PURINE METABOLISM

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Purine metabolism is essential to the body: it provides components of the nucleic acids, DNA and RNA, and the energy currency of the cell, ATP. Purine catabolism leads to the formation of a poorly soluble compound, uric acid, which can precipitate when elevated, and thereby causes gout. Our major present interests are the genetic defects of purine metabolism, and the mechanisms of action of select synthetic purine nucleoside analogues which possess therapeutic, mainly anticancer and antiviral properties.

In previous work we demonstrated that, in several tissues, the catabolism of AMP, leading to the production of uric acid and/or allantoin, proceeds only by way of AMP deaminase, a highly regulated enzyme which is normally more than 95 % inhibited. Dephosphorylation of AMP into adenosine also occurs but does not contribute to the formation of purine catabolites, owing to recycling by way of adenosine kinase. Interruption of this recycling plays a crucial role in the elevation of adenosine, a major regulatory compound, under anoxic conditions. We have also shown that a variety of rat and human tissues contain a cytosolic 5'-nucleotidase which hydrolyzes preferentially IMP and GMP, is stimulated by ATP and 2,3-bisphosphoglycerate, and inhibited by Pi. Furthermore, we have discovered that phosphorylation of aminoimidazolecarboxamide (AICA) riboside into the corresponding nucleotide, AICA-ribotide (AICAR, also termed ZMP), an intermediate of the 'de novo' pathway, results in the inhibition of key glycolytic and gluconeogenic enzymes in the liver owing to its resemblance with AMP. We have also found that ZMP stimulates AMP-activated protein kinase. This provokes phosphorylation and inactivation of acetyl-CoA carboxylase and HMG-CoA reductase, the rate-limiting enzymes of fatty acid and cholesterol synthesis, respectively (1).

Adenylosuccinate lyase deficiency

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Collaboration with the Department of Paediatrics of the University Hospital Gasthuisberg in Leuven has led us to the discovery, in 1984, of adenylosuccinate lyase (adenylosuccinase, ADSL) deficiency, the first enzyme deficiency described on the 'de novo' pathway of purine synthesis in man. This disorder causes accumulation in cerebrospinal fluid and urine of two normally undetectable compounds, succinylaminoimidazolecarboxamide riboside (SAICA-riboside) and succinyladenosine (S-Ado). These are the dephosphorylated derivatives of the two substrates of ADSL, SAICA-ribotide (SAICAR) and adenylosuccinate (S-AMP), respectively (see Figure). Affected children display variable, but mostly profound psychomotor delay, often epilepsy and/or autistic features, occasionally growth retardation and muscular wasting (2). We study the mutations that lead to ADSL deficiency (3-5), and the pathophysiologic mechanisms of the disorder.

