pletely inhibited by anti-DNase I antibody but not by anti-DNase II antibody, confirming that serum DNase activity was derived from DNase I.

The enzyme is able to diffuse more rapidly in the CAM than in agarose gel because the pore size in the CAM matrix may be larger than that in the agarose gel matrix on the SRED plate. Moreover, even a small amount of the enzyme can efficiently digest substrate DNA because the amount of DNA retained in the thin CAM is smaller than that contained in the gel. These properties of the SRED/CAM method produced better results, and the assay was more rapid and sensitive for determination of DNase I activity in this study than the conventional SRED method. When we assayed known amounts of human DNase I separately by the SRED/CAM and conventional SRED methods, we observed no significant difference between the amounts of enzyme measured by each method (n = 20; P >0.5, t-test).

We have developed ethidium bromide-SRED and SG-SRED methods for DNase activity (5, 6). The SG-SRED method is very sensitive and able to measure DNase I concentrations in the picogram range after incubation of 16 h at 37 °C (6). Because DNase I activity in serum samples derived from healthy individuals is low, a long incubation period is required for its determination by conventional SRED methods (5, 6). In our recent study, DNase I activities in serum were found to be transiently increased in patients with AMI within 2 h after the onset of chest pain, whereas only a few patients showed an increase in cardiac markers, such as creatine kinase MB and cardiac troponin T, during this period (7). As an example, in a typical case, serum DNase I activity in an 80-year-old female patient with AMI was measured periodically by this method and gave results of 30 U/L for a sample collected 2 h after onset, 9.0 U/L for a sample collected 12 h after onset, and 8.9 U/L for a sample collected 24 h after onset; the 24-h value was similar to her outpatient values (reference interval, 7.1–14.3 U/L). Thus, an abrupt increase in serum DNase I activity accompanying the onset of AMI could serve as a novel biochemical diagnostic marker for AMI in the very early phase after onset. Early diagnosis of AMI allows more appropriate and earlier therapy, such as reperfusion and thrombolysis, to be administered to patients.

In conclusion, we have succeeded in developing a sensitive and rapid SRED/CAM DNase I assay method that is convenient and reliable for determining picograms to femtograms of DNase I in 1-μL serum samples within 30 min.

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References

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Identification of Twelve Polymorphisms in the Endothelin-1 Gene by Use of Fluorescently Labeled Oligonucleotides and PCR with Restriction Fragment Polymorphism Analysis, Konstanze Diefenbach,1 Farhad Arjomand Nahad,1 Christian Meisel,1 Ingo Fietze,2 Ingolf Cascorbi,3 Karl Stungl,4 Olfter Landt,5 Reinhold Kerb,6 Ulrich Brinkmann,6 and Ture Roots1 (1 Institute of Clinical Pharmacology, 2 Sleep Medical Centre, and 3 Department of Internal Medicine, Charité, Humboldt University of Berlin, Berlin, Germany; 4 Institute of Pharmacology, Ernst Moritz Arndt University, Greifswald, Germany; 5 TIB MOLBIOL, Syntheselabor, Berlin, Germany; 6 Epidauros Biotechnology AG, Bernried, Germany; * address correspondence to this author: Institute of Clinical Pharmacology, University Hospital Charité, Campus Charité Mitte, Schumannstrasse 20/21, 10117 Berlin, Germany; fax 49-30-450-525932, e-mail christian.meisel@charite.de)

Of the three endothelin peptides, endothelin-1, -2, and -3, endothelin-1 (EDN1) is the predominant isoform in the cardiovascular system. EDN1 plays an important role in the cardiac and vascular system, to be administered to patients.

