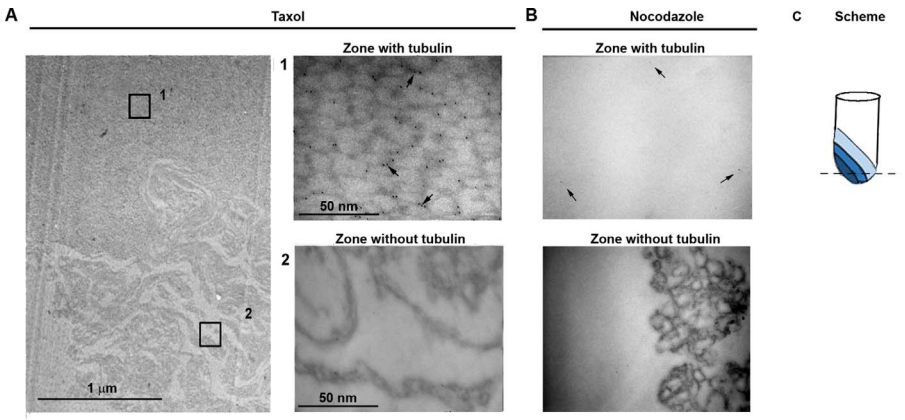


Fig. 2. TEM analysis of Sed-tub fraction. Erythrocytes were pretreated for 1 h with 10 μM taxol **(A)** or 5 μM nocodazole **(B)**. Sed-tub fraction was then isolated, fixed and embedded in LR White resin matrix, cut out, and analyzed by TEM (magnification ×4600) using mAb DM1A and IgG antibody conjugated to 5-nm colloidal gold as primary and secondary antibody, respectively. **1 and 2**: ×36,000 magnification of areas bounded by squares. Arrows: tubulin-positive. **(C)** Scheme for obtaining pellet through isolation of Sed-tub with different zones.

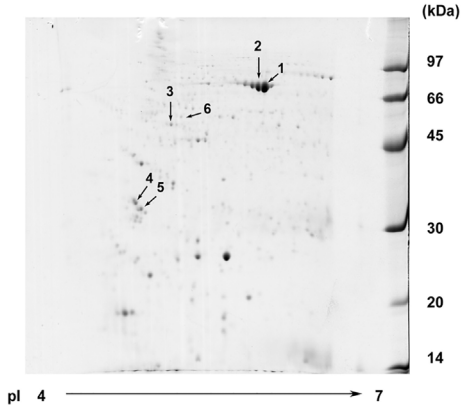
either tubulin or anti-spectrin immunoglobulin bound to sepharose beads. When erythrocyte homogenate was incubated with tubulin-sepharose beads and centrifuged, a 230-kDa polypeptide corresponding to spectrin was found in the immunoprecipitate (Fig. 4A). When the homogenate was incubated with anti-spectrin-sepharose beads, α-tubulin was observed in the immunoprecipitate (Fig. 4B). These findings indicate the presence of a protein complex containing tubulin and spectrin in human erythrocytes.

Results shows in this section demonstrated that Sed-tub fraction contains many proteins of membrane skeleton of the erythrocyte. Moreover, physical interactions between tubulin and at least one of this proteins, spectrin, was observed.

