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DEOXYRIBONUCLEASE I TYPING FROM TEARS

Junko FUJIHARA[a], Yoko HIEDA[a], Koji TAKAYAMA[a], Yuing XUE[a], Norihito NAKAGAMI[a], Shinji IMAMURA[b], Kaori KATAOKA[a] and Haruo TAKESHITA[a]

[a]*Department of Legal Medicine, Faculty of Medicine, Shimane University, Izumo 693-8501, Japan*

[b]*Criminal Investigation Laboratory, Shimane Prefectural Police Headquarters, Matsue 690-8510, Japan.*

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We have confirmed for the first time, both biochemically and immunologically, the existence of deoxyribonuclease I (DNase I) in the human tears. Isoelectric focusing of tear samples on polyacrylamide gels (pH 3.5-5), followed by dried agarose film overlay detection, was used to determine the phenotypes of tear DNase I. Because of its high degree of sensitivity and its high band resolution, this detection method allowed determination of DNase I types from tear samples of about 5 μL. Pretreatment of tear samples with the enzyme sialidase was essential for typing in order to markedly enhance the sensitivity and simplify the isozyme pattern. DNase I types in all tear samples were consistently related to types found in corresponding blood and urine samples. DNase I typing may, therefore, provide a novel discriminating characteristic in the genetic, clinical and forensic examination of tears.

Key words: forensic science, deoxyribonuclease I (DNase I), typing, dried agarose film overlay (DAFO), single radial enzyme diffusion (SRED), tears

INTRODUCTION

Deoxyribonuclease I (DNase I, EC 3.1.21.1) activity is widely distributed in various tissues and body fluids (1). Protein polymorphism of human DNase I was first demonstrated in urine by isoelectric focusing on a thin layer of polyacrylamide gel (IEF-PAGE) (2). Subsequently, similar isozymes have been detected in serum (3), semen (4), saliva (5) and perspiration (6). In addition, it has been demonstrated that there is perfect correlation among the phenotypes found in samples of these four fluids taken from the same individual. DNase I is controlled by six codominant alleles on chromosome 16 (7): three common alleles, *DNASE1*1*, **2* and **3*, have been observed at the polymorphic level (gene frequencies in the Japanese population are 0.55, 0.44 and 0.01, respectively), whereas three rare alleles, *DNASE1*4*, **5* and **6* are found at the mutational level (gene frequencies of these alleles are less than 0.01 in Japanese) (8). Since DNase I has informative gene frequencies and high stability, it is one of the most useful biochemical markers for genetic, clinical, and forensic analyses. We have successfully phenotyped DNase I from small semen stains (9), perspiration stains (10), saliva stains (11) and urine stains (12) found at crime scenes. As DNase I is widely distributed in biological fluids (1), its polymorphism is useful as an individualization marker.

Aqueous humor (vitreous humor) has been employed as alternative to blood and urine sample at autopsy, especially for toxicological analysis. To our knowledge, there are no reports on individualization utilizing aqueous humor (vitreous humor) or tear fluid which secreted from lachrymal gland. Recently, polymerase chain reaction (PCR) has been utilized to identify virus DNA in tear fluid and vitreous humor of viral ophthalmopathy patients (13-15). However, until now, no study is available extracting genomic DNA and performing genotyping from tear fluid. Only the ABO system has been used as a genetic marker for individualization of tears (16). Most tears (95% or more) are produced by the lacrimal glands. Recently, a high expression level of DNase I was reported in mouse lacrimal glands (17).

In this study, we describe results of a trial to determine DNase I phenotype and genotype from tear samples and the correlation of DNase I types among blood and urine samples from the same individual.

Correspondence: H. Takeshita, Department of Legal Medicine, Faculty of Medicine, Shimane University, Izumo, 693-8501, Japan
Tel: +81-853-20-2156
Fax: +81-853-20-2155
E-mail: htakeshi@med.shimane-u.ac.jp

MATERIALS AND METHODS

Biological samples

Sets of tear, blood, and urine samples of the three common DNase I phenotypes (1, 1-2, and 2) were collected from five healthy Japanese donors (male, 37 years; female, 33 years; female, 27 years; female 3 years; female, 2 years) with informed consent. DNase I phenotype in this study was comprised as follows: 1 donor from type 1, 2 donors from type 1-2, and 2 donors from type 2. Venous blood was drawn and placed into tubes containing heparin, and the serum was separated. Urine samples were concentrated, dialyzed and finally lyophilized, as described previously (2): a 0.1% (w/v) solution of the lyophilized material corresponding to ten-fold concentrated urine was used for DNase I typing. These solutions were treated with an equal volume of 10 units/mL *Clostridium perfringens* sialidase (Sigma, St. Louis, MO) in 50m*M* sodium acetate buffer, pH 5.0, before electrophoresis. The digest of 5-10 μL was used for isoelectric focusing on a thin layer of polyacrylamide gel (IEF-PAGE). Genomic DNA was prepared using a QIAamp DNA blood kit (QIAGEN Inc., Chatsworth, CA).

