Experimental Hematology Using Analysis of DNA Synthesizing Enzymes in Animal Models and Cell Culture

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Abstract

Thymidylate synthase (TS) and thymidine kinase (TK) are key enzymes involved in de novo and salvage pathways for pyrimidine nucleotide synthesis, respectively. High activities of TS and TK have been found in rapidly proliferating tissues of normal, fetal and neoplastic tissues. Accordingly, in an attempt to define the hematopoietic systems, we investigated the effects of various substances or conditions on rat hematopoietic cells using measurements of TS and TK activities or their mRNA expression levels. These findings were separated into the below-mentioned seven items.

(1) The hematopoietic cells first entered into cell cycle via the de novo pathway during the recovery phase of hematopoietic cells in bone marrow after the hypoplastic period induced by cyclophosphamide treatment in rats, and were secondly proliferated and differentiated via the salvage pathway. S-phase cells associated with a myeloid hematopoiesis increased earlier than those associated with an erythroid hematopoiesis in the recovery phase of rat bone marrow.

(2) Human erythropoietin increased TS and TK activities, followed by rising cell number of erythroid series in bone marrow cells.

(3) Granulocyte colony-stimulating factor (G-CSF) enhanced TK activity in bone marrow cells. Daily injections of G-CSF markedly enhanced the number of granulocytes in peripheral blood and the splenic DNA synthesis with splenomegaly.

(4) We investigated the effects of dimethyl sulfoxide (DMSO) and cytosine arabinoside (ara-C) on the growth of mouse leukemia cell line L1210 cells. The cells, which were treated with 1.5% DMSO for 96 hours, tolerated the treatment and reversed the cell cycle arrest within 36 hours. Non-high dose ara-C enhanced DNA-synthesizing enzyme activities in L1210 cells, and withdrawal of the non-high dose ara-C resulted in paradoxical cell proliferation.

(5) Thermostability of bone marrow cells of patients with acute myeloblastic leukemia using short-term cell culture was investigated. TK isozymes in bone marrow cells (leukemia cells) were separated into two types, i.e. fetal type isozyme in cytosolic cell fraction and adult type isozyme in mitochondrial cell fraction. Heating at 43℃ as a hyperthermia markedly suppressed enzyme activity of fetal type TK isozyme and number of S-phase cells. Serum TK activities of not only patients with acute leukemia but also patients in clinical end stage V and with recurrence of colorectal cancer or distant metastasis were markedly higher than those of patients without metastasis.

(6) Zinc-deficiency caused a marked reduction in the body growth rate, organ weights, plasma sex-hormone levels and hematopoiesis in rats, i.e. zinc-deficient anemia.

(7) Genetic hemochromatosis is an iron overload disorder. Patients with thalassemia major require multiple blood transfusions leading to hemochromatosis. Marked deposition of iron was noted in liver and kidney of iron-overloaded rats, resulting in damage of the proximal tubular epithelial cells in kidney and a reduction of femoral bone mineral density (BMD). Femoral BMD in male rats, but not female, was markedly reduced by iron overload, which might induce bone loss associated with renal dysfunction and hypogonadism in male rats, and circulating estradiol might be able to prevent the bone absorption in female rats.

We have investigated activities of both TS and TK enzymes on various proliferating cells in vitro. In our experimental studies, activities of these DNA synthetic enzymes were markedly elevated when the cell proliferation was accelerated. Increased speed of cell proliferation was intimately related to TK activity. From these findings TK activity can be comprehensible as one of the tumor markers. Currently our empirical data have been effectively utilized at the clinical site. Particularly the serum activity of TK enzyme has been used by the diagnosis of malignant lymphoma.

