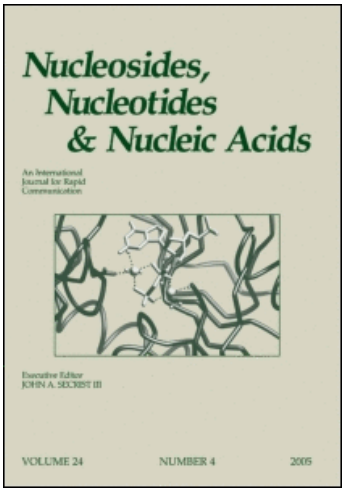

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N. Gattermann[a]; M. Dadak[a]; G. Hofhaus[b]; M. Wulfert[a]; M. Berneburg[c]; M. L. Loeffler[d]; H. A. Simmonds[e]
[a] Department of Hematology, Oncology and Clinical Immunology, Heinrich-Heine-University, Düsseldorf, Germany [b] Institute of Biochemistry, Heinrich-Heine-University, Düsseldorf, Germany [c] Department of Dermatology, Heinrich-Heine-University, Düsseldorf, Germany [d] Institute of Physiological Chemistry, Philipps-University, Marburg, Germany [e] Purine Research Unit, Guy's Hospital, London, UK

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Severe Impairment of Nucleotide Synthesis Through Inhibition of Mitochondrial Respiration

N. Gattermann,[1,*] M. Dadak,[1] G. Hofhaus,[2] M. Wulfert,[1] M. Berneburg,[3] M. L. Loeffler,[4] and H. A. Simmonds[5]

[1]Department of Hematology, Oncology and Clinical Immunology,
[2]Institute of Biochemistry and [3]Department of Dermatology,
Heinrich-Heine-University, Düsseldorf, Germany
[4]Institute of Physiological Chemistry, Philipps-University, Marburg, Germany
[5]Purine Research Unit, Guy's Hospital, London, UK

ABSTRACT

Since de-novo synthesis of pyrimidine nucleotides is coupled to the mitochondrial respiratory chain (RC) via dehydroorotic acid dehydrogenase (DHODH), respiratory chain dysfunction should impair pyrimidine synthesis. To investigate this, we used specific RC inhibitors, Antimycin A and Rotenone, to treat primary human keratinocytes and 143B cells, a human osteosarcoma cell line, in culture. This resulted in severe impairment of de novo pyrimidine nucleotide synthesis. The effects of RC inhibition were not restricted to pyrimidine synthesis, but concerned purine nucleotides, too. While the total amount of purine nucleotides was not diminished, they were significantly broken down from triphosphates to monophosphates, reflecting impaired mitochondrial ATP regeneration. The effect of Rotenone was similar to that of Antimycin A. This was surprising since Rotenone inhibits complex I of the respiratory chain, which is upstream of ubiquinone where DHODH interacts with the RC. In order to avoid unspecific effects of Rotenone, we examined the consequences of a mitochondrial DNA mutation that causes a specific complex I defect. The effect was much less pronounced than with Rotenone, suggesting that

*Correspondence: N. Gattermann, Department of Hematology, Oncology and Clinical Immunology, Heinrich-Heine-University, 40225 Düsseldorf, Germany.

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complex I inhibiton cannot fully explain the marked effect of Rotenone on pyrimidine nucleotide synthesis.

Key Words: Pyrimidine nucleotide synthesis; Dehydroorotic acid dehydrogenase; Mitochondrial respiratory chain; Respiratory chain defect; Mitochondrial DNA mutations; Myelodysplastic syndromes.

INTRODUCTION

Leflunomide and Brequinar inhibit de novo pyrimidine synthesis at the level of dihydroorotic-acid dehydrogenase (DHODH) which is located in the inner mitochondrial membrane (Fig. 1), and is coupled to the mitochondrial respiratory chain.[1] DHODH uses ubiquinone as the proximal and cytochrome c oxidase as the ultimate electron transfer system.

Therefore, RC dysfunction should impair pyrimidine biosynthesis.[2] This is supported by the fact that rho-0-cells, lacking mitochondrial DNA and thus a functioning respiratory chain, are pyrimidine auxotrophs requiring uridine for growth.[3,4] Our interest in the consequences of RC dysfunction stems from the finding of acquired clonal mitochondrial DNA mutations in the bone marrow of patients with myelodysplastic syndromes (MDS).[5] Mutations of mitochondrial DNA (mtDNA) can cause respiratory chain dysfunction because all 13 protein genes of mtDNA encode subunits of the RC. Myelodysplastic syndromes are clonal hematopoietic stem cell disorders, characterized by ineffective hematopoiesis leading to peripheral cytopenias despite a hypercellular bone marrow. The bone marrow cells are dysplastic and genetically unstable. About 25% of cases develop into acute myeloid leukemia.

