Brief Report

Ineffective hematopoiesis linked with a mitochondrial tRNA mutation (G3242A) in a patient with myelodysplastic syndrome

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Abstract

In a patient with refractory anemia with excess blasts (RAEB), a somatic mutation of mitochondrial transfer RNA\textsuperscript{Leu(UUR)} was detected in bone marrow cells. Heteroduplex analysis indicated that 40-50 percent of mitochondrial DNA (mtDNA) molecules in the bone marrow (BM) carried the novel G3242A mutation. The proportion of mutant mtDNA was higher in CD34+ cells than in the unfractionated sample. Surprisingly, the mutation was not detectable by heteroduplex analysis in the peripheral blood (PB). However, PB CD34+ cells selected by immunomagnetic beads harboured the mutation with a proportion of approximately 50%. In hematopoietic colony assays, CD34+ cells from BM and PB yielded only colonies with wildtype mtDNA. These results indicate that the mtDNA mutation in CD34+ cells was associated with a maturation defect. Mitochondrial tRNA mutations impair mitochondrial protein synthesis, thereby causing dysfunction of the mitochondrial respiratory chain. We propose that this effect contributed to ineffective hematopoiesis in our patient.

Running title:

Ineffective hematopoiesis linked with mtDNA mutation
Introduction

In bone marrow cells of patients with myelodysplastic syndrome (MDS), mitochondria often show ultrastructural abnormalities, including pathological iron accumulation in the mitochondria of erythroblasts. This suggests that mitochondrial dysfunction may contribute to the pathophysiology of MDS.\textsuperscript{1,2} We are finding acquired, clonally expanded mutations of mitochondrial DNA in the bone marrow of MDS patients. Recently, we made an observation that supports the functional relevance of such mutations.

Materials and methods

Case report

A 65-year old male patient was diagnosed with refractory anemia (RA) in June, 2000. Ringed sideroblasts were not seen and the result of cytogenetic examination was normal. The patient became transfusion dependent in April, 2002. Repeat bone marrow biopsy in July, 2002, showed progression to RAEB (20% blasts). Starting in October, 2002, the patient was treated with gradually increasing doses of thalidomide (max. dosage of 400 mg/d). By the end of January, 2003, WBC had remained unchanged (2000/µl), but platelet count had improved from 25,000 to 40,000/µl and the patient had become independent of RBC transfusions (Hb 11g/dl). The marrow blast count had decreased from 18% (Sept. 2002) to 8% (Jan. 2003). Hematological parameters subsequently continued to improve.

A bone marrow sample obtained in July, 2002, was stored and later scanned for mutations of the mitochondrial genome. A heteroplasmic mtDNA mutation was identified. On follow-up (November 2002) peripheral blood was examined, which did not show the mutation on heteroduplex analysis. This discrepancy was confirmed by negative findings in further blood samples and a positive finding in material from an archived bone marrow smear (September, 2002). Analysis was then extended to
CD34+ cells from blood and bone marrow, as well as lymphocytes and platelets, all obtained on a further follow-up examination in January, 2003.

**Mutation scanning**

Heteroplasmy, i.e. coexistence of mutant and wildtype mtDNA, is typical of mtDNA diseases. Heteroduplex analysis is a suitable method for scanning the mitochondrial genome, since it usually permits the detection of less than 10% mutant mtDNA. To cover the circular mtDNA completely, we amplified 67 overlapping mtDNA segments by PCR. Amplification products were denatured and renatured to allow heteroduplex formation through hybridization. Heteroduplex detection was carried out with denaturing HPLC (the WAVE™ system, Transgenomic, Crewe, UK).