3.3. Chronological tracking of tubulin in precursors of human erythrocytes

The results presented in Figs. 1–4 indicate that tubulin is a component of a sedimentable nontubular macrostructure in erythrocytes. Many previous studies have demonstrated the presence in hematopoietic precursors of erythrocytes of tubulin organized into microtubules. However, the localization of tubulin following loss of the cell nucleus (enucleation process) is not well understood (Kalfa and Zheng, 2014; Kobayashi et al., 2016; McGrath et al., 2008; Migliaccio, 2010; Palis, 2012; Ubukawa et al., 2012). We therefore investigated the presence and distribution of tubulin in cells at various stages of erythroid differentiation, including reticulocytes, which originate following enucleation and subsequently mature into erythrocytes in peripheral blood circulation. Hematopoietic progenitor stem cells (CD34+, lineage-) from umbilical cord blood were isolated, and hematopoietic differentiation was induced *in vitro*. To induce differentiation into erythroid lineage, cells were cultured in medium supplemented with EPO and SCF. Levels of differentiation in various cell types were assessed periodically by immunophenotype analyzed by flow cytometry and Wright staining and morphological observation (Fig. 5). Various typical cell types were observed during hematopoietic maturation, from hematopoietic progenitor cells to mature reticulocytes. The effectiveness of the differentiation procedure used was demonstrated by the variability of cell types during the culture period (Fig. 5). As shown in Fig. 5A, at culture onset, erythroid progenitors (CD34+ CD38+ CD71+ CD45RA- cells) corresponded to around 8% of the total progenitor population (CD34+ CD38+ cells). On the other hand, erythroid precursors (CD34- CD38- CD235a+ CD33- cells) corresponded to around 21% of the total precursor/mature cells. After 13 days of culture under our culture conditions, erythroid progenitors corresponded to more than 50% of the CD34+ CD38+ cell population, whereas almost 100% of the total cells in culture corresponded to erythroid precursors (Fig. 5A, lower panels). These results indicate that

A



B

Spot No,	pI	MW (kDa) In the 2D gel	Score	Results	MW (kDa) theoretical
1	6,1	67	100	albumin-like	52
			97	PRO2619	56
			96	Chain A, Crystal Structure Of The Ga Module Complexed With Human Serum Albumin	55
2	6.0	73	88	albumin-like	52
			84	unnamed protein product	59
			83	albumin, isoform CRA_b	59
3	4.7	54	99	alpha-I spectrin	61
			70	spectrin, alpha, erythrocytic 1 (elliptocytosis 2), isoform CRA_a	177
			60	spectrin, alpha, erythrocytic 1 (elliptocytosis 2), isoform CRA_c	178
4	4.4	35	101	unnamed protein product	38
			99	ACTB- actin cytoplasmic 1	40
			97	actin, beta	40
5	4.5	32	70	actin, alpha, cardiac muscle, isoform CRA_c	30
			61	actin, alpha cardiac muscle 1	42
			61	unnamed protein product	42
6	4.8	51	84	Chain P, Crystal Structure Of The Cytoplasmic Domain Of Human Erythrocyte Band-3 Protein	42
			77	band 3 anion transport protein	101
			77	unnamed protein product	101

Fig. 3. Partial proteomic analysis of proteins present in Sed-tub fraction. **(A)** 2-D gel electrophoresis of proteins, with Coomassie Blue staining. **(B)** MALDI-TOF-MS sequencing of labeled proteins in spots.

the culture system used in this study preferentially favoured the growth and maturation of cells of the erythroid lineage. These observations were confirmed by the morphological analysis (Fig. 5B). Indeed, our smears observations indicated that, at culture onset, 90% of the cells showed a lymphoblastic phenotype, whereas erythroid precursors corresponded to 4% of the cells. By day 12, around 65% of the total cells in culture corresponded to nucleated erythroid precursors and 15% corresponded to enucleated erythroid cells. By day 13, 85% of the cells corresponded to enucleated erythroid cells. It is noteworthy, however, that although the patterns were similar, there were some differences between the morphology of the cells and the immunophenotype, since the percentages were not identical (i.e., erythroid precursors corresponded to > 98% of total cells, by flow cytometry, vs around 90% of total cells, by morphology). This is a condition repeatedly found when comparing the morphology and immunophenotype of cultured cells (unpublished observations). Under this culture conditions content of hemoglobin (red; autofluorescence), α-tubulin (green; DM1A and Fit-c), DNA (blue; DAPI), and superposition of the three colors, were analyzed and results are shown in Fig. 6. On day 1, fluorescence corresponding to tubulin was observed as a thin peripheral ring (because the cells were small, with very low activity, and little cytoplasm), and hemoglobin was absent. On day 7, the predominant cells were pro-erythroblasts (which have active metabolism), and tubulin fluorescence was visualized accordingly as a well-defined microtubule network. As differentiation continued, cell size and nucleus size decreased and microtubules were restructured. By day 13, late orthochromatic erythroblasts were abundant, a diffuse network of microtubules was present, tubulin was localized mainly around the nucleus, and hemoglobin synthesis was significantly increased (Fig. 6; compare pro-erythroblast to orthochromatic erythroblast). Significant numbers of reticulocytes and pyrenocytes were also present by day 13, and intense fluorescence corresponding to tubulin was observed in reticulocytes (Fig. 6). In contrast to a previous report (Liu et al., 2010), we found that following enucleation most of the tubulin, like hemoglobin, was in reticulocytes, and localized uniformly throughout the cell enucleation. Tubulin content was very low in pyrenocytes (excluded nucleus wrapped by plasma membrane).

