TUBULIN AND ITS ASSEMBLY PROMOTING FACTOR(S) DURING MATURATION OF AVIAN ERYTHROCYTES

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1. Introduction

In recent years many studies have implicated the participation of tubulin–microtubule system in cell shape and development [1–4]. The great bulk of this work has relied on the effect of microtubule disaggregating conditions like colchicine and low temperature, which cause readily visible structural changes when cells are exposed to these conditions. In avian erythrocyte system, it has been shown that the shape of immature erythrocytes is altered by low concentration of colchicine and cold, whereas, mature erythrocytes remain unaffected indicating the role of tubulin–microtubule system in cell maturation. Electron microscopic studies have revealed that the microtubules in chick red blood cells (RBC) are reduced in number by 4–6-fold as maturation proceeds [2]. However, it is not known whether microtubule assembly promoting factors are also reduced during this process. Further, it has not been unequivocally established how total tubulin pool changes during different stages of maturation. We have therefore, monitored the contents of tubulin and the factor(s) promoting microtubule assembly, in mature and immature erythrocytes of chicks and compared the polymerizing capability of DEAE-purified RBC tubulin with that of brain tubulin.

2. Materials and methods

[*ring C, methoxy*-^{3}H]Colchicine (spec. act. 5 Ci/mmol) was obtained from New England Nuclear Corp. GTP (grade II S) and colchicine were products of Sigma.

Goat brain tubulin was purified according to [5] using DEAE-cellulose (Whatman DE 52) chromatography instead of DEAE–Sephadex A-50, in PMG buffer (10 mM phosphate (pH 7.0), 10 mM $MgCl_2$ and 0.1 mM GTP). The active fractions as judged from [^{3}H]colchicine binding assay, were pooled and concentrated by overnight dialysis against 8 M glycerol in buffer A (0.1 M MES (pH 6.4), 1 mM EGTA, 0.5 mM $MgCl_2$ and 1 mM GTP). Chick erythrocyte tubulin was prepared by a similar method to [6]. Phosphocellulose chromatography of DEAE-purified tubulin was done according to [7].

To obtain blood rich in immature RBC, white leghorn chicks (10–12 weeks age group) were made anemic by subcutaneous injection of neutralized phenylhydrazine hydrochloride at 10 mg/kg body wt daily for 4–7 consecutive days; blood was collected either from wing veins or by decapitation of chicks using EDTA or citrate as anticoagulant. The number of RBC/ml blood were counted in a Neubauer hemacytometer. Cytoplasmic proteins were released from washed RBC pellets by freeze–thawing in an equal volume of buffer A; the lysate was centrifuged at 100 000 × *g* for 60 min and the supernatant was termed as RBC extract.

Colchicine binding was evaluated by the DE 81 (Whatman) filter disc method [5] as modified [8]. Protein was determined by the method in [9] using bovine serum albumin as standard.

Tubulin polymerization was monitored at 37°C by turbidometry according to [10] at 600 nm since the addition of RBC extracts imparts a red color to the solutions.

DNA was estimated by the method in [11].

3. Results and discussion

3.1. *Colchicine binding activity and RBC maturation*

The peripheral blood of chicks contains mostly the mature RBC, whereas, when anemia is induced in chicks by phenylhydrazine injection, the RBC appearing in the circulation are mostly immature. Blood was drawn from the control (untreated) as well as from the experimental (treated) chicks on different days, RBC counts in blood and the colchicine binding activity of 0–55% $(NH_4)_2SO_4$ fraction of the cytoplasmic proteins of washed RBC were determined since, the crude lysates did not have detectable colchicine binding activity [6,12]. Figure 1 shows that during anemic condition (as evident from a drop in RBC counts), the colchicine binding activity of RBC lysate/mg DNA equivalent RBC increased markedly and became 3–4-times the control value on day 7, when >80% of the RBC in blood were immature. Upon withdrawal of phenylhydrazine injection, the animals reverted to normal condition (when RBC counts increased) and the colchicine binding activity of RBC lysate also decreased to the original value.

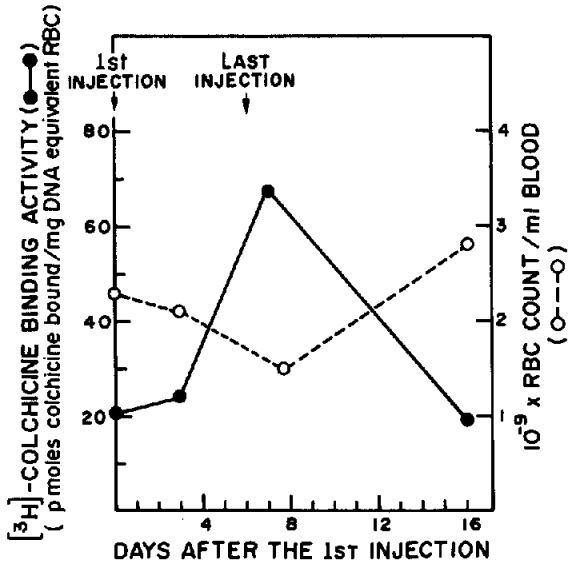

Fig.1. Colchicine binding activity in the cytoplasmic proteins of mature and immature RBC of chicks. Blood was drawn at different days from control and phenylhydrazine-treated chicks. Colchicine binding activity was measured in 0–55% $(NH_4)_2SO_4$ fraction of the RBC cytosol after incubation with [^{3}H]colchicine (1 μM) at 37°C for 1 h.

