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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