To investigate structural organization of tubulin during the enucleation process and after loss of the nucleus, we obtained images (Z-stack series) by confocal microscopy (Fig. 7; Videos 1 and 2 in Suppl. Material). Tubulin was present in each plane of reticulocytes, indicating that cytosolic intracellular tubulin is maintained in these cells. Tubulin adopted different structures during the enucleation process. Microtubules were present in early stages of differentiation, *e.g.*, in pro-erythroblasts (compare Fig. 7A to Fig. 6). Tubulin was subsequently reorganized into some yet-unknown structure, and was still present in reticulocytes and erythrocytes. Distribution of tubulin differed between reticulocytes and erythrocytes. The typical submembranous ring seen in erythrocytes was not observed in reticulocytes. This ring structure is

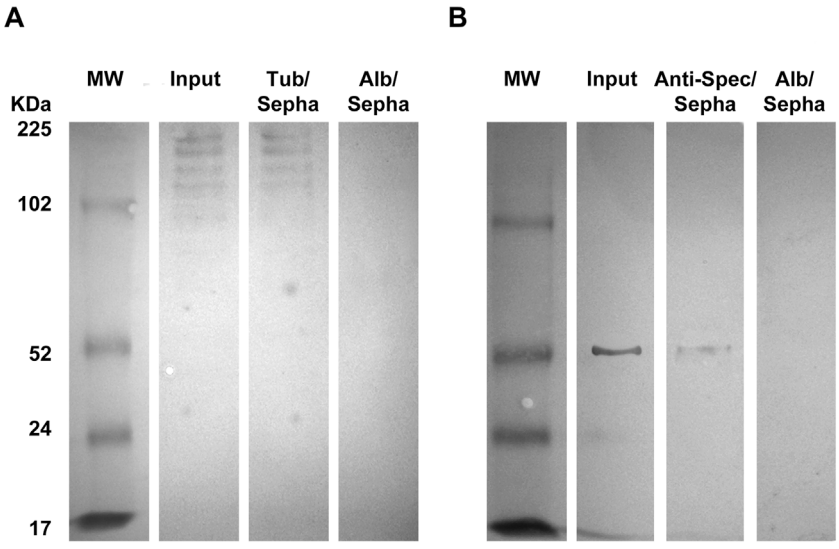


Fig. 4. Physical interaction between tubulin and spectrin. Erythrocytes from 2 mL blood were lysed, and an aliquot from 1 mL lysate was mixed with 0.3 mL packed tubulin-sepharose beads **(A)**, or anti-spectrin-sepharose beads **(B)**, and incubated at 20 °C for 30 min. Samples were centrifuged, and precipitated material was washed. A fraction of packed beads was resuspended in Laemmli buffer, incubated at 100 °C for 6 min, and centrifuged. Aliquots of soluble fractions were subjected to SDS-PAGE and then to simultaneous immunoblot staining with anti-spectrin **(A)** or mAb DM1A **(B)**. A control to eliminate nonspecific interaction was run in parallel using albumin-sepharose. Volumes of analyzed samples were calculated to represent equivalent amounts of total proteins.