Key words —— Hematopoietic cells, DNA synthesis, thymidylate synthase, thymidine kinase

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Thymidylate synthase (TS; EC 2.1.1.45) is the enzyme responsible for the de novo synthesis of deoxythymidine monophosphate (dTMP) by catalysing the methylation of deoxyuridine monophosphate (dUMP) with the concomitant conversion of N^5,N^{10}-methylenetetrahydrofolic acid to 7,8-dihydrofolic acid. TS activities have been studied in regenerating liver[1], in bone marrow cells[2] and in neoplastic cells[3]. Thymidine kinase (TK; EC 2.7.1.21) catalyses the formation of dTMP by the phosphorylation of thymidine *via* the salvage pathway[4, 5]. High TK activities and the presence of TK isozymes have been found in rapidly proliferating tissues[4-14]. TK isozymes have been separated by acrylamide gel electrophoresis[4] and affinity[10] or DEAE-cellulose[11] column chromatography. Accordingly, in an attempt to define the hematopoietic systems, we investigated the activities of TS and TK, or the expression levels of TS and TK mRNA, and the cell cycle by flow cytometric (FCM) DNA analysis and bromodeoxyuridine (BrdU)-immunocyto- chemistry during the recovery phase of hematopoietic cells in bone marrow after the hypoplastic period induced by cyclophosphamide (Cy) treatment in rats[15].

The hematopoietic cells first entered into cell cycle *via* the *de novo* pathway in pyrimidine nucleotide synthesis, and were secondly proliferated and differentiated via the salvage pathway. S-phase cells associated with a myeloid hematopoiesis increased earlier than those associated with an erythroid hematopoiesis in the recovery phase of rat bone marrow.

Thus, to define the effects of recombinant human erythropoietin (rEPO) on hematopoietic organs, we investigated TS and TK activities, and BrdU-immunohisto-chemistry during the recovery phase of hematopoietic cells in bone marrow and spleen after the hypoplastic period induced by Cy-treatment in rats[16]. Treatment with rEPO increased TS and TK activities and cell number of erythroid series in bone marrow cells; it also increased organ weight and S-phase cells in the spleen, followed by an augmentation of the number of erythrocytes and a rise in the hemoglobin and hematocrit levels in peripheral blood with or without Cy-treatment.

Next, we investigated the effects of macrophage (M) and granulocyte (G) colony-stimulating factor (CSF) on the hematopoietic cells of rats treated with Cy[17]. The additive treatments using M- and G-CSF enhance TK activity, but not TS activity, to 1.5- and 3.0-fold, respectively. TK activity in rats given M- or G-CSF for 7 days in the absence of Cy-treatment was increased to over 1.5 times that of the control.

Spontaneous ruptures of the spleen have been observed in donors and patients with malignancy during mobilization of peripheral blood stem cells. Thus, we investigated the morphological and histological alteration of the spleen, and mRNA expression levels and activities of TS and TK in the splenic cells of rats treated with recombinant human G-CSF (rhG-CSF)[18]. Daily injections of rhG-CSF for 5 or 7 days slightly enhanced the splenic weight. Single or 3-day treatment with rhG-CSF markedly enhanced the number of granulocytes in peripheral blood. Expression levels of TS and TK mRNA in the splenic cells were significantly increased 6 hours after rhG-CSF treatment. Early expression of TS and TK mRNA in the splenic cells may indicate a reseeding of hematopoietic cells from the bone marrow. Daily injections with rhG-CSF enhanced the TS and TK activities in the splenic cells. As continuous elevations of the splenic DNA synthesis and splenomegaly are suggestive of a possible splenic rupture, the monitoring of peripheral granulocytes and splenic size is necessary during the rhG-CSF treatment.