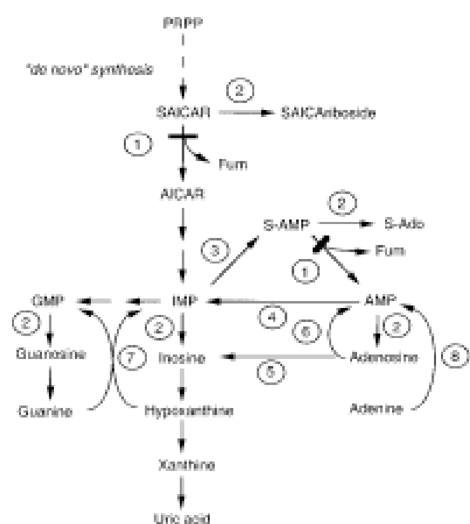

Fig. 1. Pathways of purine metabolism. The *ten-step synthetic route*, often termed 'de novo' pathway, leads from phosphoribosyl pyrophosphate (PRPP) to IMP. From IMP, the nucleoside monophosphates, AMP and GMP, and the corresponding di- and triphosphates (not shown) are formed. The *catabolic pathway* starts from the nucleoside monophosphates and, in man, produces uric acid, a poorly soluble compound. In lower mammals, uricase (not shown) converts uric acid into allantoin, which is much more soluble. The *salvage pathway*, composed of two enzymes, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase, converts the purine bases, guanine, hypoxanthine and adenine, into the corresponding nucleoside monophosphates. Adenosine kinase can also be considered a salvage enzyme. AICAR, aminoimidazolecarboxamide ribotide; Fum, fumarate, S-Ado, succinyladenosine; SAICAR, succinylaminoimidazolecarboxamide ribotide; S-AMP, adenylosuccinate. (1) adenylosuccinate lyase; (2) cytosolic 5'-nucleotidase; (3) adenylosuccinate synthetase; (4) AMP deaminase; (5) adenosine deaminase; (6) adenosine kinase; (7) hypoxanthine-guanine phosphoribosyltransferase; (8) adenine phosphoribosyltransferase. Bars indicate the defect in adenylosuccinate lyase deficiency.

Mutation analysis

ADSL deficiency has been diagnosed in more than 60 patients worldwide. In accordance with the variability of the clinical picture, 38 different mutations in the ADSL gene have been identified to date in 40 unrelated families (see Adenylosuccinate Lyase Mutations Database at http://www.icp.ucl.ac.be/adsldb/). The majority are missense mutations. In about half of the families, the patients are compound heterozygotes. Most frequently encountered, accounting for about one third of the alleles investigated, is a R426H mutation, which has been found in the homozygous form in 9 families, and in the compound heterozygote form in 5 families.

A - 49 T>C change in the 5' untranslated region (5'UTR) of the ADSL gene, found in 2000 in an ADSL-deficient patient in whom one of the ADSL genes displayed a normal coding sequence, was diagnosed in two additional unrelated patients. Studies of the influence of this mutation on gene expression (5) confirmed that it provokes 80 % reduction of the capacity of the promoter region, containing a 400 bp sequence upstream to the initiation ATG, to lead to expression of luciferase activity. Measurements of luciferase mRNA levels showed that the amount of messenger was also reduced 3- to 4-fold with the mutated construction.

Further investigation has shown that the mutation affects the binding of NRF-2, a known activator of transcription. The functional importance of the mutation was corroborated by gel-shift experiments that showed that RCO4-NRF-2 oligonucleotides competed very well the WT-ADSL oligonucleotide but not the ADSL oligonucleotide with the - 49 T>C mutation. The observation that, in binding assays with nuclear proteins, the oligonucleotide probe with the – 49 T>C mutation revealed a complex of higher molecular weight, suggests that the mutation might also allow the binding of an unknown repressing factor. Our findings indicate that a mutation of a regulatory region of the ADSL gene might be an unusually frequent cause of ADSL deficiency, and suggest a role for NRF-2 in the gene regulation of the purine biosynthetic pathway.

Pathogenetic studies

The symptoms of ADSL deficiency could a priori be due to a distal deficiency of purine, particularly adenine nucleotides, and/or to a toxic effect of proximally accumulating SAICAR and S-AMP, and/or of their

dephosphorylated derivatives, SAICA-riboside and S-Ado, respectively.

The observation that the levels of SAICA-riboside are comparable in severely and mildly retarded patients, whereas those of S-Ado are markedly higher in the latter, has led to the hypothesis that SAICA-riboside is the neurotoxic compound, and that S-Ado could counteract its noxious effects. However, we could not demonstrate a cytoxic effect of the succinylpurines on cultured rat neurones. A recent search for interactions of the succinylpurines with 30 membrane receptors by binding analysis has also given negative results.

These findings have led us to consider the possibility that the intracellular accumulation of the substrates of the enzyme might be toxic. To test this hypothesis, construction of ADSL-deficient models was initiated. Several approaches are under way to obtain ADSL-deficient cells: creation of dominant negative mutants by overexpression of mutated ADSL in mammalian cells, expression of antisense RNAs to repress endogenous ADSL, and inhibition of ADSL by compounds such as adenylophosphonopropionate. We are also in the process of creating an ADSL-deficient neuronal cell line by knock-in through insertion of the most frequent mutation, R426H, in the murine gene. Mouse N1E-115 neuroblastoma cells have been efficiently transfected with the modified ADSL gene using lipofection, and we are screening the neomycin-resistant recombinant clones for homologous recombination.