This experiment suggests that there is ~3–4-fold decrease in the level of tubulin in chick RBC during erythrocyte maturation.

3.2. *Assembly promoting factor(s) in chick RBC*

Competence for self assembly of tubulin from different stages of brain development has been studied extensively [13–15]; it has been shown that tubulin present in crude supernatants of fetal brain at an early stage of development is unable to undergo in vitro polymerization efficiently, due to lack of sufficient amount of a factor responsible for microtubule formation. Unlike in brain, the content of tubulin in avian erythrocytes is very low [6,16] and therefore, it was not possible to study the in vitro microtubule assembly in crude supernatants of chick RBC. However, as it has been reported from several laboratories, that, DEAE-purified brain tubulin possesses a little or no competence for self assembly [17,18], we have checked whether crude RBC extract contains any assembly promoting factor, that could stimulate the polymerization of DEAE-purified brain tubulin. For this purpose, tubulin was isolated and purified by the procedure in [5] using PMG buffer and was concentrated by dialysis against 8 M glycerol in Shelanski buffer condition [19] for polymerization studies. Figure 2 depicts that DEAE-purified brain tubulin could be reassembled into microtubules without the addition of any exogenous factor(s) when present in Shelanski polymerizing buffer*. However, the addition of increasing amounts of mature chick RBC extract to this DEAE-purified brain tubulin augmented the microtubule assembly in vitro. This increase in absorbance due to the addition of added RBC extract was indeed due to true polymerization as evidenced by its sensitivity towards cold (fig.2). The extent of polymerization appears to be linear function of the amount of the RBC extract added (inset) in a manner similar to that found on addition of microtubule associated proteins to tubulin purified by phosphocellulose chromatography [3,7,17],

* No polymerization could be obtained when tubulin was concentrated against 8 M glycerol in PMG buffer. Even reconstitution with 1 mM GTP, lowering Mg^{2+} to 0.5 mM and adjustment of ionic strength equivalent to 0.1 M MES buffer by adding appropriate amount of NaCl, failed to initiate polymerization

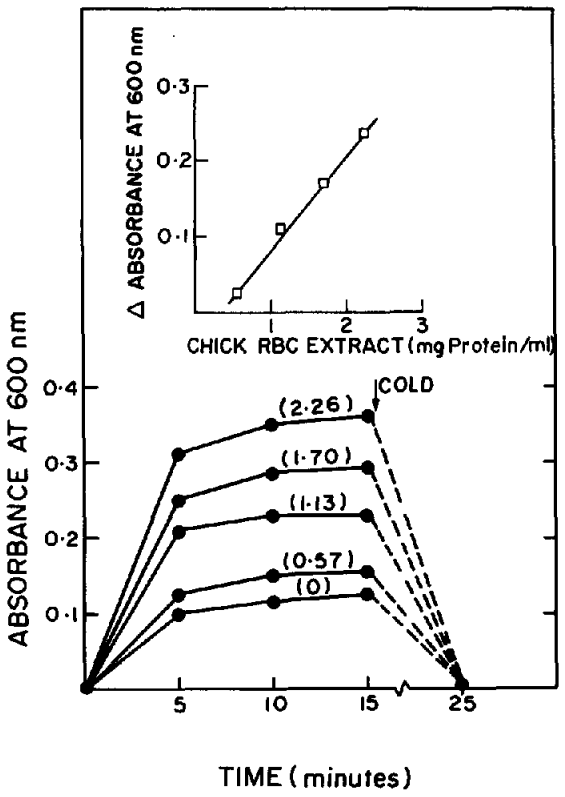

Fig.2. Enhancement of polymerization of DEAE-purified brain tubulin by chick RBC extract. DEAE-purified brain tubulin (2.16 mg/ml) was incubated at 37°C for the indicated period in the presence of varying concentrations of protein of chick RBC extract as shown in the parentheses. The linearity of the added RBC extract with ΔA_{600} for 15 min is shown in the inset.