DNase I phenotyping method

Isoelectric focusing was performed as previously described (6). In short, gels measuring 0.5 (thickness) by 90 (width) by 120 (length) mm were prepared using the following materials: 1.4 mL of acrylamide-*N, N'*,-methylenebisacrylamide (19.4 %, w/v; 0.6%, w/v), 1 mL of distilled water, 2.3 mL of sucrose-glycerol (20%, w/v), 280 μL of Ampholine 3.5-5 (Pharmacia Biotech, Uppsala, Sweden), 10 μL of *N, N, N', N'*,-tetramethylethylenediamine and 50 μL of 1.2 % (w/v) ammonium persulfate. Wicks were formed from strips of filter paper and soaked in the electrode solution; 1.0*M* phosphoric acid (H_3PO_4) at the anode and 2% (v/v) Ampholine 5-7 (Pharmacia Biotech) at the cathode. A sample was applied to the gel with a Whatman 3MM filter paper at a distance of 20 mm from the cathode wick. A Multiphor apparatus (Pharmacia Biotech) was used to run the gel at *V*max 1000 V, *I*max 10 mA, *P*max 3W for 4h under cooling at 12℃.

Visualization of DNase I was achieved using the dried agarose film overlay (DAFO) method (5). For this, the reaction mixture consisted of 0.05 mg of ethidium bromide and 0.05 mg of salmon testis DNA (type III, Sigma) per 1 mL of 100mM sodium cacodylate buffer, pH 6.5, which contained 0.2 mM calcium chloride ($CaCl_2$) and 2.0 mM magnesium chloride ($MgCl_2$). To the reaction mixture, an equal volume of 2% (w/v) molten agarose in distilled water at 50℃ was added, mixed, and poured immediately onto a horizontal Agafix sheet (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After solidification at room temperature, the agarose gel was dried completely in an incubator at about 50℃ to produce a dried agarose film. After the IEF-PAGE run, the dried agarose film was placed carefully in full contact on top of the focused gel. The IEF-PAGE gel was incubated in contact with the agarose film at 37℃ and the progress of DNase I action was observed under UV transillumination (312 nm). Ethidium bromide produces fluorescence with intact but not with degraded DNA digested by DNase I. After incubation for optimal development, the film was removed from the gel and then observed or recorded photographically. DNase I types in serum and urine samples were analyzed as previously described (2, 3).

DNase I genotyping method

Genotyping assay for detection of *DNASE1* polymorphism from the DNA samples was carried out according to a previously described system (18). In brief, the mismatched PCR method was employed to discriminate the *DNASE1*1* from the *DNASE1*2* allele. The sequence including the nucleotide substitution generating the mutation was amplified using a pair of primers, U-1(5'-ATCGTGGTTGCAGGGAT GCTGCCTC-3') and D-1(5'-AGTTCAACAGGTGT GGGGAG-3'). Followed by amplification with PCR, 5 μL PCR-product was digested with 40 U Xho I in a 20 μL reaction volume at 37℃ for 2 h. The digests (5 μL) were separated in 8 % polyacrylamide gel in x 1 TBE buffer at a constant voltage (200V). Patterns on the gels were visualized by silver staining as described previously (19).

Activity assay and characterization of tear DNase I

Tear DNase I activity was determined by the single radial enzyme diffusion (SRED) method (1). Effects

of G-actin, anti-human DNase I antibody and divalent cations on tear DNase I activity were examined as described previously (20).

RESULTS AND DISCUSSION

Human tears consist of various secretions that are mixed in the conjunctival sac and protein concentration has been shown to be in the range of 136-592 mg/100 ml in studies of normal tears from adults (21, 22). Recently, Napirei et al. reported the lacrimal gland and eye to be DNase I-expressing organs (17). DNase I types are of value in terms of discrimination potential (2,3) and the high stability of the enzyme protein. In this study, we observed high activity levels of DNase I in ten tear samples using SRED method. Enzyme activity was completely inhibited by 1mM ethylenediaminetetraacetate (EDTA) and also by 1mM ethyleneglycol bis (aminoethyl ether) tetraacetic acid (EGTA) even in the presence of 10 mM $MgCl_2$. G-actin, which is known to be a potent inhibitor of bovine-pancreatic and human DNase I (20), completely inhibited the activity of tear DNase I. Furthermore, the activity was completely abolished by anti-human DNase I antibody, but not by anti-human DNase II antibody. It was revealed that these catalytic and immunological properties of tear DNase I are very similar to those of urinary and serum enzymes (2,3). This is the first study to confirm the presence of DNase I activity in human tears.