Dimethyl sulfoxide (DMSO) is known as a cryoprotectant and a lipophilic solvent for cells in vitro, and has been used as a convenient cryoprotectant for stem cells in stem cell

transplantation using allogenic peripheral blood or umbilical cord blood[19]. As the stem cells have a multipotency, clarification of the extent of cell proliferation after transplantation is difficult. Accordingly, we investigated the effects of DMSO on mouse leukemia L_{1210} cells[20]. DMSO gradually induced G_0/G_1 arrest in mouse leukemia L_{1210} cells with good cell viability. After removal of DMSO, the cells proliferated appropriately, resulting in the expression of TS and TK mRNA within 6 hours, and the cells entering into S phase within 12 hours. The sequence was followed by the marked activation of both enzymes within 24 hours and the increase of BrdU-immunoreactive (S-phase) cells with rapid cell proliferation within 36 hours. That is, mouse leukemia L_{1210} cells, which were treated with 1.5% DMSO for 96 hours, tolerated the treatment and reversed the cell cycle arrest within 36 hours.

Next, we investigated the withdrawal effects of various concentrations of cytosine arabinoside (ara-C), which was one of the potent agents in the treatment of acute myeloid leukemia, on the growth of the same cell line L_{1210}[21]. The three concentrations of ara-C were established to be 2.0 x 10^3 ng/ml as a high dose, 100 ng/ml as a middle dose and 5.0 ng/ml as a low dose, respectively. In the analysis by flow cytometry, high dose ara-C arrested the cell cycle in the G_0/G_1 phase. Middle and low doses ara-C induced a block in the S-phase, that was not completely blocked by the low dose. Analysis of DNA fragmentation revealed that ara-C dose-dependently induced apoptosis, which was only slightly induced by the low dose. We measured activities of cellular TS and TK after 24-hour culture. Low and middle doses, but not high dose, ara-C markedly enhanced TS activity to 2.9- in low and 5.3-fold in middle doses ara-C, and TK activity to 1.3- in low and 2.2-fold in middle doses, respectively, compared with those of the control. The cells accumulated in the S-phase by 48-hour culture with low dose ara-C and markedly proliferated compared to that of the control in ara-C-free medium. That is, non-high dose ara-C enhanced DNA-synthesizing enzyme activities in L_{1210} cells, and withdrawal of the non-high dose ara-C resulted in paradoxical cell proliferation. These results indicate that an insufficient dose of ara-C may induce cells into S-phase, resulting in the proliferation of leukemic cells.

Hyperthermic treatment has been used for human malignancies, since its clinical application is based on the observation that malignant cells are more sensitive to hyperthermic killing than the normal counterparts at temperature of 41℃ to 44℃[22]. There is rather few information available on the thermal sensitivity of human hematopoietic or leukemic cells[23]. Thus, we investigated thermostability of bone marrow cells of patients with acute myeloblastic leukemia in the aspect of DNA -synthesizing enzymes[24]. TK isozymes in bone marrow cells were separated into two types, i.e. fetal type isozyme in cytosolic cell fraction and adult type isozyme in mitochondrial cell fraction. Heating at over 43℃ as a hyperthermia markedly suppressed enzyme activity of fetal type TK isozyme and number of S-phase cells labeled with BrdU, but not activities of adult type TK isozyme and TS. These findings indicate that hyperthermia may regulate the proliferation of S-phase cells by the suppression of salvage synthesis of DNA.

Serum TK activities of patients in different clinical stages (I to V) of colorectal cancer were investigated[25]. There were no differences between serum TK activities in normal control group and colorectal cancer groups of clinical stage I to IV. However, in the end stage V, 31% of the cases showed high TK activities in sera, and in the group with recurrence of colorectal cancer, 88% of the cases showed high TK activities. Serum TK activities of patients with distant metastasis such as liver, lung or bone metastasis were markedly higher than those of patients without metastasis.

