Short Communication

Heteroplasmic ratio of the A3243G mitochondrial DNA mutation in single pancreatic beta cells

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Abstract

Aims/Hypothesis. To examine whether there is a high content of mutated mitochondrial DNA in individual pancreatic beta cells from a patient with the A3243G mitochondrial DNA mutation.

Methods. Tissues were available from a patient with diabetes and the A3243G mutation including pancreatic tissue. We quantified the amount of mutated mitochondrial DNA in tissue homogenates and single pancreatic beta cells using hot last cycle PCR.

Results. The percentage ratio of mutated to wild-type mtDNA was high in tissues such as muscle and brain (>60%), but surprisingly low in both pancreatic islets

and in individual beta cells from these islets. The islets were smaller in the patient than in control subjects in keeping with a decreased beta-cell mass.

Conclusions/interpretation. These observations suggest that either the beta cells show increased sensitivity to the effects mtDNA mutations on respiratory chain function, and/or cells with a high mutant load are preferentially removed leading to a progressive decrease in the islet beta-cell mass. [Diabetologia (2003) 46:296–299]

Keywords Mitochondrial DNA, A3243G mutation, pancreatic beta cells.

Current evidence suggests that mitochondrial DNA (mtDNA) abnormalities account for approximately 1% of all cases of diabetes [1]. The most commonly described mtDNA defect in patients with diabetes is the A3243G transition in the tRNA^{Leu(UUR)} gene. Impaired insulin secretion is a key feature of diabetic patients with mtDNA defects. Studies in pancreatic beta-cell lines have shown that mitochondrial function is pivotal for several components of insulin secretion [2]. Since defects of mtDNA result in low respiratory

chain activity, it is logical that insulin secretion will be impaired in these patients. However, there are many unresolved issues, not least why pancreatic beta cells should be so susceptible to mtDNA defects compared to other metabolically active cells in the body.

Mitochondrial genetics is complicated by multiple mitochondrial genomes within individual mitochondria and thus several hundreds or thousands of copies within an individual cell. The defects of mtDNA described in patients with diabetes are heteroplasmic, which is a mixture of mutated and wild-type DNA within an individual cell. The percentage ratio of mutated to wild-type mtDNA seems to be crucial since a biochemical defect is only seen in cells if there is a high ratio of mutated compared to wild-type mtDNA. In most patients with mtDNA defects there is involvement of post-mitotic metabolically active tissues in which the amount of mutated mtDNA is much higher than in dividing tissues. We were particularly keen to see if this was also true for pancreatic beta cells and if this could explain the common occurrence of diabetes

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Abbreviations: mtDNA, mitochondrial DNA; COX, cytochrome c oxidase; SDH, succinate dehydrogenase.

in our patients. For these studies we had the opportunity to study the percentage ratio (heteroplasmy) in several different tissues from one patient with the A3243G mutation and diabetes.

Subjects and methods

Clinical details and tissue preparation. The patient was a 46-year-old man who had recurrent stroke-like episodes, seizures and cognitive decline. On investigation he was found to have the A3243G mutation. Diabetes was diagnosed by OGTT and managed initially by dietary modification, and impaired insulin secretion was confirmed by IVGTT. He died 1 year after the diagnosis of diabetes. Pancreatic tissue was obtained at post mortem examination (pm delay <4 h). Control pancreatic tissue was obtained during surgical removal of the pancreas for ampullary carcinoma from non-diabetic patients of similar age. Tissue was rapidly frozen in super-cooled isopentane and stored at -80°C .

The study was approved by the Newcastle and North Tyne-side Joint Ethics Committee.

Histology and histochemistry. Frozen sections of pancreas and muscle were cut at 10 μm thickness. Cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) activities were examined histochemically. Islets were identified in pancreatic sections immunohistologically with a mouse anti-human insulin antibody (dilution 1/100, Sigma I2018) by the indirect immunoperoxidase method using rabbit peroxidase conjugated anti-mouse antibody (Dako P0206). Antibody binding was visualised with 3,3'-diaminobenzidine.

DNA extraction and quantification of the A3243G mutation. DNA was extracted from tissues using standard techniques. Whole islets, beta cells and acinar cells were identified on the pancreatic sections following immunohistological staining for insulin. Islets and individual cells were isolated, lysed and PCR amplified [3]. Quantification of the amount of the A3243G allele in tissue and cells was done by hot last cycle PCR [3].