Features of systemic lupus erythematosus in Dnase1-deficient mice. Nat Genet 1995;25:177–81. of the human EDN1 gene (6836 nucleotides), which is located on chromosome 6p23-p24. The mature 21-amino acid EDN1 is generated by subsequent enzymatic cleavage of the big-EDN1 (1). Eight variants of EDN1, which
may influence the hereditary risk of cardiovascular diseases, including coronary heart disease, hypertension, and ventricular arrhythmia (5–12), have already been located and examined:

- T/A transversion at position −1398 (from the start of transcription), which is in complete allelic association with the G/A transition at position −1396 (T −1396A, G −1396A) (13)
- T/G transversion at position −1370 (T −1370G) (8)
- Insertion A in the 5′-untranslated region (exon 1) at position +138 (+138/ex1ins/delA) (6, 8, 11)
- G/A transition in intron 1 at position +1932 (G-46/in1A) (8)
- T/C transition in intron 2 at position +3539 (T-37/in2C) (8)
- G/A transition in exon 3 at position +3660 (codon 106), which produces a synonymous change (Glu106Glu) (14)
- G/A transition in intron 4 at position +4395 (G+356/in4A) (9, 10)
- G/T transition in exon 5 at position +5665 (codon 198; Lys198Asn) (5, 7, 8, 12)

By sequencing the EDN1 gene of 56 unrelated Caucasians systematically, we identified four novel common genetic variations:

- T/G transversion in intron 2 at position +2176 (T+30/in2G)
- Insertion T in exon 4 at position +5567 (−38/in4ins/delI)
- T/C transition in the 3′-untranslated region (exon 5) at position +6438 (T+834/ex5C)
- T/C transition in the 3′-untranslated region (exon 5) at position +76485 (T+881/ex5C)

Genotyping methods for large numbers of samples have been reported for only 3 of the 12 partly novel polymorphisms (G+356/in4, +138/ex1ins/delA, and Lys198Asn) (5–7, 9, 11, 12). We therefore established standard PCR-restriction fragment length polymorphism (RFLP) methods for the 12 polymorphisms listed above and a melting peak analysis method using fluorescent probes (LightCycler assays) for the rapid detection of possibly functionally important EDN1 polymorphisms [except for +138/ex1ins/delA, for which a TaqMan assay already exists in our laboratory (11)]. To minimize assay costs, methods were optimized for minimal amounts of hybridization probes and restriction enzymes.

High-molecular-weight DNA from 300 healthy Caucasian volunteers was extracted from EDTA-blood with use of the MagnaPur DNA-Isolation Kit (Roche Diagnostics). Primers and restriction enzymes for PCR-RFLP methods and primers and hybridization probes for melting peak analysis (see the Data Supplement accompanying the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue2/) were chosen according to the EDN1 sequence (OMIM*131240; GenBank accession no. J05008), using Oligo Primer Analysis Software 6.0 and LightCycler Probe Design Software.

In some PCR-RFLP assays (G-1396A, +138/ex1ins/delA, and T+30/in2G; see the online Data Supplement), primer mismatches were incorporated to create recognition sites for restriction enzymes. One of the PCR-RFLP assays was established for simultaneous detection of two polymorphisms (T+834/ex5C and T+881/ex5C). All PCRs were performed in a total volume of 20 μL. The reaction mixture contained 0.2 μM each of the primers (TIB MOLBIOL), 0.1 mM each of the deoxynucleotide triphosphates (BioTherm), 2 μL of 10× Taq Buffer (BioTherm), 1 μL of the sample (containing 30–50 ng/μL genomic DNA), 1 U of Taq Polymerase (BioTherm), and assay-specific amounts of MgCl₂ (1.2–2.0 mM; see the online Data Supplement). The PCR was performed in a Thermocycler (GeneAmp PCR System 9700; Perkin-Elmer) with the following conditions: initial denaturation at 95 °C for 120 s, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at assay-specific temperatures for 30 s (see the online Data Supplement), extension at 72 °C for 60 s, and a final extension at 72 °C for 7 min. For RFLP analysis, a 20-μL aliquot containing 10 μL of the PCR product was digested with 1 (T+30/in2G), 2 (+138/ex1ins/delA), 5 (G+356/in4, T+834/ex5C, and T+881/ex5C), 6 (G-1396A), or 10 U (G-46/in1A) of assay-specific restriction enzymes (MBI Fermentas GmbH; see the online Data Supplement) at the enzyme-specific temperatures overnight. Digestion products (see the online Data Supplement) were visualized by SYBR Green staining after electrophoresis in 3% agarose gels.