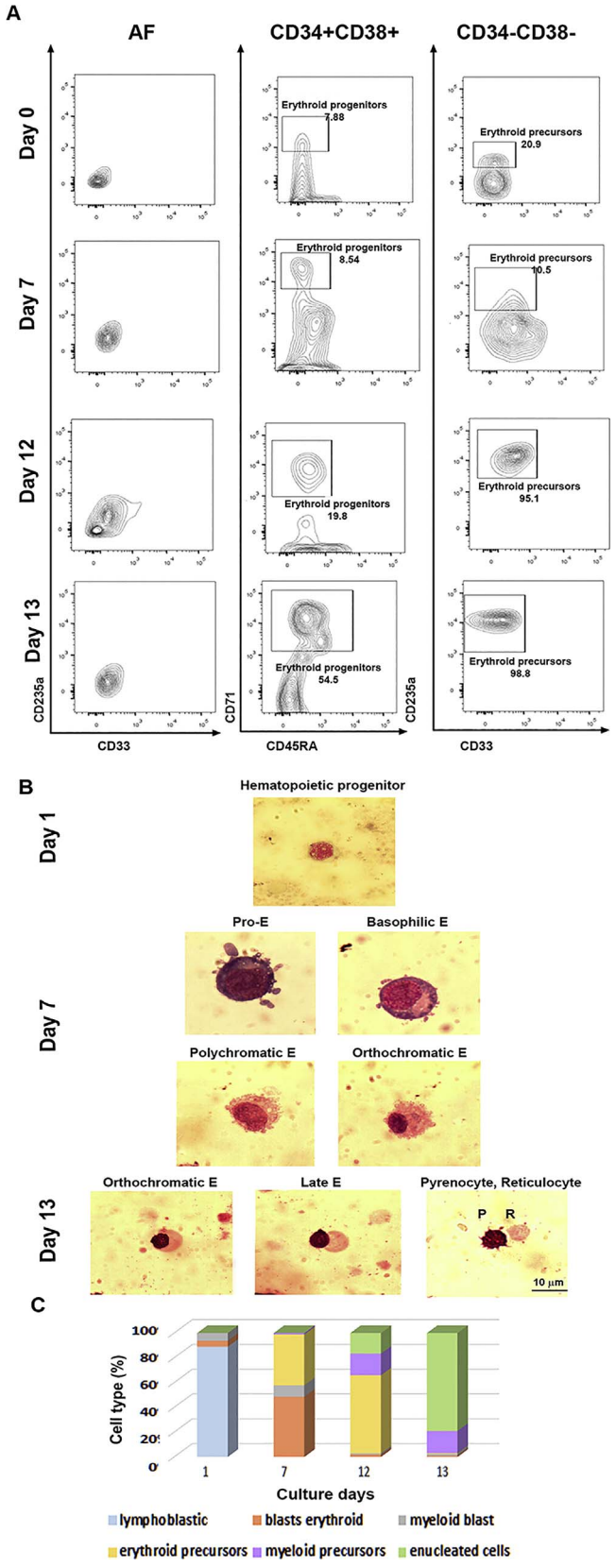


Fig. 5. Induction of hematopoietic progenitor cell differentiation. Hematopoietic progenitor cells (CD34+, lineage-) were isolated from umbilical cord blood and differentiated as described in M & M. **(A)** The different maturation stages were evaluated at days 0, 7, 12 and 13 by flow cytometry, based on the immunophenotipic criteria previously described by Doulatov and colleagues (Doulatov et al., 2012). Erythroid progenitors were defined as CD34+ CD38+ CD71+ CD45RA- cells, whereas erythroid precursors were defined as CD34- CD38- CD235a+ CD33-. AF, autofluorescence. **(B)** Representative images showing the morphology of each one of the cell stages analyzed were photographed at the indicated times after Wright staining. **(C)** Frequency of the different cell types and stages analyzed on days 1, 7, 12 and 13.

presumably adopted at the time cells enter the bloodstream.

Data clearly shows that tubulin is not only present in erythrocytes, but is also present in each precursor analyzed in this work. Structure of tubulin was different in each stage, from microtubules in more undifferentiated precursors to soluble tubulin in reticulocytes. Moreover, this work shows by the first time that, after enucleation process, tubulin is not lost with the nucleus but the most tubulin remains in the reticulocyte.

