Review

Physiological significance and molecular genetics of red cell enzymes involved in the ribonucleotide metabolism

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Abstract: At the final maturation process red blood cells (RBC) are enucleated, becoming unable to synthesize nucleic acids as well as proteins. RBCs survive approximately 120 days in circulation using glucose as the sole energy source. Most crucial RBC functions depend on ATP to sustain physiological homeostasis. It is thus quite important that generation of ATP by glycolysis and replenishing of adenine nucleotide pools by the reaction, which is catalyzed by adenylate kinase (AK1). In turn, ribosomal RNA is degraded during remodeling of reticulocytes, and pyrimidine ribonucleotides become unnecessary for RBC viability. Thus they should be dephosphorylated by pyrimidine 5'-nucleotidase (P5N-I) and finally transported outside RBCs. There have been reported that hereditary deficiency of AK1 and P5N-I may cause shortened RBC life span, i.e. hemolytic anemia. In this review, we summarize physiological importance of these enzymes, which are involved in ribonucleotides metabolism during RBC maturation.

Key words: Erythrocyte; reticulocyte; pyrimidine 5'-nucleotidase; adenylate kinase; hemolytic anemia; gene mutations.

Introduction. At the final stage of differentiation, erythroid cells are enucleated, and organelles such as mitochondria, ribosomes, lysosomes, endoplasmic reticulum and Golgi apparatus are eliminated or decayed. Among them mitochondria and ribosomes still remain for a few days after enucleation, i.e. a stage of reticulocytes. The name of reticulocytes is derived from the network structure of the precipitated ribosomal RNA, which can be visualized by staining with basic dyes such as brilliant cresyl blue, new methylene blue or azure B.

The metabolism of reticulocytes are characteristic in the following respects: Firstly, reticulocytes are able to produce ATP by oxidative phosphorylation, resulting that the ATP production reaches to about 75 times more than mature RBCs.[1),2)] Secondly, reticulocytes can utilize both fatty acids and amino acids for energy-yielding substrates. Finally, there are biochemical systems, by which mitochondria and ribosomes are eliminated. The breakdown of mitochondria is mediated by erythroid-specific lipoxygenase,[3)] whereas abundant ribosomal RNA is biochemically catabolyzed into ribonucleotides by ribonuclease.[4),5)]

An ATP-dependent proteolytic system in reticulocytes may attack the ribosomal proteins, rendering the ribosomal RNA susceptible to ribonuclease.[2)] Digestion of ribosomal RNA with ribonuclease yields ribonucleotides in reticulocytes. Purine ribonucleotides then are utilized to replenish adenylate pools for ATP synthesis.[6)] On the other hand, pyrimidine ribonucleotides such as UMP or CMP are not reused, and finally dephosphorylated by pyrimidine 5'-nucleotidase (P5N-I).[7),8)] These ribonucleosides can be transported toward outside of cells (Fig. 1); as a result, the pyrimidine ribonucleotides are normally unable to detect. In the mature RBCs, most ribonucleotides consist of adenosine phosphates such as AMP, ADP and ATP, and guanylates are reported to exist less than 3%.[9),10)] Adenosine is salvaged to AMP, however other purine moiety cannot be converted to nucleotides.[11),12)] Adenylate kinase (AK1) has an important role on maintenance of adenine nucleotide pools.

These metabolic reactions are characteristic in the mature RBCs, which lose machinery to synthesize

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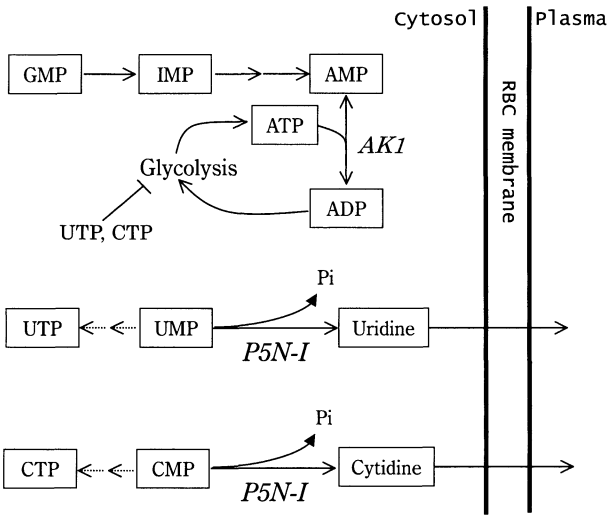

Fig. 1. Metabolic pathway of ribonucleotide in red blood cells. Abundant ribosomal RNA is degraded into ribonucleotides during the reticulocyte stage. In mature RBCs, most ribonucleotides are adenosine mono-, di- or tri-phosphate, which are converted each other by adenylate kinase (AK1) and finally utilized in glycolysis. On the other hand, UMP or CMP are dephosphorylated by pyrimidine 5'-nucleotidase (P5N-I), and then transported to outside of RBCs. In the P5N-I deficiency, accumulated CDP, CTP, UDP or UTP may competitively inhibit glycolysis as well as pentose phosphate shunt activity.