One of the dysplastic features in erythroid precursor cells is mitochondrial iron overload. We attribute this phenomenon to respiratory chain dysfunction, caused by mtDNA mutations.[6] A defective respiratory chain will consume less oxygen, thus

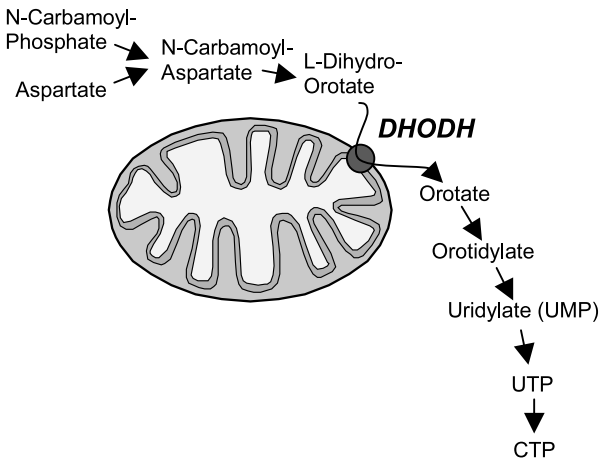

Figure 1. Pyrimidine synthesis pathway.

leaving more oxygen than usual in the mitochondrial matrix. This leads to oxidation of ferrous iron (Fe^{2+}) which is imported into mitochondria for heme synthesis. Since ferric iron (Fe^{3+}) is unsuitable for heme synthesis, it will accumulate in the mitochondrial matrix.

The bone marrow in MDS often shows megaloblastic changes, which are not caused by vitamin B12 or folate deficiency, but, considering the link between the RC and pyrimidine nucleotide synthesis, may instead be caused by respiratory chain defects.

MATERIALS AND METHODS

143B cells are a human osteosarcoma cell line which served as the parent cell line for establishing rho-0 cells. Rho-0 cells are completely devoid of mitochondrial DNA, as a result of long-term treatment with ethidium bromide. Transmitochondrial cell lines can be established by repopulating rho-0 cells with exogenous mitochondria, e.g. by fusion with platelets from patients carrying a particular mtDNA mutation.[4]

Such transmitochondrial cell lines were used to analyze the effect of a respiratory chain complex I defect on pyrimidine nucleoside synthesis (see Fig. 3).

Foreskin derived normal human *keratinocytes* were cultured in normal serum free keratinocyte medium (Gibco, Germany) supplemented with 50 μg/ml bovine pituitary extract (BPE) and 5 ng/ml epidermal growth factor (EGF), 1% Penicillin, Streptomycin and 1% Glutamin at 37°C in 5% CO_2 to near confluency. Subsequently, they were grown for one week in the presence of Antimycin A or Rotenone.

Nucleotide extraction: Cells were harvested, counted and washed with HBSS. Cells were then transferred to 1.5 ml Eppendorf polypropylene tubes and centrifuged (1200 rpm, 10 min), washed with 1 ml HBSS and centrifuged again. The supernatant was pipetted off and the cell pellet disrupted with 200 μl 10% trichloroacetic acid (TCA)

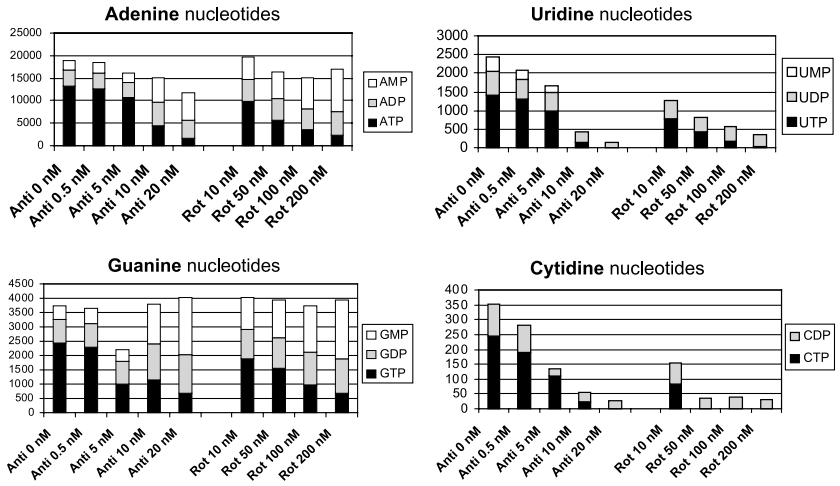

Figure 2. Nucleotide concentrations (pmol/10^6 cells) in human keratinocytes treated for one week with various concentrations of respiratory chain inhibitors Antimycin A or Rotenone.