4. Discussion

Tubulin content and composition in erythrocytes, which have no nucleus, has been studied by many groups during the past five decades. In one of the earliest reports, van Deurs and Behnke (1973) used TEM to show that microtubules are not present in erythrocytes. More recently, Goodman's group has used proteomic analysis to demonstrate the presence of various tubulin isoforms in human erythrocytes (Goodman et al., 2007, 2013). Our group discovered how to obtain tubulin operationally from three distinct fractions in human erythrocytes: cytosolic fraction (Cyt-tub), membrane fraction (Mem-tub), and a fraction sedimented at 100,000 × *g* (Sed-tub). We also showed that erythrocytes contain multiple tubulin isotypes, including acetylated, detyrosinated, and tyrosinated (Amaiden et al., 2011). There is a dynamic balance of tubulin between the Sed-tub and Mem-tub fractions, which can be modified depending on the needs of the cell (Nigra et al., 2016). Our knowledge of tubulin in the Mem-tub fraction has increased greatly during the past decade, whereas structural and functional properties of tubulin in the Sed-tub fraction (hereafter also referred to as "Sed-tub" for convenience) remain largely unknown. The present study is part of an ongoing effort to better understand these properties. Sed-tub in erythrocytes is an organized structure which forms connected rings in combination with other cytoskeletal proteins, although erythrocytes do not contain typical microtubules. Sed-tub content is dynamic, and the presence of tubulin in this fraction is essential for maintaining integrity of the connected-ring structure, presumably through interaction with other proteins. This concept is supported by several observations: (i) Sed-tub content is altered by taxol and nocodazole (drugs that modify microtubule assembly/disassembly state) (Fig. 1); (ii) the connected-ring structure remains organized in the presence of Sed-tub, but is disrupted when tubulin is removed from this fraction (Fig. 2); (iii) 2-D electrophoresis and MALDI-TOF-MS revealed the presence in Sed-tub fraction of several proteins (spectrin, actin, band-3 domain) typical of the membranous cytoskeleton; (iv) immunoprecipitation experiments showed that tubulin and spectrin form a complex together (Fig. 4).

Studies by our group have revealed dynamism of tubulin distribution among the three fractions in erythrocytes under various experimental conditions, including treatment with taxol/nocodazole (Amaiden et al., 2011, 2012), high glucose concentrations (Amaiden et al., 2015; Nigra et al., 2016; Rivelli et al., 2012), increased levels of certain lipids (diacylglycerol, phosphatidic acid) (Monesterolo et al., 2015), and certain medical conditions (diabetes, hypertension) (Amaiden et al., 2015; Nigra et al., 2016; Rivelli et al., 2012). Such dynamism is related to changes in erythrocyte deformability (Amaiden et al., 2015; Monesterolo et al., 2015; Nigra et al., 2016), illustrating the importance of tubulin balance among the three fractions; the rheological properties of erythrocytes determine their ability to perform their normal physiological functions. Modification of deformability through regulation of Sed-tub content can potentially be used to normalize certain symptoms of hypertension and diabetes. Studies are in