Table I. Erythroenzymopathies associated with hereditary hemolytic anemia discovered in our laboratory (Oct., 2002)

Enzyme	Families	Cases
Hexokiness	1	1
Glucose phosphate isomerase	11	14
Phosphofructokinase	5	7
Aldolase	1	2
Triosephosphate isomerase	1	2
Phosphoglycerate kinase	3	3
Enolase	1	1
Pyruvate kinase	93	102
Glucose 6-phosphate dehydrogenase	100(151)*)	118(177)
Glutathione peroxidase	1**)	1
Glutathione synthetase	1	1
Glutamylcysteine synthetase	2	2
Adenylate kinase	3	3
Pyrimidine 5'-nucleotidase	14	18
Adenosine deaminase (overproduction)	2	2(4)
Total	239(290)	277(335)

*)Include cases without hemolysis, **)Heterozygote.

nucleic acids as well as proteins. It was noteworthy that physiological importance of these enzymes, AK1 and P5N-I, in the RBC metabolism has been unveiled by the discovery of enzymopathies that were responsible for shortened RBC life span, i.e. hemolytic anemia. Both AK1 and P5N-I deficiency are relatively rare among hemolytic anemia due to RBC enzyme defects (Table I), however, it seems quite important to understand physiological importance of purine and pyrimidine metabolism in RBCs. In this review, we summarized current understandings of ribonucleotides metabolism in RBC and recent advances of the molecular genetics of both P5N-I and AK1.

Pyrimidine-specific 5'-nucleotidase in human RBCs. The 5'-nucleotidases (5'-ribonucleotide phosphohydrolase; E.C. 3.1.3.5, 5NT) comprise enzymes those hydrolyze either 5'-ribonucleotide or 5'-deoxyribonucleotide to form nucleoside and inorganic orthophosphate (Pi).[13] 5NT is commonly distributed in bacteria, yeast, plant or animal tissues. In higher organisms, 5NT activity can be demonstrated in both cytosolic and membrane-bound fractions. Most 5NT dephosphorylate both purine and pyrimidine 5'-monophosphates with variable specificity. However, human RBCs contain an isozyme, which catalyzes only pyrimidine 5'-monophosphates such as 5'-CMP and 5'-UMP.[7),8)] This pyrimidine-specific 5NT, P5N-I, has firstly been demonstrated through the biochemical studies of hereditary hemolytic anemia due to deficiency of RBC P5N-I activity.[7)] The P5N-I is a cytosolic 5NT, and acts as a monomer of 36-kDa subunit.[14),15)] This enzyme has limited substrate specificity to pyrimidine 5'-monophosphates, CMP and UMP, while another cytosolic pyrimidine nucleotidase (P5N-II) in RBCs is able to use 2'- and 3'-nucleotides as substrates.[16)-18)] P5N-I is considered to function during reticulocyte maturation, since RNA degradation produces pyrimidine monophosphates those are unable to reuse in mature RBCs.

Severe deficiency of P5N-I in RBCs results in accumulation of pyrimidine nucleotides, which cannot transport outside cells as long as they remain phosphorylated. Increased pyrimidine di- or triphosphates such as CDP, UDP, CTP or UTP may interfere the reactions those require either ADP or ATP, resulting in impaired glycolysis. In addition, CTP was reported to be a competitive inhibitor for glucose-6-phosphate (G6P) and a noncompetitive inhibitor for NADP, resulting that hexose monophosphate shunt activity was suppressed at the pathological concentration of CTP and UTP in the P5N-I deficiency.[19)]

Recently, P5N-I has been reported to be identical with a protein called p36, which is inducibly expressed in B-lymphocytes[15)] (GenBank AF312735). The p36 is shown as a protein moiety in lupus inclusion, which appears in lymphocytes or endothelial cells under pathogenic conditions such as systemic lupus erythematodes

or AIDS.[20),21)] Amici *et al.* determined partial amino acid sequence of RBC P5N-I and subsequent database analysis resulted that primary structure of the P5N-I (GenBank NM016489) was almost identical with p36 with slight divergence at the amino terminal region. Since the p36 gene is transcriptionally activated by stimuli of interferon α, speculating that the p36 may play a role in immune response. Physiological function of the p36 remains investigated.

