cations in the stability data published previously for this assay [3]. Both CIS tracers showed similar N-terminal specificity consistent with other published data [4]; assay specificity may be determined by the solid phase.

Samples obtained from a population of children with renal insufficiency were used as a model system for high concentrations of both endogenous Oc and circulating fragments. Interference for antibodies reporting Oc species in each assay was assessed by observing dilution linearity from this system. We think that this interference most likely results from the recognition of circulating fragments of Oc in the serum by component antibodies that limit capacity to report the osteocalcin species that each assay is capable of reporting. When samples were diluted according to the manufacturers’ protocol, observed recoveries at a 1:8 dilution were: DSL Active >700%, Nichols >250%, CIS ELSA-OST(1–43) >200%. Observed mean (±range) at 1:8 for both CIS ELSA-Ost-Nat(1–49) and the INCSTAR N-tact Osteo SP were 100% (±20%). Because of the lack of linear results from the dilution experiments, the assays that lack linearity must be viewed with extreme caution when it comes to clinical utility.

The calibrations of all kits were assessed as unknown values in the INCSTAR assay in an effort to normalize all of the calibrations with the following results. The average values in the INCSTAR assay in an effort to normalize all of the assays that lack linearity must greatly improve the clinical utility of this analyte.

The calibrations of all kits were assessed as unknown values in the INCSTAR assay in an effort to normalize all of the calibrations with the following results. The average percent (expected/observed) was: CIS kits ~27%, Nichols +32%, DSL +50% at 1.0 ng/mL to ~20% at 25.0 ng/mL. INCSTAR standards are intact (1–49) purified from human bone and calibrated to quantitative amino acid analysis verified in independent laboratories. Complete assessment of specificity and limitations of Oc immunoassays will greatly improve the clinical utility of this analyte.

References


Since the molecular basis of the first mitochondrial DNA (mtDNA) disorder, Leber hereditary optic neuropathy (LHON), was established [1], mtDNA mutations have become increasingly more recognized as an important cause of genetic disease. The most common mutations identified are A3243G, A8344G, T8993G/C, G11778A, and large mtDNA deletions. The A3243G mutation accounts for 80% of patients with MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) [2], and the A8344G mutation accounts for 80% of patients with MERRF (myoclonic epilepsy and ragged-red fibers) [3, 4]. Substitution of T8993 with G or C causes NARP (neuropathy, ataxia, retinitis pigmentosa) [5, 6], and the G11778A mutation is responsible for >50% of patients with LHON [1]. MtDNA deletions and rearrangements are found in Kearns–Sayre syndrome and chronic progressive external ophthalmoplegia patients.

Most pathogenic mtDNA mutations are heteroplasmic, with normal and mutant DNA coexisting in the same cell or tissue. The phenotypic expression of a pathogenic mutation depends on tissue-specific energy requirements [7]. Accordingly, age of onset, tissues involved, and clinical severity vary greatly between individuals, even within a given family, depending on the proportions of mutant and normal mtDNA. Direct mtDNA mutation analysis allows for identification of asymptomatic relatives who harbor the mutant mtDNA. Prediction of future disease expression is assisted by quantitative determination of the proportion of mutant mtDNA in the relevant tissues.

DNA-based molecular diagnosis of mtDNA disorders has been routinely performed on blood or biopsied skeletal muscle [8, 9]. Because of variation in percentage of mutant mtDNA among different tissues, mutant mtDNA may not be detectable in blood. Nevertheless, it is difficult to justify obtaining muscle biopsies from multiple asymptomatic at-risk family members. In this setting, the examination of alternative tissues such as cheek cells or hair follicles is especially relevant. Studies of MELAS mutant heteroplasmy in hair follicles have been reported [10, 11]. Although the use of hair follicles still plays an important role in mutation analysis of mtDNA [10, 11], at present the data are insufficient to validate this method.

Here we describe the use of hair follicles and cheek cells for quantitative screening of mutant mtDNA in affected and asymptomatic family members; the patients studied represented different mitochondrial diseases. Patients in whom mtDNA disorders were suspected were referred to the Molecular Genetics Laboratory at Children’s Hospital Los Angeles for mutation analysis. Probands and asymptomatic family members; the patients studied to justify obtaining muscle biopsies from multiple asymptomatic at-risk family members. In this setting, the examination of alternative tissues such as cheek cells or hair follicles is especially relevant. Studies of MELAS mutant heteroplasmy in hair follicles have been reported [10, 11]. Although the use of hair follicles still plays an important role in mutation analysis of mtDNA [10, 11], at present the data are insufficient to validate this method.