Quantification of mtDNA in single cells. Single beta cells isolated from islets (sections 10 μm) stained with anti-insulin antibody were lysed and the amount of mtDNA per single pancreatic beta cell was calculated by comparing the radioactivity incorporated in the templates with that incorporated in the standards by the method used [4].

DNA sequence analysis of single cell PCR products. Extracted DNA from single cells was amplified using the M13-tailed PCR primers L3218 and H3353 [5] to amplify the region containing the A3243G mutation.

Image analysis of anti-insulin labelled pancreatic tissue sections. Pancreatic sections from both the patient and the four control subjects were stained with anti-insulin antibody (counterstained with haemalum). Images were captured using a JVC KY-F55B digital camera under identical conditions. Image analysis was carried out using the UTHSCSA ImageTool program (v.2) (University of Texas Health Science Centre at San Antonio, Texas, USA available from <http://ddsdx.uthscsa.edu/dig/itdesc.html>).

Statistical analysis. Comparisons were made using Student's *t* test, and differences were considered statistically significant if there was a *p* value of less than 0.05.

Table 1. Percentage ratio of heteroplasmy of the mitochondrial DNA A3243G point to mutation in tissues from the patient

	Patient
Skeletal Muscle	60%
Occipital Cortex	78%
Cerebellum	66%
Liver	60%
Pancreas	31%
Kidney	75%
Myocardium	58%
Blood	8%

Results

Investigation of the A3243G mutation in various tissues and organs. The percentage ratio of heteroplasmy of the A3243G mutation was measured in various tissues obtained at post-mortem (Table 1). The results obtained are for whole tissue homogenates and are consistent with other reports showing the tissue-specific differences in the amounts of mutated mtDNA. High percentage ratios were found in post-mitotic tissues such as skeletal muscle and brain. A surprising finding was the relatively low ratio in the pancreas. However, in a homogenate the predominant cell type will be acinar cells and thus would not reflect the situation in the islets.

A3243G mutation in whole islets and acinar tissue. To investigate the situation further we studied the percentage ratio of heteroplasmy of the A3243G mutation in single isolated pancreatic islets and acinar tissue. The ratio of mutated to wild-type mtDNA was low in the islets ($20 \pm 9\%$ for 5 islets) and acinar tissue ($27 \pm 5\%$ for six acinar tissue samples).

A3243G mutation in single pancreatic cells. One possible explanation for the above result is that there was marked variability in the percentage heteroplasmy between different individual pancreatic beta cells. We therefore investigated the possibility of measuring the percentage ratio of mutated to wild-type mtDNA in individual beta cells. Firstly, we wanted to assess the feasibility of this by measuring the total amount of mtDNA within individual beta cells. The mean amount of mtDNA per beta cell obtained for the patient and a control subject was $0.028 \pm (\text{SEM}) 0.014$ pg, and 0.059 ± 0.022 pg (vs patient, $p=0.25$), respectively. We then assessed the sensitivity of the PCR and restriction digest to accurately quantify the percentage ratio of heteroplasmy with this amount of mtDNA. Using muscle homogenate DNA, we were able to show the assay was quantitative over a range of 0.025 pg–1 μg of DNA.

To enable us to correlate the percentage heteroplasmy in the individual beta cell with that in the whole islet, we isolated three individual beta cells from each islet. We then took the rest of the islet and

Fig. 1A, B. Percentage ratio of heteroplasmy (**A**) and size of islets (**B**) in a patient with A3243G mitochondrial DNA mutation. **A** The percentage heteroplasmy for individual beta cells (empty column; $n=3$) followed by that for the islet from which they were obtained (filled column) is given for 8 islets. **B** The size of the islets (n =number) from the patient was compared with those from 4 control subjects and shown to be smaller (Student's t test; $p<0.0001$)

isolated DNA and measured the percentage heteroplasmy in the remaining islet tissue. These results confirm that the amount of mutated mtDNA is relatively low in the islets and this was reflected in the low percentage ratios of heteroplasmy found in the individual beta cells (Fig. 1).

Quantification of A3243G in individual muscle cells.

In view of these findings we wished to examine whether changes in other tissues were the same or corresponded with what we believe is more typical of this syndrome. Single muscle fibres which showed both COX positive and deficient histochemistry were isolated, lysed and analysed by RFLP-PCR using identical conditions to those used for the pancreatic single cells. In the muscle from the patient we found the percentage ratio of mutated to wild-type mtDNA in COX-positive fibres was $60\% \pm 15$, and in COX deficient fibres $93\% \pm 0.4$.