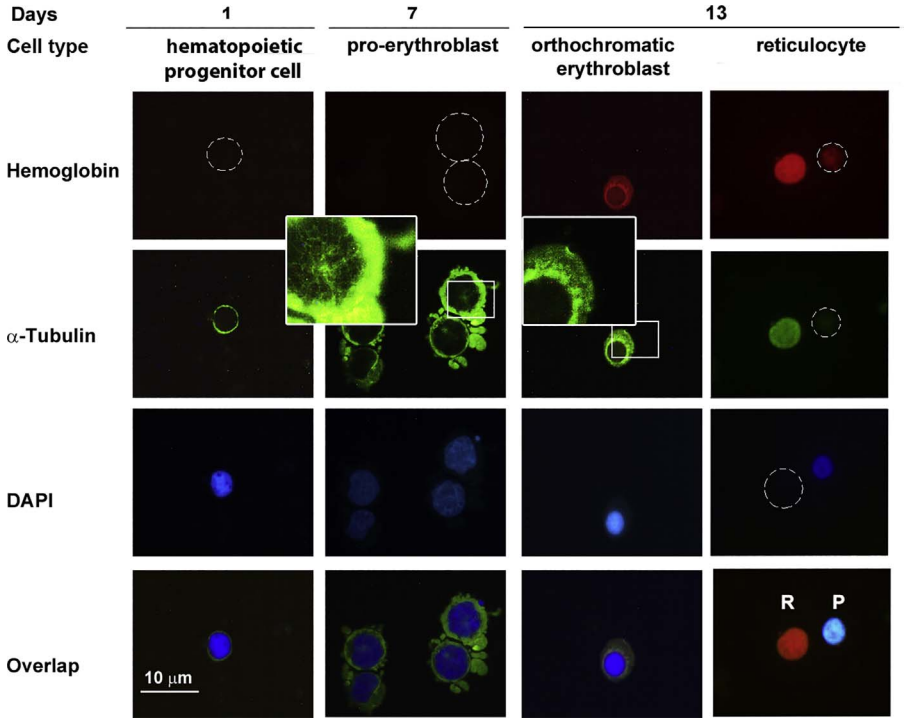


Fig. 6. Tracking of tubulin during erythroid differentiation. Hematopoietic progenitor cells as in Fig. 5 were differentiated, and samples at days 1, 7, and 13 of culture were analyzed by indirect immunofluorescence microscopy to observe hemoglobin (red: autofluorescence), α-tubulin (green: mAb DM1A and Fit-c), DNA (blue: DAPI) and overlap of the three colors. White dashed line: position of cell or nucleus. P: pyrenocyte. R: reticulocyte. Images shown are from a representative experiment from three independent replicates.

progress to elucidate this possibility, with focus on Sed-tub in erythrocytes of diabetic and hypertensive patients.

The essential role of tubulin dynamism among the three erythrocyte fractions may reflect the ability of tubulin to form complexes with various proteins, *e.g.*, NKA and PMCA in the membrane (Amaiden et al., 2011; Casale et al., 2001; Monesterolo et al., 2015), or protein in Sed-tub fraction (present study, Fig. 4). Along the same line, Bennett and Baines (2001) reported that the N-terminal, membrane-binding, cytoplasmic domain of ankyrin is capable of interacting with various membrane proteins (NKA, erythrocyte anion exchanger AE1), tubulin, spectrin, and clathrin. Many important physiological changes are induced by interactions between proteins; *e.g.*, interactions with ATPases affect ion transport through modification of enzyme activity. The physiological importance of tubulin/spectrin complex remains to be clarified. One possibility is that erythrocyte deformability can be altered by modification of this complex, since spectrin is a key component of the erythroid cytoskeleton.

Structure of the Sed-tub fraction is largely unknown. Results of the present study show that actin and band-3 are major protein components of this fraction (Fig. 3), suggesting structural similarity to the membranous cytoskeleton of erythrocytes, which has the same proteins (Lux, 2016). 2-D electrophoresis and MALDI-TOF-MS did not detect tubulin in Sed-tub fraction (Fig. 4), indicating that its level must be very low; however, the level is evidently sufficient to maintain organization of the connected-ring structure described above (Fig. 2). The ability of tubulin to affect rheological and biochemical properties of erythrocytes at low levels suggests that it may function as a molecular signal.

Because erythrocytes have no nucleus, all proteins present in the cells originated prior to the enucleation process. To understand disorders of tubulin content or distribution related to various diseases, it is therefore necessary to track the origin of tubulin in progenitor cells. For this purpose, we developed a protocol to induce *in vitro* differentiation of hematopoietic precursors from umbilical cord blood (Fig. 5). Using this protocol, we discovered that tubulin was present at each stage of erythroid differentiation, including reticulocytes (which originate after enucleation). However, tubulin undergoes polymerization to form typical microtubules only in early stages (Fig. 6: pro-erythroblast); in later stages, it assumes more disorganized structures. There was a clear tendency during the enucleation process for tubulin to move toward the side of the cell opposite the nucleus, whereby tubulin was inserted into