Genomic DNA was extracted from peripheral blood samples with a salting-out procedure [12]. Hair follicles (~10–20) obtained from various areas of the scalp were digested for 16 h at 37°C with 20 μg of proteinase K in 100 μL of 50 mmol/L Tris buffer, pH 8.5, containing 1 mmol/L EDTA and 5 mL/L Tween 20 [10]. Subsequently, the digested mixture was heated to 95°C for 10 min to
inactivate proteinase K, and 10 μL of the heated digest was used directly for PCR. Cheek cells collected with a cytology brush were washed and vortex-mixed with 1–3 mL of phosphate-buffered saline. The cell suspension was centrifuged, and DNA was extracted from the cell pellet by the same protocols as were used for hair follicles.

Oligonucleotides homologous to heavy-strand position 3116–3134 and light-strand position 3335–3333 of the mtDNA sequence were used as primers for PCR amplification of the MELAS region. An A to G mutation at 3243 positions a HaeIII site, which we used to identify mutants. The forward primer used for MERRF was 5’CTACCCCCCTCTAGAGCCCACTG, which has been modified to create a BglII site in the presence of a mutation. The primers used for NARP syndrome were 5’TATGATTAATGTTGCGCAATTTACTG-TAAAGCCGTGTTGG3’, which has been modified to create a MspI site in the presence of a mutation. The primers used to identify mutation carriers were identified.

We amplified 100 ng of total DNA in a 25-μL reaction mixture containing 1× Mg-free Taq DNA polymerase buffer, 200 μmol/L of each dNTP, 1.5 mmol/L MgCl2, 1 μmol/L of each primer, and 1 unit of Taq DNA polymerase. The reaction mixture was heated to 94 °C for 4 min, followed by 25 cycles of 1 min of denaturation at 94 °C, 1.5 min of annealing at 55 °C, and 3 min of extension at 72 °C. In the final cycle, the reaction was held at 94 °C for addition of 2 μCi of [α-32P]-dCTP to each tube, followed by a final extension time of 8 min. Quantitative analysis of mutant mtDNA was performed according to published procedures [6, 10, 13]. The PCR products were digested with 10 units of restriction enzyme at 37 °C for 2 h and loaded onto a 16-cm 120 g/L acrylamide:bisacrylamide (29:1 by wt.) gel. Electrophoresis was carried out at 15 W for 1 h in a Tris–borate–EDTA buffer. The radioactivity in each fragment was measured with a Betascope 603 (Betagen, Waltham, MA) blot analyzer, and the percentage of mutant mtDNA was determined. Mitochondrial DNA deletion was determined by Southern transfer analysis.

Affected and at-risk family members were screened for mtDNA mutations by routine molecular analysis on blood. Given that the proportion of mutant mtDNA can vary from tissue to tissue, additional tissues were analyzed. Hair follicles and cheek cells were selected as alternative, readily available tissues that could be obtained noninvasively. Results of quantitative studies indicated that the proportion of mutant mtDNA in blood correlated with that in hair follicles and cheek cells (Fig. 1). The slopes of these positive correlations are 1.05 for hair follicles and 1.01 for cheek cells (r = 0.955 and 0.965, respectively). Other than the three patients who have >90% of mutant mtDNA in both blood and muscle, the percentage of mutant mtDNA in muscle (□ in Fig. 1) is notably higher than that in the other tissues tested for all but one patient. When the mutant load is high (>90%) in blood, as in the T8993C/G mutation, which generally requires higher mutant load than A3243G mutation to cause pathogenic expression, the variation in mutant heteroplasmy among different tissues is minimized.

Although muscle is clearly the more relevant tissue, the most common specimen submitted for routine molecular diagnosis of mtDNA disorders is blood. Given the heterogeneous distribution of mutant mtDNA, it is important to correlate mutant heteroplasmy among various tissues. Besides blood, we also selected hair follicles and cheek cells for comparative studies. For patients who have heteroplasmic mutant mtDNA, identification of the mutation is only the first step in sorting out an often-complex clinical picture, which subsequently should involve determination of the proportion of mutant mtDNA. For accurate quantification, we use a “last cycle hot PCR” method followed by Betascope 603 measurement of the amount of radioactivity present in the DNA bands corresponding to the mutant and normal DNA fragments [14]. Utilizing this approach, we demonstrated good correlation between the proportions of mutant mtDNA in hair follicles, cheek cells, and blood, consistent with the findings of comparative studies of mutant mtDNA in blood and muscle [15] (although in most of the patients in our study, the mutant mtDNA was still higher in muscle than in the other tissues studied). These results indicate that hair follicles and cheek cells are reliable tissues to be used for mutation analysis of mtDNA, and that pooling hair follicle samples minimized the discrepancies of single hair follicle analysis [10].