Anti-leukaemic properties of 2-chloro-2'-deoxyadenosine

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In 1997, a collaborative study of the antileukaemic nucleoside, 2-chloro-2'-deoxy-adenosine (CdA), was started with the Department of Haematology of the University Hospital Saint-Luc. This adenosine deaminase-resistant deoxyadenosine analogue displays remarkable therapeutic properties in indolent lymphoid malignancies including hairy cell leukaemia and B-cell chronic lymphocytic leukaemia (B-CLL). Nevertheless, resistance is also observed, and CdA does not confer a survival advantage when compared to more conventional therapies such as alkylating agents. The aims of the project are to understand the mechanisms that lead to resistance to CdA, and to improve its therapeutic efficacy by searching for synergisms with other compounds (6).

To exert is antileukaemic effect, CdA has to be phosphorylated by deoxycytidine kinase (dCK) into CdAMP, followed by conversion into CdADP and CdATP. The latter, the active metabolite of CdA, has been shown to interfere with a variety of enzymes involved in DNA synthesis and repair, including ribonucleotide reductase and DNA polymerases α and β. Moreover, CdATP can be incorporated into newly synthesized DNA, causing chain termination. Together, these actions result in the progressive accumulation of DNA strand breaks, leading to initiation of apoptosis by mechanisms which are not yet entirely clear. 2-Chloroadenine, the major catabolite of CdA, was found to be actively phosphorylated, but poorly cytotoxic (7).

To improve our understanding of the mechanisms by which CdA induces apoptosis in B-CLL cells, we investigate EHEB cells, a continous cell line derived from a patient with B-CLL. This cell line was found resistant to CdA, and the mechanisms responsible for this resistance are now analyzed. We have also initiated a study of the regulation of dCK, a key enzyme in the activation of CdA and of several other nucleoside analogues used in anticancer or antiviral therapy.

Resistance to CdA of EHEB cells

The EHEB cell line was found to be less sensitive (10- to 1000-fold) to the nucleoside analogue CdA than other human lymphoblastic cell lines. Moreover, DNA synthesis, measured by thymidine incorporation into DNA, was unexpectedly increased in EHEB cells, up to 2-fold, after a 24 h-incubation with 10 μM CdA (8). Analysis by flow cytometry, using double labelling with propidium iodide and bromodeoxyuridine, has shown that CdA, in EHEB cells, provokes an increase in the proportion of cells in S phase, synthesizing actively DNA. These results contrast with those reported in other leukemic cell lines sensitive to CdA, like CCRF-CEM cells, in which CdA inhibits DNA synthesis and provokes an accumulation of most cells in either early S phase or at the G1-S border. Kinetics and synchronisation experiments have shown that CdA stimulates the progression of EHEB cells from G1 to S phase, rather than blocking them in S phase. This led us to study the effect of CdA on proteins regulating the G1/S checkpoint of the cell cycle, and firstly on the phosphorylation of the retinoblastoma (Rb) protein, which is increased during the

G1/S transition. We have observed that CdA enhances the phosphorylation of Rb in EHEB cells, whereas it decreases it in CCRF-CEM cells. The p53 status of this cell line was determined and found unmutated. Additional preliminary experiments have shown that CdA decreases p21 expression in EHEB cells. In conclusion, we show a new mode of cellular response to CdA, implying modification of the cell cycle regulation leading to enhanced DNA synthesis. We propose that this peculiar effect might be implied in some types of yet unexplained resistance of leukaemic cells to CdA.