**Cell fractions**

Platelet-rich plasma was obtained from citrated blood samples by centrifugation at 100g for 15 minutes. Contaminating white blood cells were removed by centrifugation at 200g for 10 minutes. Mononuclear cells (MNC) were obtained from blood and bone marrow through density gradient centrifugation using Lymphoprep 1.077 (Nycomed, Oslo, Norway). From the MNC interphase, CD34+ cells, T-lymphocytes (CD3+), and B-lymphocytes (CD19+) were isolated with immunomagnetic beads according to the manufacturer's instructions (Miltenyi, Bergisch Gladbach, Germany). Total DNA was isolated from the respective cell fractions using the QIAamp blood kit (Qiagen, Hilden, Germany).

**Colony assays**

CD34+ cells were plated in quadruplicate (2x10^4 cells per dish) in a volume of 1 mL serum-free methylcellulose medium with recombinant cytokines: stem cell factor, GM-CSF, IL-3, IL-6, G-CSF, and erythropoietin (MethoCult H4436; StemCell Technologies, Vancouver, Canada). After 12 days of incubation at 37°C and 5% CO₂, 50 colonies
derived from bone marrow and peripheral blood, respectively, were picked under an inverted microscope. DNA from colonies was extracted using the QIAamp blood kit (Qiagen, Hilden, Germany). Because of the small amount of DNA from colonies, carrier DNA was added (5µg Poly-dA-dT, Sigma-Aldrich, Steinheim, Germany).

**Results and Discussion**

According to our experience so far, heteroplasmic mtDNA mutations can be detected in about 50% of MDS patients. In the patient under consideration, a heteroduplex pattern was detected in a segment of mtDNA including nucleotide positions 2762-3389. DNA sequencing confirmed a novel heteroplasmic mutation G→A at nt3242. The mutation was not detectable in the patient's buccal mucosa cells. The mutation was unique among 80 patients with MDS whose mtDNA from bone marrow samples was scanned by heteroduplex analysis.

Figure 1 shows the results of heteroduplex analysis in different cell fractions. Unfractionated bone marrow yielded a heteroduplex pattern on several occasions. The proportion of mutant mtDNA appeared to be lower in January, 2003, than in July or September, 2002. This might be due to more extensive admixture of peripheral blood cells (representing wild type mtDNA). However, as the patient had been treated with thalidomide since October, 2002, and showed hematological improvement as well as a reduction in bone marrow blasts (from nearly 20% to less than 10%), it is reasonable to conclude that the MDS clone probably decreased in size.

Bone marrow CD34+ cells showed a more prominent heteroduplex finding than whole bone marrow (January, 2003). CD3+ and CD19+ lymphocytes from the marrow yielded a single homoduplex peak representing wildtype mtDNA. In the blood, CD34− MNC, as well as platelets, also showed a single wildtype homoduplex peak. In contrast, PB CD34+ cells, isolated by immunomagnetic beads, gave a clear heteroduplex signal. The
small number of circulating CD34+ cells explains why a heteroduplex pattern was not
detectable in whole blood samples.

The hematopoietic colony assays yielded approximately 25-30 erythroid bursts/colonies,
10 mixed colonies, and 15 granulocyte-macrophage colonies per 10^4 CD34+ cells
seeded. When mtDNA was PCR-amplified from individual hematopoietic colonies,
heteroduplex analysis always showed a single homoduplex peak, irrespective of the
type of colonies harvested (22 x CFU-GEMM, 7 x BFU-E, 11 x CFU-E, 10 x CFU-GM).
The homoduplex peaks derived from colonies represented wild type mtDNA, because
mixing PCR product from each colony with PCR product from wild type mtDNA always
yielded a single homoduplex peak on dHPLC analysis (data not shown).

Figure 2 shows how the mitochondrial tRNA mutation may be related to bone marrow
pathology. First, the mutation must have occurred in a transformed stem cell with
considerable self-renewal capacity. Otherwise it is hard to explain how mutant mtDNA
came to represent around 50% of mtDNA molecules in BM and PB CD34+ cells.

Second, the mutation must be compatible with survival and proliferation in the stem cell
compartment, because selection against affected clonogenic cells would else have
eliminated the mutation. Third, strong selective pressure against cells harbouring the
G3242A mutation must be operative in the differentiation/maturation compartment,
since the proportion of mutant mtDNA was low in mature cells reaching the circulation.
Mixing experiments indicated that in our hands the detection limit was about 3%.