Exclusion of nuclear pseudogene amplification as a cause of the low percentage ratio of heteroplasmy in pancreatic beta cells. In a report from this laboratory [5], the presence of a pseudogene containing a sequence similar to that around the site of the A3243G mutation was reported and amplification of this pseudogene, in preference to the mtDNA could have explained some of our results. The sequence generated from both single cell preparations ($n=8$) and pooled cell (2–3 cells, $n=4$) preparations was identical to the Cambridge reference sequence, which confirms that the products were derived from mtDNA and not the nuclear pseudogene.

Estimation of the size of islets and islet histochemistry. Islets in pancreatic sections were identified following staining with anti-insulin sera and the size of the beta-cell mass in islets was calculated. Images were captured under identical conditions and analysed (Fig. 1). This shows that the patient has a smaller beta-cell area in their islets in comparison with the four control subjects ($p<0.0001$). The islets from the patient showed no evidence of a histochemical defect when analysed for COX and SDH activity.

Discussion

We believe that our results are surprising in terms of the low percentage heteroplasmy of the A3243G mutation in islets and individual pancreatic beta cells. We predicted that we would find high amounts of mutated mtDNA in islets and that this would explain the high incidence of diabetes in patients with the A3243G mutation. Our studies have in fact shown the opposite, with the pancreatic tissue from our patient, including the islets, containing a remarkably low percentage ratio of mutated to wild-type mtDNA. There are several potential explanations for our findings. The first is that our results are artefacts of the techniques used. We believe that this is not the case since we have included the appropriate controls at each step. The amount of mutation observed was not due to the amplification of pseudogenes. The technique of single-cell lysis followed by PCR and restriction digest in a different tissue (skeletal muscle) gives results identical to those found by other authors [6].

The second possible explanation is that the patient studied was in some way atypical of patients with the A3243G mutation. We do not believe this to be the case. Our patient had the expected high percentage heteroplasmy in post mitotic tissues such as brain and muscle similar to other patients described with the A3243G mutation.

A third possible explanation is that beta cells are particularly sensitive to the respiratory chain defect produced by the A3243G mutation. Evidence is emerging from magnetic resonance spectroscopy that

there could be a biochemical defect even at very low levels of the A3243G mutation [7]. This evidence is in conflict with some of the results from studies on cultured cells and in COX-deficient muscle fibres but might in fact reflect the situation *in vivo*. Thus, even the relatively low percentage ratios of heteroplasmy might cause impaired insulin secretion.

The fourth explanation relates to changes seen in islet size. One observation from our study is that the islet size is smaller in the patient compared to the control subjects. This is similar to the observations made in other patients with mitochondrial diabetes [8, 9] and suggests that decreased beta-cell mass is a factor in the development of diabetes. It is interesting to relate these changes to those observed in a mouse model of mitochondrial diabetes [10]. Pancreatic islets from 7-week-old diabetic animals contained COX deficient beta cells indicating a defect of respiratory chain function. However, when older diabetic animals were studied, the COX deficient beta cells were no longer evident and the beta-cell mass was decreased. This suggests a loss of beta cells with a high mutant load, although there was no evidence of increased apoptosis. The absence of COX deficient beta cells together with the decreased islet size in our patient raises the possibility that the same mechanisms could be operating.

Previous studies of patients with the A3243G mutation and diabetes have been rather limited. Specifically, other studies have not had the opportunity to study the amount of mutated mtDNA in individual beta cells. In a patient of similar age to ours but with insulin-dependent diabetes, higher percentage ratios of mutated to wild-type mtDNA were found in islet (63%) compared to acinar (32%) tissue [8]. However, this difference in the percentage ratios of heteroplasmy between acinar and islet tissues was not observed by ourselves nor by other investigators [9].

Finally, we have to ask what impact these findings have on diabetes as a whole. There is good evidence that with age, mtDNA acquires mutations and these mutations can reach amounts that impair mitochondrial function. It is likely that these mutations accumulate to a greater extent in highly metabolically active tissues such as pancreatic beta cells. Under these cir-

cumstances, the sensitivity of the beta cells to the effects of these mutations on cellular function and overall beta-cell mass may be very important to the age-related increase in the prevalence of Type 2 diabetes.

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