Regulation of dCK activity

Since dCK activates numerous nucleoside analogues used in anticancer and antiviral therapy, knowledge of its regulation can be expected to allow optimization of the activation of these analogues. Recently, it has been shown by others and by us that dCK activity can be increased by various genotoxic agents, including CdA, aphidicolin, etoposide, and UV-C irradiation. This activation is not explained by an allosteric effect or by an increase of the quantity of enzyme. A post-translational activation of dCK by intracellular signalling pathways was suggested. To unravel the mechanism of the activation of dCK by CdA, we first investigated the effect of a variety of activators and inhibitors of protein kinases on the basal activity of dCK and on its activation by CdA. We discovered that dCK can be activated by several inhibitors of protein kinases, including genistein, an unspecific inhibitor of protein tyrosine kinases, AG-490, an inhibitor of the protein tyrosine kinase JAK-2, PD-98059, U0126, SB 203580 and dicoumarol, which are inhibitors of various MAP kinases. We also observed that these inhibitors potentiated the activating effect of CdA. In contrast, sorbitol which is known to activate the MAP kinase pathway was found to decrease dCK activity and to counteract its activation by CdA. On the other hand, we have shown that dCK activity can be markedly increased in intact EHEB cells by incubation with okadaic acid, an inhibitor of protein phosphatase PP2A. Taken together, these results do not allow to identify the pathway by which dCK is activated, but clearly indicate that its activity can be regulated by protein kinase(s) and phosphatase(s). This was confirmed by our observation that dCK, activated or not by CdA, can be inactivated in a crude cell extract by purified PP2A. This last result also demonstrates that activation of dCK results from its phosphorylation. We try now to overexpress dCK in HEK 293 or lymphoblastic cells in order to verify if dCK can be effectively labelled with [^{32}P]orthophosphate and if this labelling can be increased by activators of dCK.

Activation of dCK and DNA repair after UV-C irradiation in B-CLL cells

In mammalian cells, various forms of DNA damage elicit a repair response that includes a DNA re-synthesis step which takes place after recognition and removal of the lesions. In resting cells, DNA repair synthesis is usually investigated by measuring incorporation of labelled thymidine into DNA in the presence of hydroxyurea. In a first study, we had investigated the effect of CdA on DNA repair elicited by UV-C irradiation. We observed that CdA inhibits DNA repair synthesis in B-CLL lymphocytes and was able to potentiate UV- C cytotoxicity (9). We also found that the incorporation of deoxycytidine into DNA was much more stimulated by UV-C irradiation than that of dThd. This led us to investigate the effect of UV-C on deoxynucleoside phosphorylating enzymes. The activity of dCK was significantly increased, up to 2.6-fold, 30 min after irradiation with 30 J/m^2 of UV-C, whereas thymidine kinase (TK) activity was not augmented. Activation of dCK by UV-C light was caused neither by a change in the concentration of a low molecular weight metabolite, nor by an increase of the amount of dCK protein, suggesting that it results, like dCK activation by CdA, from a post-translational modification of the enzyme. Preincubation of the cells with the growth factor receptor inhibitor suramin, prevented activation of dCK by UV-C. This polysulfonated drug also completely suppressed not only the increase of the incorporation of dCyd into DNA elicited by UV-C irradiation, but also that of dThd. Our results strongly suggest that both activation of dCK and upregulation of DNA repair synthesis by UV-C are mediated by a membrane-activated signal transduction pathway. We propose that activation of dCK, initiated at the membrane level, could be the prerequisite to DNA repair synthesis induced by UV-C irradiation in B-CLL lymphocytes (10).

Drug sensitivity profiles

With standard treatments of B-CLL, clinical response is variable, depending on drug and patient. To tailor therapy on a more individual basis, we develop an *in vitro* assay which evaluates the sensitivity of the patients' lymphocytes to various drugs (nucleoside analogues, alkylating agents, steroids, anthracyclin, etc.). The lymphocytes will also be characterised by conventional cytogenetics, hybridisation *in situ* (FISH), and molecular biology techniques. By revealing correlations

between chemoresistance profiles and genetic anomalies, these studies might also allow identification of mechanisms of resistance.

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