Zinc is required for many biological functions including DNA synthesis, cell division, gene expression and the activity of various enzymes in all animals. A deficiency of zinc due to nutritional factors and several disease states is now recognized as a disorder, e.g. zinc-deficient anemia. The high phytate content of cereal proteins is known to decrease the availability of zinc, thus the prevalence of zinc-deficiency is likely to be high in a population consuming large quantities of cereal proteins[26]. Zinc-deficient Bangladesh infants showed improvements in rate of growth and a reduced incidence of acute lower respiratory infections after zinc supplementation, which produced highly significant, positive responses in height and weight[27]. Zinc-deficiency has had negative effects on

growth rate, food intake, specific organ weights, hematological parameters, and serum levels of zinc, copper and iron, especially in rats fed the lowest zinc level[28]. Thus, we investigated the effects of a low-zinc diet on body growth, organ weights, hemograms, plasma biochemical markers, plasma levels of hormones, and the BrdU-incorporation and TS and TK mRNA expression into the prostate cells in growing rats[29]. The low -zinc diet caused a marked reduction in the body growth rate and weights of the spleen, liver, kidney, testis, ventral prostate and femur, along with a marked decline in the plasma concentration of zinc (less than 60% of the control), compared with the control diet. It resulted in a low hematopoiesis (pancytopenia), hypoalbuminemia and hypocholesterolemia, though it did not affect the plasma concentration of glucose. Although there were few differences in plasma biochemical markers, plasma levels of luteinizing hormone and testosterone were markedly reduced by six-week's feeding of a low-zinc diet. In prostate, BrdU-immunoreactive (S-phase) cells, TS and TK mRNA expression levels were markedly affected by a zinc deficiency.

Genetic hemochromatosis is an iron overload disorder, and osteopenic and osteoporotic. Femoral neck bone mineral density (BMD) appears to fall with rising hepatic iron concentrations. A critical role for iron in mediating tissue injury is played via hydroxyl radical formation in nephrotoxicity. Accordingly, we investigated the effects of a colloidal iron overload on renal function, organ siderosis, and femoral bone in male rats[30]. Iron overload reduced body growth, and increased the weights of the liver and spleen. Marked deposition of iron was noted in liver and kidney. Activities of lactate dehydrogenase and alkaline phosphatase were decreased, and the concentrations of blood urea nitrogen and creatinine were increased with the reduction in plasma calcium and inorganic phosphorus levels, i.e. functions of the liver and kidney might be affected by reactive oxygen species. Damage to the proximal tubular epithelial cells of the kidney and a loss of connectivity of cancellous bone in the epiphysis and of trabecular bone in the metaphysis of the distal femur were observed in iron-overloaded rats with a reduction of femoral BMD, i.e. reabsorption of calcium from the proximal tubular epithelial cells of the kidney might be affected and urinary discharge of calcium might be elevated. It was suggested that iron overload gave rise to osteoporosis combined with renal dysfunction and liver iron overload syndrome.

Next, patients with thalassemia major require multiple blood trasfusions leading to hemochromatosis which is an iron overload disorder, resulting in osteoporosis and/or osteopenia which are more frequent and severe in males than females. Thus, we investigated the effects of a colloidal iron overload on femoral bone loss and gender-based differences therein in rats31). The rate of increase of iron accumulation by iron overloading into liver and kidney appeared to be more elevated in male rats than the females. The reduction in plasma levels of calcium and alkaline phosphatase activity were much greater in the male rats, and circulating levels of estradiol were much higher in the female rats. Plasma testosterone levels were slightly reduced by iron overload in males. Femoral BMD in male, but not female, was markedly reduced by iron overload. It is suggested that iron overload induces bone loss associated with renal dysfunction and hypogonadism in male rats, and circulating estradiol can prevent the bone absorption in female rats. Either genetically or therapeutically, iron overload may give rise to osteoporosis combined with renal dysfunction, liver iron overload syndrome and in part hypogonadism in male mammals.

We have investigated activities of both TS and TK enzymes on various proliferating cells in vitro. In our experimental studies, activities of these DNA synthetic enzymes were markedly elevated when the cell proliferation was accelerated. Increased speed of cell proliferation was intimately related to TK activity. From these findings TK activity can be comprehensible as one of the tumor markers. Currently our empirical data have been effectively utilized at the clinical site. Particularly the serum activity of TK enzyme has been used by a diagnosis of malignant lymphoma.