The mean activity of human tear DNase I was determined to be 0.22 ± 0.18 (mean $\pm$ SD) $\times 10^{-3}$ units per mL of tear liquid (range, 0.12 to 0.30), which was estimated simultaneously in terms of the 47.8 ± 22.9 units/mg protein. Although the activity levels of tear DNase I per unit volume appears to be extremely lower than those for urine, semen, and saliva, the mean activity level per mg of tear protein is higher than those of semen and saliva (Table 1). We concluded that the enzyme activity level in tears was sufficiently high for phenotyping by our method using a combination of the IEF-PAGE and zymogram methods because we previously succeeded in typing DNase I from saliva or semen samples having a similar level of enzyme activity.

When typing tear samples, three serum samples of known types 1, 1-2, and 2 should be simultaneously applied to the IEF-PAGE gel as a control. The IEF-PAGE pattern of tear DNase I was similar to those of serum and urine DNase I in each type, as shown in Fig. 1A. This finding was supported by the fact that a mixture of serum and tears of Type 1 (or Type 2) produced a single main isozyme band indistinguishable from that of Type 1 (or Type 2) serum on the gel (data not shown). Pretreatment of tear samples with sialidase was essential for precise and sensitive typing of tear DNase I as it is for DNase I typing in other biological fluids (2-6). DNase I types

Table 1. DNase I activity in human biological fluids

Biological Fluid	Activity*		Reference
	units/mL liquid	units/mg protein	
Tear	$(0.22 \pm 0.18) \times 10^{-3}$	47.8 ± 22.9	This study
Saliva	0.56 ± 0.56	18.7 ± 12.6	(5)
Semen	0.35 ± 0.17	32.1 ± 17.1	(4)
Serum	$(4.40 \pm 1.80) \times 10^{-3}$	65.0 ± 27.0	(1)
Urine	0.60 ± 0.22	6000 ± 2200	(1)
Sweat	$(0.98 \pm 0.53) \times 10^{-3}$	29.1 ± 12.0	(6)

*Activities are given as mean value ± SD.

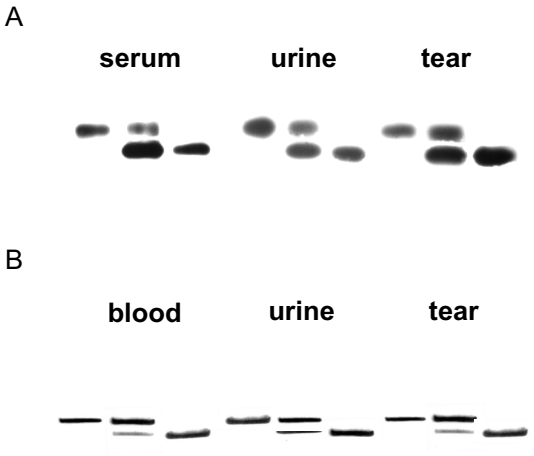

Fig. 1. (A) Isoelectric focusing (pH 3.5-5) patterns of three common DNase I phenotypes from desialylated serum (lanes 1, 2 and 3 from left to right), urine (lanes 4, 5 and 6) and tear (lanes 7, 8 and 9) samples detected by activity staining using the DAFO method (see Materials and Methods). Anode is at the top. Paired samples obtained from the same donors: lanes 1, 4 and 7, type 1; lanes 2, 5 and 8, type 1-2; lanes 3, 6 and 9, type 2. (B) The electrophoretic patterns of mismatched PCR product followed by Xho I digestion. Genomic DNAs were obtained from donors with three common phenotypes. Blood (lanes 1, 2 and 3 from left to right), urine (lanes 4, 5 and 6) and tear (lanes 7, 8 and 9) samples were obtained from same individuals as Fig.1 (A).

were examined in several sets of serum and urine from the same donors. The type of each donor was determined from the tear samples, and the results agreed with the donors' serum and urine types in each case; no example of non-correlation of DNase I typing among corresponding serum and urine samples was found. Genotyping assay for detection of *DNASE1* polymorphism in tears was consistent with the IEF-PAGE phenotyping result of all tear samples (Fig.1B). Additionally, in preliminary experiment, Short tandem repeat (STR) analysis was also possible from human tear fluids (data not shown).

Up to now, limited genetic marker systems have been found in human tears. Among them, only the ABO system is commonly used for the individualization of tears. The present findings indicate that the DNase I isozymes in tears, sweat, serum, semen, and urine are comparable, and that typing of DNase I may therefore provide a novel discriminating characteristic in tear samples.

CONCLUSION

The DNase I, which can be detected by traditional but accepted and well established methods with an unquestionable genetic basis, is very effective and indispensable for the individualization. The combination of conventional phenotyping by IEF and genotyping method using PCR is important for enhancing the usefulness of DNase I polymorphism as a tool in anthropological and clinical surveys. Because of the relatively high activity level and favorable gene frequency in tear DNase I, use of DNase I polymorphism may provide information for practicing genetic, clinical and forensic biologists.

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