Heteroplasmic mtDNA mutations are subject to random segregation, which can give rise
to daughter cells harbouring predominantly normal or predominantly mutant mtDNA. In
our patient it seems that very few cells arrived at a proportion of mutant mtDNA low
enough for the cells to escape destruction. It is also possible that the G3242A mutation
in the preleukemic stem cell was homoplasmic rather than heteroplasmic, thus
precluding the generation of daughter cells with low-level heteroplasmy. In that case, the heteroduplex pattern obtained in the CD34+ fraction must be attributed to a mixture of normal and abnormal progenitor cells. Since we have no chromosomal marker, we cannot determine the proportion of healthy polyclonal CD34+ cells in our patient.

Irrespective of the mutation being homoplasmic or heteroplasmic at the cellular level, it appears obvious that selection eliminated differentiating cells harbouring the mutation. It is tempting to speculate that the majority of maturing cells died by apoptosis, probably triggered by mitochondrial dysfunction.

The G3242A mutation, changing a conserved nucleotide, is immediately adjacent to the A3243G mutation which causes an hereditary disease called MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like symptoms) \(^3,^4\). The MELAS mutation impairs mitochondrial protein synthesis, thereby leading to respiratory chain (RC) dysfunction. MELAS patients are not known to have ineffective hematopoiesis. However, the proportion of mutant mtDNA in their leukocytes is significantly lower than in skeletal muscle (e.g. 58% vs. 84%) \(^5\). This suggests that, in the highly proliferative hematopoietic system, the MELAS mutation is also subject to negative selection.

Our findings suggest that hematopoietic stem cells are less vulnerable to the effects of mitochondrial dysfunction than their maturing progeny. This appears plausible since stem cells are mainly concerned with self-maintenance. Their energy requirements may be met by glycolytic ATP production, without much oxidative phosphorylation in the mitochondria. In contrast, differentiating cells appear much more dependent on mitochondrial activity, not just for ATP regeneration. In erythropoietic cells, for example, the major metabolic pathway, heme synthesis, is strongly dependent on mitochondrial respiratory chain activity \(^6-^8\). Erythropoiesis may therefore be particularly vulnerable to the consequences of mtDNA mutations.
MtDNA mutations with a mild effect on mitochondrial function may be detectable in the peripheral blood because they are sufficiently compatible with progenitor cell maturation. At present, our collection of DNA from patients with MDS is based on bone marrow cells. In the future, we shall try to collect blood samples in parallel, since comparison of mutant mtDNA proportions in blood vs. bone marrow (or mature blood cells vs. PB CD34+ cells) may be useful in assessing the severity of mtDNA mutations in the hematopoietic system.

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References

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**Figure 1**

**Heteroduplex analysis with denaturing HPLC of mtDNA fragment 2762-3389 at 59°.**

PB: peripheral blood; BM: bone marrow; PRP: platelet rich plasma.

* homoduplex peak, representing homoduplex wildtype as well as homoduplex mutant mtDNA. The small shoulder in the homoduplex peak was also present in normal controls.

** heteroduplex peak, representing the heteroduplex species formed by wildtype and mutant mtDNA strands after denaturation and slow renaturation of the PCR product. Since heteroduplex molecules contain a destabilizing physical “bubble” of the two DNA strands at the site of the mismatch, they denature at lower temperature than the corresponding homoduplex. Because of the resulting increase in single-strandedness, heteroduplex molecules more easily desorb from the HPLC column bead surface and thus have a shorter retention time.
Figure 2
Random segregation of mitochondrial DNA as a determinant of cell fate in an MDS clone harbouring a mtDNA mutation. For explanation, see text. Oval forms represent mitochondria (black: with mutant mtDNA). Red crosses denote apoptotic cells eliminated by macrophages.
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