and centrifuged for 1 min at 13,000g. The supernatants were transferred to new 1.5 ml Eppendorfs and TCA in the supernatants back-extracted with water-saturated diethyl ether to pH 5.0. Extracts were frozen at −20°C until analysis by HPLC with in-line diode-array detection as described.[1]

RESULTS AND DISCUSSION

RC inhibition resulted in no significant decrease in the total amount of purines, but a redistribution from triphosphates to monophosphates was noted, which we interpret as resulting from impaired ATP regeneration. The pyrimidines not only showed a decrease in the triphosphates but also a strong decrease in their total amount (Fig. 2). Results were very similar with the osteosarcoma cell line 143B (data not shown). UDP sugars were also decreased with RC inhibition in both 143 cells and keratinocytes (data not shown).

It was surprising that the effect of Rotenone was similar to that of Antimycin, because Rotenone blocks the RC at complex I, i.e. upstream of coenzyme Q (ubiquinone). We had the opportunity to check whether a pure complex I defect affects pyrimidine synthesis (Fig. 3).

In contrast to what we had seen with rotenone, we found no drastic effect even with mitochondria known to have undetectable complex I activity (CT4 cells). This suggests that complex I inhibition cannot fully explain the marked effect of Rotenone on pyrimidine synthesis. A possible explanation relates to the fact that Rotenone has some structural similarities to coenzyme Q. It is therefore conceivable that in the

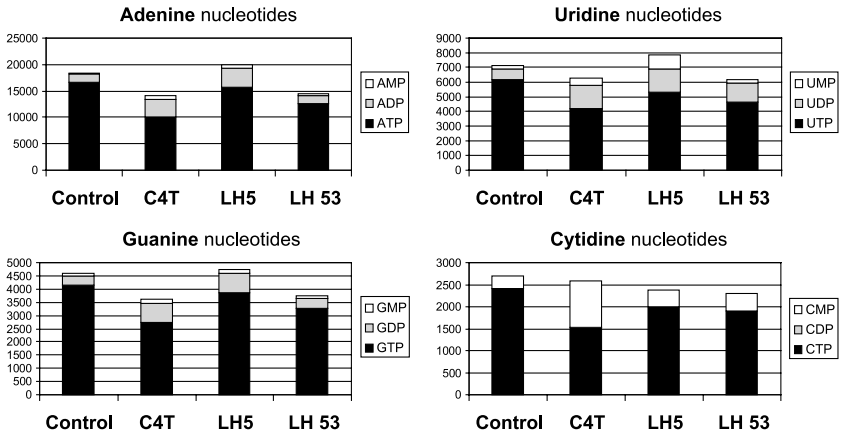

Figure 3. Nucleotide concentrations in rho-0 cells transformed with mutant mitochondria from a patient with complex I defect resulting in different complex I activities. The 143B osteosarcoma cells (parent cell line of rho-0 cells) served as a control. C4T cells, LH5 cells and LH53 cells are transmitochondrial cell lines characterized by an mtDNA mutation affecting complex I of the RC. C4T cells have undetectable complex I activity. LH5 cells have ∼ 50% complex I activity. LH53 cells carry wildtype mtDNA and have normal complex I activity.

mitochondrial membrane Rotenone somehow competes with coenzyme Q for the interaction with DHODH.

In summary, we have shown that inhibition of mitochondrial respiration causes a breakdown of purine triphosphates, probably as a result of impaired ATP regeneration, and a decrease in the total amount of pyrimidines, as a result of co-impairment of DHODH. We are currently investigating whether mitochondrial DNA mutations in MDS, by causing RC dysfunction, also lead to diminished pyrimidine synthesis. This would help to explain ineffective hematopoiesis in these disorders, as well as increased mutation rates and genomic instability, which contribute to the risk of leukemic transformation. However, it may not be easy to demonstrate impaired pyrimidine synthesis in MDS, because the effects of mtDNA mutations cannot be expected to be as strong as the effect of complete RC inhibition with Antimycin A or Rotenone.

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