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動物モデルと細胞培養系におけるDNA合成系酵素解析を用いた実験血液学

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要旨

ピリミジン代謝のデ・ノボ経路およびサルベージ経路に位置するチミジル酸合成酵素 (TS)，チミジン・キナーゼ (TK) は DNA 合成の活発な細胞で酵素活性の増加が認められ，増殖の速い細胞では特異な TK 分子種も存在する．そこで血液関連分野における実験系として実験動物と細胞培養系を用い，上記酵素活性およびその mRNA 発現について検討し，結果を７項目に分類してその意義を考察した．

(1) エンドキサンによって人為的にラット骨髄造血を抑制し，その後の造血細胞回復過程を観察した結果，赤血球系造血の回復より早期に白血球系の造血が活発となり，それぞれの段階において造血細胞の S 期細胞増加とともに，まず TS 活性増加がおこり次いで TK 活性増加の増加が起こることが確認された．

(2) エリスロポイエチンにより人為的にラット赤血球系造血細胞の増加を促進させた結果，TS，TK 活性の消長から，骨髄の赤血球系造血細胞の増加，脾臓での S 期細胞の増加発現後に末梢血の赤血球数増加がみられた．

(3) 顆粒球コロニー刺激因子 G-CSF をラットに投与し顆粒球系細胞の造血刺激を行った．末梢血顆粒球の増加に伴い，脾臓重量の増加が起こり，脾臓細胞の TS,TK 活性の顕著な増加が認められこの造血刺激因子が脾腫を誘発することを明らかにした．

(4) 白血病細胞株 L1210 を用いた実験では，凍結保護剤 DMSO 添加，白血病治療薬 Ara-C 添加を行い，細胞周期に与える影響を TS,TK 活性の動態とあわせて検討して DMSO が L1210 細胞を細胞静止期 G_0 に誘導し，DMSO 除去後 36 時間以内に再度細胞回転に転入させることを確認した．また Ara-C の細胞回転に及ぼす影響を検討したが，通常投与量もしくは低投与量では直接的殺細胞効果はなくすべての細胞を S 期に留めるにすぎないことが明らかとなった．

(5) 急性白血病患者から得られた血清ならびに骨髄の白血病細胞について TS，TK 活性を測定し，主に血清 TK 活性は白血病の病勢と関連することを確認した．骨髄細胞は 43℃の加温により，TK 胎児型分子種の酵素活性と，S 期細胞を減少させることが判明し白血病温熱療法の可能性が確認された．

(6) 亜鉛欠乏は貧血をきたすことで知られるが，亜鉛欠乏ラットで，造血ホルモンともされる男性ホルモンの低下ならびに造血能の低下を観察した．

(7) ラットに鉄過剰投与して作成したヘモクロマトーシスでは，腎に沈着した鉄が腎遠位尿細管からのカルシウム再吸収を阻害して骨量減少と性機能低下を引き起こした．この変化は雄ラットで顕著であったが，雌ラットではエストロゲンによる骨吸収抑制のため，骨量減少は認められなかった．

DNA 合成系酵素として知られるＴＳ，ＴＫ活性を様々な細胞増殖系とくに血液細胞を中心に測定した．行ったすべての実験系で細胞増殖の亢進時に両酵素活性の増加が観察された．しかしながら増殖速度はＴＳ活性よりＴＫ活性の増加と密接に関連した．このことからＴＫ活性は腫瘍性細胞増殖のマーカーとしての意義を持つといえる．現在われわれの実験データが臨床現場において活用されており，特に血清中のＴＫ活性測定値は悪性リンパ腫の腫瘍マーカーとして検査診断の一助をなしている．

キーワード

造血細胞，DNA 合成，チミジル酸合成酵素，チミジン・キナーゼ