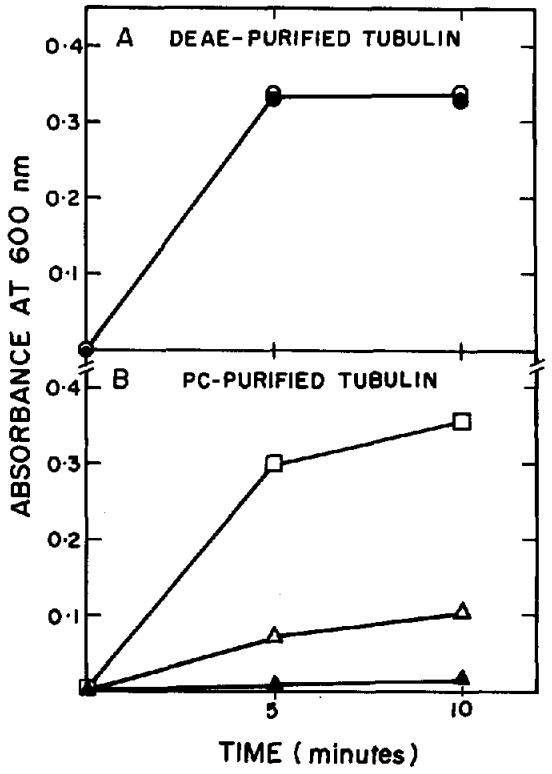

Fig.3. Polymerization of DEAE-purified chick RBC tubulin. (A) DEAE-purified chick RBC tubulin (1.75 mg/ml) was incubated at 37°C in the absence (•) and presence (○) of chick RBC extract containing 2.5 mg/ml protein. (B) Phosphocellulose (PC) purified RBC tubulin (1.12 mg/ml) was incubated at 37°C: (▲) PC tubulin only; (□) PC tubulin + chick RBC extract (2.5 mg/ml); (△) PC tubulin + 100 μl of the dialysed 0.8 M NaCl wash from phosphocellulose column.

indicating that some accessory factor(s) responsible for microtubule formation might be present in chick RBC. The contribution of this added RBC extract towards total tubulin is neglected since no colchicine binding activity was detectable in the added extract. In contrast, tubulin purified from chick RBC extract by an identical procedure was itself capable of polymerization quite efficiently and, to our surprise, no enhancement of polymerization was observed upon addition of the chick RBC extract into it (fig.3A). This is expected only when the DEAE-purified RBC tubulin contains stoichiometric proportion of polymerizing factor. Critical concentration of DEAE-purified brain tubulin has been found to be 1.4 mg/ml which reduced to 0.8 mg/ml when chick RBC extract was added; on the other hand, the value was 0.5 mg/ml for DEAE-purified chick RBC tubulin, with and without added chick RBC extract. However, the DEAE-purified chick RBC tubulin after passage through a phosphocellulose column, lost its ability to polymerize. As shown in fig.3B, the ability could be partially restored upon addition of an aliquot of dialysed high-salt wash from the phosphocellulose column to the phosphocellulose-purified tubulin, indicating that the DEAE-purified preparation indeed contained trace amount of polymerizing factor(s), which was not detectable even in overloaded gel (not shown).

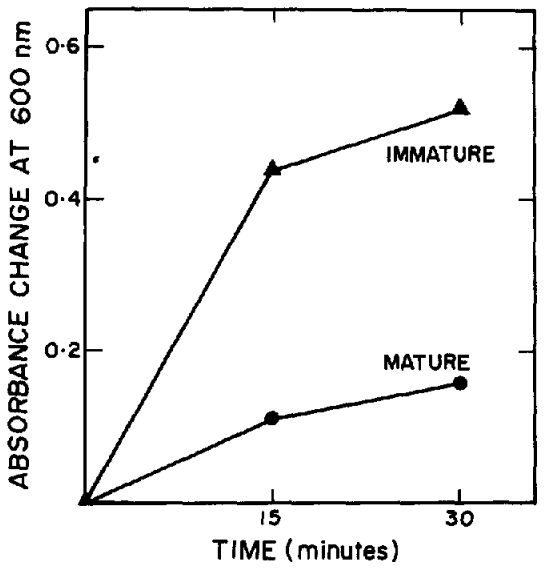

Fig.4. Promotion of microtubule assembly by addition of mature and immature chick RBC extracts in DEAE-purified brain tubulin. DEAE-purified brain tubulin (2.7 mg/ml) was incubated at 37°C with 17 μg DNA equivalent mature (•) and immature (▲) RBC extract. ΔA_{600} over the control (tubulin alone) was plotted against incubation time.

3.3. *Assembly promoting factor(s) and RBC maturation*

The question arises as to whether the capacity of chick RBC extract to facilitate the microtubule formation varies with the stage of erythrocyte maturation. Red blood cells were therefore, isolated from the blood of normal and phenylhydrazine-induced anemic chicks. These mature and immature cell populations were then tested for their ability to promote tubulin assembly using DEAE-purified brain tubulin. As shown in fig.4, the turbidity change was higher with the extract of immature RBC than with the mature one. It is evident from the calculations that the polymerization promoting activity/mg DNA equivalent of chick RBC in the immature stage was ~3–4-times that in the mature stage. We conclude that in chick RBC the level of tubulin as well as the factor(s) responsible for its assembly decreases in a parallel way as the cells become mature.

Acknowledgement

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