Hybridization probes for LightCycler assays were labeled with LightCycler Red640 or Red705. One hybridization probe pair could be used to detect two polymorphisms simultaneously (Lys198Asn and Glu106Glu). PCR was carried out in glass capillaries in a total volume of 20 μL. The reaction mixture contained assay-specific amounts of each primer (0.2–0.25 μM; see the online Data Supplement), anchor and sensor (0.05–0.1 μM; see the online Data Supplement), 0.1 mM each of the deoxynucleotide triphosphates; 30 mg/L bovine serum albumin, 50 mL/L dimethyl sulfoxide, 2 μL of 10× Taq Buffer, 1 μL of the sample, 1 U of Taq polymerase, and assay-specific amounts of MgCl₂ (2.0–3.0 mM; see the online Data Supplement).

Conditions were optimized as follows: Conditions for the denaturation steps and extension temperatures were always the same. For the T-1370G PCR, the initial denaturation was at 95 °C for 120 s, followed by 30 cycles of denaturation for 0 s at 95 °C, annealing for 20 s at 53 °C, and extension for 40 s at 72 °C. For melting curve analysis, the conditions were 20 s of denaturation at 95 °C and 20 s of annealing at 32 °C, after which the temperature was continuously increased up to 70 °C (ramp rate, 0.15 °C/s).

For the T-37/in2C PCR, the initial denaturation was followed by 35 cycles of denaturation for 10 s, annealing at 58 °C, and 20 s of extension. The conditions for melting curve analysis were denaturation followed by annealing for 20 s at 40 °C, after which the temperature was continuously increased up to 80 °C (ramp rate, 0.2 °C/s).

For the −38/in4ins/delI PCR, initial denaturation was
followed by 35 cycles of denaturation for 10 s, annealing at 58 °C, extension for 25 s. The conditions for melting curve analysis were denaturation followed by 20 s of annealing at 35 °C, after which the temperature was continuously increased up to 65 °C (ramp rate, 0.1 °C/s).

For the PCR for Lys198Asn and Glu106Glu, initial denaturation was followed by 43 cycles of denaturation, 20 s of annealing at 60 °C, and extension for 30 s. For melting curve analysis, denaturation was followed by 20 s of annealing at 49 °C, after which the temperature was continuously increased up to 75 °C (ramp rate, 0.1 °C/s).

Fluorescence was recorded during the heating. The melting curves (F/T) were converted to melting peaks (ΔF/ΔT); their analysis showed distinct temperatures for all polymorphisms (see Fig. 1 and the online Data Supplement).

Genotyping results obtained by PCR-RFLP analysis and by melting peak analysis with fluorescent probes correlated perfectly with DNA sequencing (Prism™ 310 Genetic Analyzer; Applied Biosystems).

We have established reliable and cost-efficient methods for the detection of 12 EDN1 polymorphisms. The melting peak analysis with fluorescent probes allows rapid detection of the common functional polymorphisms. High-throughput analysis can be performed by transferring the assays (using the same primers and hybridization probes) to a LightTyper instrument.

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IgA Antibodies against Tissue Transglutaminase in the Diagnosis of Celiac Disease: Concordance with Intestinal Biopsy in Children and Adults, Mª Jesus Llorente,1 Mercedes Sebastián,2 Mª Jesus Fernández-Aceñero,3 Gemma Prieto,3 and Santiago Villanueva1 (Departments of 1 Biochemistry, 2 Pediatrics, and 3 Surgical Pathology, Hospital General of Móstoles, Móstoles, Madrid, Spain; * address correspondence to this author at: c/Hilarión Esclava 28 6-A, Madrid 28015, Spain, e-mail mgg10167@teleline.es)

Celiac disease (CD), an immune enteropathy caused by gluten-containing foods in genetically susceptible individuals, is usually diagnosed during childhood, but delayed diagnosis in adulthood is not uncommon (1). One-half of cases show atypical forms of the disease, e.g., iron-deficient anemia unresponsive to iron or persistent hypertransaminasemia (2–4).

Patients with CD often have high circulating concentrations of anti-endomysium antibodies (EMAs), the main disease marker (5), and anti-gliadin antibodies (AGAs), the most effective marker for children <3 years (6, 7). Tissue transglutaminase (tTG) has been identified as the autoantigen of CD (8).

The first assays for tTG antibodies used antigen from guinea pigs (9–15). Assays that used recombinant human tTg (rh-tTG) improved sensitivity and specificity (16–21), but it is not well established in prospective studies whether the clinical effectiveness of rh-tTG is similar for children and adults.