The P5N-I is also called either as 5'-nucleotidase-cytosolic III or uridine 5'-monophosphate hydrolase, so the gene is symbolized as NT5C3 or UMPH1. The NT5C3 has been localized on chromosome 7 (7p15.3) by BLAST database search.[22)] Chromosome locus of NT5C3 is found as a close proximity of NRF3 (nuclear factor erythroid 2-related factor 3), GCK (glucokinase) or the gene cluster of HOXA (homeo box gene A). The gene consists of 10 exons and spans approximately 48 kilo base pairs. Alternative splicing of exon 2 produces two distinct polypeptides of 286 and 297 amino acid residues. There are two pseudogenes on chromosome 4 and 7 (OMIM #606224).

The persistent thymidine nucleotidase and deoxyribonucleotidase activity has been shown in subjects with P5N-I deficiency, suggesting that distinct isozyme exists in human RBCs.[17),23)] This isozyme, P5N-II (UMPH2), was later characterized[24)] and shown to be localized in the region 17q23.2-q25.3.[25)] The cytosolic deoxyribonucleotidase has been cloned[26)] (GenBank NM014595). However, it remains still unclear whether human RBC UMPH2 is identical with the deoxyribonucleotidase or not.

P5N-I activity has been shown to change during embryonic development,[27)] and it correlated with intracellular concentration of RBC UTP. P5N-I activity of human fetuses from the 17 to 23 weeks of gestation as well as extremely low birth weight infants have been shown as higher than adults.[28),29)] Activation of β-adrenergic or A2A-adenosine receptors has been reported to cause transcription-dependent de novo synthesis of P5N-I. Both receptors are found on erythroid cells, suggesting that the P5N-I activity may be hormonally controlled.[30)] Recent observation shows that hypoxia during development causes erythroid β-adrenergic receptor stimulation, subsequently initiates the activation of several key metabolic adaptation such as elevation of carbonic anhydrase activity and 2,3-diphosphoglycerate synthesis,[31)] suggesting that activation of several genes those are important RBC maturation may be under common control mechanism.

The 5'-flanking region of the NT5C3 gene were included in sequence of a human chromosome 7 clone RP11-81010 (GenBank AC074338) and it showed that there were multiple recognition sites for the transcription factor Sp-1, implicating that the NT5C3 may be expressed ubiquitously. Mechanisms responsible for transcriptional activation of the P5N-I gene during erythroid maturation remains further studied.

P5N-I has phosphotransferase activity,[15),32)] which is capable of transferring phosphate from the pyrimidine nucleoside monophosphate, UMP and CMP, to nucleoside acceptors, including azido-deoxythymidine (AZT), cytosine arabinofuranoside (AraC) and 5-fluoro-deoxyuridine (5FU), all of which are commonly used as pyrimidine analogues in chemotherapy. Since kinetic studies showed that P5N-I had higher phosphotransferase activity for oxynucleosides with respect to deoxynucleosides, suggesting that overexpression of the enzyme may be related in drug resistance to above mentioned pyrimidine nucleoside analogues.

A murine homologue of the p36 has been cloned and designated as lupin.[33)] The gene expression was detected at the 7th embryonic day, and subsequently increased through gestation. In adults, the gene is highly expressed in brain, heart, spleen, kidney and blood. The authors found that homologous sequences are expressed in *Caenorhabditis elegans, Drosophila* and Zebrafish, indicating that the lupin (p36, NT5C3) is evolutionary conserved.

Molecular basis of the P5N-I deficiency. Since the first description by Valentine and colleagues in 1974,[7)] P5N deficiency (OMIM #606224) has so far been reported in 51 cases from 36 unrelated families.[34)] Compared to other common RBC enzyme deficiencies such as pyruvate kinase (PK) or glucose-6-phosphate dehydrogenase (G6PD) deficiency, chronic hemolytic anemia due to P5N deficiency is mild and often well compensated. Basophilic stippling in RBCs is diagnostically significant in P5N deficiency,[35)] and also useful for diagnosis of lead poisoning, since P5N is known as one of enzymes, which are sensitive to lead.[36)]

Marinaki *et al.* has recently reported three gene mutations of the P5N-I deficiency.[22)] We analyzed eight Japanese families of the P5N deficiency, and identified four novel P5N-I gene mutations.[37)] Since the tertiary structure of the P5N-I has not been elucidated, the molecular analysis of the P5N-I deficiency may clarify the functional domain as well as the structure-function correlation of the enzyme.