Noninvasive sampling is especially advantageous when screening asymptomatic, at-risk family members. However, the lack of identifiable mutant mtDNA in blood from known mutation-positive individuals has been reported [8, 16]. In such cases, depending on the clinical scenario, it may be advisable to proceed with more-invasive testing, especially when carrier status is in question. This phenomenon is demonstrated in one of our patients with mtDNA deletion (Fig. 1, □ on y-axis).

In conclusion, our results demonstrate that the propor-
tion of mutant DNA in hair follicles and cheek cells correlate well with that in blood. Hair follicles and cheek cells are convenient, noninvasive sources of tissue that, in addition to blood, are suitable for the quantification of mutant mtDNA in probands when the relevant tissues are unavai

able or when a suspected mutation is not detectable in blood of at-risk members of known mtDNA mutation-containing kindreds. Thus qualitative and quantitative mtDNA analysis can assist in genetic counseling and anticipatory guidance in individuals at risk for mitochondrial disease.

We thank the clinicians and patients for participation in this study.

References


Plasma Renin Activity: Temperature Optimum at ~45 °C. Joop H.A. Roding*, Ton Weterings, and Cees van der Heiden (BCO Analytical Services, PO Box 2176, 4800 CD, Breda, The Netherlands; *author for correspondence: fax +31 765 737777, e-mail info@BCO.NL)

Renin (EC 3.4.23.15) catalyzes the proteolytic degradation of angiotensinogen to angiotensin-I. Plasma renin activity can be assessed by detection of the amount of angiotensin-I produced under well-defined incubation conditions. Despite numerous studies of incubation conditions [1–8], the temperature optimum of the renin enzyme activity and the effect of the assay temperature on the reproducibility of renin activity measurement have not been reported. Here we draw attention to the temperature effect on the human plasma renin activity over the range between 30 and 50 °C.

Blood samples from two men, coded A and B, ages 34 and 40 years, were collected in 10-mL EDTA-containing evacuated polyester tubes (Venoject II, type VP-100DK; Omnilabo Nederland, Breda, The Netherlands) and mixed for 1 min. Within 10 min of collection, each sample was centrifuged at ambient temperature for 15 min at 1300 g. The plasma obtained was separated from the blood cells within 10 min thereafter and, with the use of polyethylene pipettes, was transferred into polystyrene tubes in small aliquots. Also used were an EDTA-anticoagulated plasma selected without conscious bias from left-over laboratory (“waste”) samples (coded C) and a RIANEN Renin kit (see below) control sample (coded D). Subjects’ samples were stored at ~−18 °C until analysis; the kit control sample was reconstituted with ice-cold distilled water; aliquots were stored in polystyrene tubes as instructed in the package insert. Samples were thawed at room temperature within 1 h of assay.

Renin activity was measured with the RIANEN (NEA-104, 105; Du Pont NEN Research Products, Boston, MA) test kit, including the renin kit control. Reconstitution and dilution was performed with doubly distilled, pyrogen-free water (B. Braun NPBI, Oss, The Netherlands). Into noncoated tubes kept on melting ice were added consecutively 500 μL of sample, 10 μL of dimeracpol solution, 10 μL of 8-hydroxyquinoline solution, and 1 mL of maleate buffer, pH 6.0. After mixing, we obtained split samples for incubation at two temperatures by transferring 1.0 mL of each sample into a second tube. The first series was incubated at ~4 °C for 60 min; the second series was incubated for the same period at a fixed temperature (~0.3 °C) selected from the range of 30–50 °C. After this incubation period, all samples were put on melting ice. From each, 100 μL was transferred into each of two duplicate tubes, followed by addition of 100 μL of 125I-labeled angiotensin-I solution and 100 μL of antisemur solution, and mixed thoroughly. Similarly, 100 μL of calibrators (in duplicate) were mixed with 100 μL of 125I-labeled angiotensin-I solution and 100 μL of antisemur solution. After incubation for 2 h at ambient temperature, 500 μL of second antibody solution was added to each sample tube and mixed thoroughly. After