The aims of this study were (a) to evaluate the potential utility of rh-tTG IgA (rh-tTGA) compared with EMAs and AGAs for CD diagnosis in children and adults; (b) to analyze the concordance between rh-tTGA and small intestine biopsy (IB); and (c) to analyze the association of hypertransaminasemia and ferropenia with untreated CD.

We prospectively selected 2570 patients with clinical suspicion of CD. Most patients (73.4%) were referred by the Pediatrics Department. From this cohort of patients we analyzed consecutively all patients fulfilling the inclusion and exclusion criteria. Inclusion criteria included histologic analysis of an IB and determination of serum markers for CD; the exclusion criterion was IgA deficiency. Both the IB and the immunologic markers were analyzed blindly and independently.

The patients were classified into two groups. Group I consisted of 61 patients diagnosed with classic CD based on the results of the IB and obvious clinical and serologic response to a gluten-free diet. Group II contained 64 patients with clinical suspicion of CD but a normal IB and was considered the control group. The most common presentations in the control group were failure to thrive (29%) and gastrointestinal symptoms (27%).

We also included 86 first-degree relatives of celiac patients, all asymptomatic. In this group we diagnosed six new cases of CD, who were then incorporated in group I.

In our series 146 IBs were performed. Full-thickness jejunal biopsies were obtained by endoscopy or Watson-Crosby capsule (children). The histopathologic findings were classified according to internationally accepted criteria as normal mucosa, partial villous atrophy (slight, moderate, or severe), and subtotal villous atrophy (22). We considered all cases with at least a moderate villous atrophy as diseased, and biopsies showing slight atrophy or unspecific changes were considered as normal.

rhTGA (expressed in the eukaryotic baculovirus system) was determined by enzyme immunoassay (Celikey; Pharmacia Diagnostics). The antibody concentration was expressed in arbitrary units (AU/mL).

IgA-AGAs were measured by fluorescent enzyme immunoassay (Pharmacia Diagnostics). IgA-EMAs were measured by indirect immunofluorescence with monkey esophagus as substrate, and IgA was measured by turbidimetry in a Hitachi-Modular (Roche Diagnostics). Aspartate aminotransferase, alanine aminotransferase, and ferritin were measured in the same analyzer.

A descriptive analysis (frequencies, medians, and percentiles) was performed; we also compared the quantitative variables (Mann-Whitney U-test) and analyzed correlation with the Spearman ρ coefficient. The concordance rate was estimated with the κ index. We constructed ROC curves and calculated sensitivity, specificity, and likelihood ratios (LRs) at selected marker concentrations.

In our series, similar to other reports (2), 67% of the CD cases were women. Forty-seven patients (77%) were children (31 females and 16 males; age range, 1–17 years), and 14 (23%) were adults (10 women and 4 men; age range, 19–70 years). The frequency of CD among patients’ relatives was 7%, similar to the rates reported in other studies (23).

Serum rh-tTGA was significantly higher in celiac patients [median, 100 (5th–95th percentiles, 21.9–100) AU/mL] than in controls [0.3 (0.1–4.4) AU/mL] or asymptomatic relatives [0.33 (0.1–1.7) AU/mL; Fig. 1].

The sensitivity, specificity, and LRs obtained for the serologic markers of CD are summarized in Table 1. We detected two false-positive (FP) results for rh-tTG at a cutoff of 7.5 AU/mL. One was a 1-year-old boy with long-standing diarrhea (EMA titer, 1/5; AGA concentration, 1.1 mg/L; rh-tTG concentration, 9.6 AU/mL); IB showed intraepithelial lymphocytosis without villous atrophy or crypt hyperplasia. The final diagnosis was a postenteritis syndrome with transitory intolerance to lactose. The rh-tTGA value normalized to 1.9 AU/mL after 7 months. The other FP was an 8-year-old girl with hypertransaminasemia of undetermined etiology (rh-tTGA concentration, 23 AU/mL; EMA titer, 1/40; AGA concentration, 2.6 mg/L) with a normal IB. The serologic markers and the transaminases were within reference values 3, 6, and 18 months later.