Adenylate kinase isozymes and AK1 gene. Adenylate kinase (ATP: AMP phosphotransferase, E.C.2.7.4.3) catalyzes the interconversion of Mg – ATP + AMP ↔ Mg – ADP + ADP, and is a ubiquitous enzyme, which has three isozymes, AK1-3.[38)-40)] AK1 is a cytosolic enzyme, which is expressed in skeletal muscle, brain and RBCs. AK2 activity is present in the intermembrane space of mitochondria of liver, kidney and heart. Finally, AK3 is also a mitochondrial enzyme, which resides in the mitochondrial matrix of liver as well as heart. Human AK1 gene locus is on chromosome 9 (9q34.1).[41),42)] The gene (GenBank J04809) is 12-kilobases long and has 7 exons.[43)] AK1 has been reported to be proximal to the breakpoint that creates the Philadelphia chromosome in chronic myelogenous leukemia (CML). Subsequently it is found that ABL1, which is fused with BCR1 in CML cells, is also located on 9q34.1. There is another pathogenic genes near AK1 locus, hamartin, a causative gene for tuberous sclerosis-1 (TSC1).

Disorders of purine metabolism in RBCs include deficiency of adenosine deaminase (ADA, OMIM #102700), purine nucleoside phosphorylase (PNP, OMIM #164050), hypoxanthine phosphoribosyl transferase (HPRT, OMIM #308000), adenine phosphoribosyl transferase (APRT, OMIM #102600) and adenylosuccinase (OMIM #103050), all of which do not cause deleterious effects on RBC survival.[6),44)] One exception is the case of hereditary non-spherocytic hemolytic anemia due to RBC AK1 deficiency (OMIM #103000), and another one is erythroid-specific overproduction of ADA (OMIM #102730).

Biochemistry and molecular genetics of AK1 deficiency. As previously mentioned, phosphorylation of AMP by AK1 is the only available pathway by which the RBCs can synthesize ADP.[45)] Thus ADP formation by AK1 and subsequent synthesis of ATP by the Embden-Meyerhof glycolytic pathway are pivotal reactions for RBCs to maintain homeostasis. Additionally AMP formation by use of plasma adenosine is catalyzed by adenosine kinase, providing an essential salvage reaction.[12)] RBC AK1 deficiency is a quite rare disorder that has been described only in 8 unrelated families so far.[34)] Enzymatic analysis of AK1 deficiency showed that partial deficiency (44% of normal control) of RBC AK1 caused moderate hemolysis in one case [46)] and that in the other instance almost complete (1/2000 of normal level) loss of the activity did not account for any hematological abnormality.[45)] Discrepancy between residual RBC AK1 activity and clinical manifestation made the investigators doubt whether decreased AK1 activity alone is causative for shortened RBC life span.

Gene mutations responsible for AK1 deficiency have been identified in three independent cases.[43),47),48)] The Japanese subject with partial AK1 deficiency was found to be heterozygous for a missense mutation 382CGG → TGG (Arg128Trp).[43)] The proband had another single base change at Ivs3 (-4) ggag → agag. Whether the heterozygous mutation alone is capable of inducing hemolysis remains unsolved. The Ivs change may affect aberrant splicing, though the authors described this as a neutral polymorphism.

Secondly, a homozygous missense mutation 491TAT → TGT (Tyr164Cys) was identified in an Italian girl. The RBC AK1 activity in the proband is almost undetectable. Both 491G and 382T are located in the exon 6 of AK1 gene, suggesting that this region encodes crucial amino acid residues.[47)]

Recently, a nonsense mutation at codon 107, 319CGA → TGA, of the AK1 gene has been revealed in two siblings of Italian origin.[48)] The proband showed mild chronic hemolytic anemia as well as psychomotor impairment. The mutation causes truncation of the AK1 polypeptide, resulting in complete loss of AK1 activity in the affected RBC. Psychomotor impairment has been reported also in a French boy with severe AK1 deficiency.[49)] The residual AK1 activity was about 3.5 percent of normal value, being associated with fully compensated hemolytic anemia.

Since the scarcity of AK1-deficient cases, it is still unclear that AK1 gene mutation alone account for hemolytic anemia. Accumulation of the mutation analysis of AK1 deficiency may elucidate the molecular pathology of AK1 deficiency as well as the structure-function correlation of the enzyme.

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