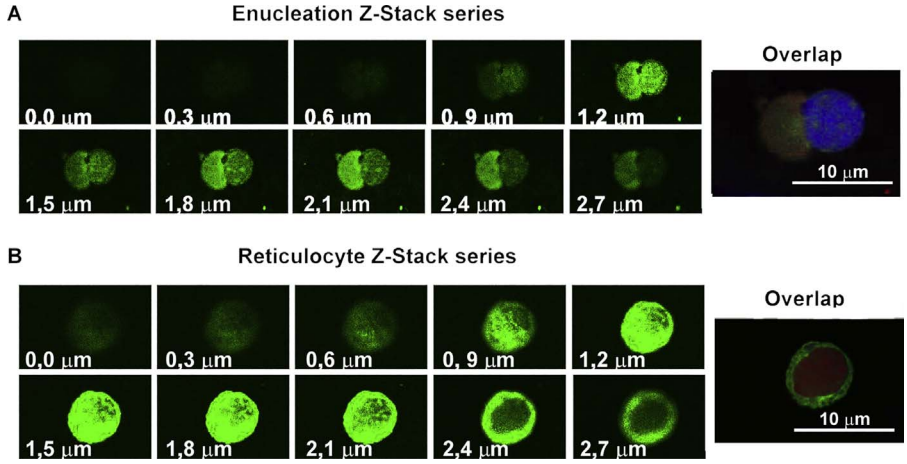


Fig. 7. Tracking of intracellular tubulin during enucleation process. Hematopoietic progenitor cells as in Fig. 5 were differentiated, and samples at day 13 of culture were analyzed by confocal microscopy to observe α-tubulin (green: mAb DM1A and Fit-c), and overlap between α-tubulin, DNA (blue: DAPI) and hemoglobin (red: autofluorescence). **(A)** Images (Z-stack series) of an orthochromatic erythroblast during enucleation process. **(B)** Images of a reticulocyte. Images shown are from a representative experiment from three independent replicates.

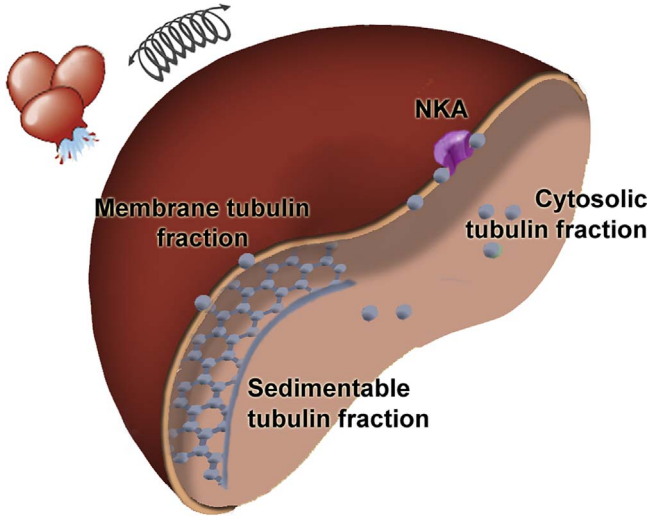


Fig. 8. Proposed scheme of erythrocyte Sed-tub function. Tubulin in human erythrocytes is distributed in three distinct fractions (Cyt-tub, Sed-tub, Mem-tub) as described in the text. Tubulin within these fractions is able to interact with and inhibit the activity of certain enzymes, including Na^+,K^+-ATPase (NKA), thereby increasing osmotic fragility and reducing erythrocyte deformability. The Sed-tub fraction functions as a protein network that assumes a connected-ring structure (see Discussion) and is located underneath the plasma membrane.

the reticulocyte; very little tubulin was observed in the excluded nucleus (Fig. 7). Thus, tubulin during the early genesis of human erythrocytes displays microtubular behavior similar to that in other cells; however, as differentiation continues, microtubule structure is disrupted as the nucleus is lost. In the mature erythrocyte, tubulin is found in three distinct fractions: Mem-tub, Cyt-tub, and Sed-tub. On the basis of results of the present and previous studies, we propose a model shown schematically in Fig. 8, in which the Sed-tub fraction functions as a protein network underlying the membranous cytoskeleton, with the ability to affect rheological and biochemical characteristics of erythrocytes.

5. Conclusions

In erythrocytes, tubulin is a dynamic protein that moves between the Mem-tub and Sed-tub fractions depending on cell state. The Sed-tub fraction displays a non-uniform connected-ring structure, with tubulin localized in the connection areas. Tubulin, though present at low level, is essential for maintaining integrity of the Sed-tub fraction, most likely through interaction with other proteins (*e.g.*, spectrin). During the process of hematopoietic differentiation, tubulin shifts from typical microtubule structure (in pro-erythroblasts) to a disorganized structure (in later stages). Following enucleation, tubulin is retained in reticulocytes. A model is proposed for function of the Sed-tub fraction in erythrocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2017.08.012.

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