Analysis of a Variable Number of Tandem Repeats in a Heart Disease Gene by Capillary Electrophoresis with Laser-Induced Fluorescence Detector

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Capillary electrophoresis with laser-induced fluorescence detector (CE-LIF) was successfully applied to the analysis of the variable number of tandem repeats (VNTR) in a human apolipoprotein B gene (APOB). Apolipoprotein B VNTR alleles containing more than 35 repeat units are a significant risk factor for heart disease. Thus, we developed a method for accurately determining the number of repeat units (16 bp) in the VNTR using capillary electrophoresis. The CE-LIF technique gave excellent resolution of APOB alleles differing by 2 or 4 repeat units over the range 600 to 1000 bp.

The recommended conditions for the analysis of APOB VNTR loci by CE-LIF are as follows: effective length of capillary (100 μm i.d., 360 μm o.d.), 50 cm; running buffer, 50 mM Tris–borate 0.5% methylcellulose and 0.1 μM fluorescent dye YO-PRO-1; electric field, 150 V/cm.

Key words capillary electrophoresis; VNTR; apolipoprotein B; heart disease; polymerase chain reaction

Human apolipoprotein B gene (APOB) maps to the short arm of chromosome 2, spans 42 kbp, and contains variable number of tandem repeats (VNTR) located immediately downstream from the APOB. The APOB VNTR alleles generally contain 25—52 repeats of a basic 16 bp unit. These alleles differ with respect to the number of 16 bp repeat units.[1-3] A genetic linkage study of the APOB locus has indicated that large alleles containing more than 35 repeat units are more common in myocardial infraction patients and represent a serious risk factor for coronary heart disease.[3] These alleles are also associated with elevated levels of total serum cholesterol and APOB among patients and with elevated levels of total serum triglycerides among controls.[3]

The accurate determination of the DNA fragment size of polymerase chain reaction (PCR)-amplified APOB alleles is useful for the DNA diagnosis of coronary heart disease. To achieve this, a method for resolving mixtures of alleles comprising 16 bp repeat units over the range 500—1000 bp needs to be developed, because the PCR amplification of the APOB VNTR alleles of 25—52 repeat units produces 500—1000 bp DNA fragments.[1,2] Capillary electrophoresis with a laser-induced fluorescence detector (CE-LIF) can determine the size of PCR-amplified DNA fragments with high accuracy, resolution, and speed.[4-10] Slab gel electrophoresis is the conventional method for determining the DNA fragment size of PCR products, but is time-consuming, labor-intensive, and nonquantitative. In the present paper, therefore, we report a method for the rapid resolution of PCR products and the determination of APOB VNTR allele size using CE-LIF.

Experimental

A P/ACE system 2050 capillary electrophoresis apparatus with a laser source (Beckman Instruments, Inc., Fullerton, CA, U.S.A.) was used with negative source polarity. The laser source was operated at a power of 3 mW, excitation was at 488 nm and a 560 nm bandpassfilter was used as an emission cut-off filter. A DB-17 coated capillary (360 μm o.d., 100 μm i.d.; J&W Scientific, Folsom, CA, U.S.A.) of 50 cm effective length and 57 cm total length was used.

The running buffer solution used in the present study was 50 mM Tris–borate. To this buffer solution was added 0.5% methylcellulose. Just prior to CE analysis, 1 μl fluorescent dye solution (1-(4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylidene]-quinolinium)-3-trimethylammonium propane diiodide (YO-PRO-1)), which is easily intercalated into the double strand of DNA, was added to 10 ml running buffer solution.

A 100 bp DNA ladder was purchased from GENSURA LABORATORIES INC. (CA, U.S.A.). Methylcellulose was purchased from Sigma (St. Louis, MO, U.S.A.). DNA samples were diluted 10- to 100-fold with Mili-Q water and stored at −18 °C until use. All other chemicals were of analytical reagent or electrophoretic grade from Wako (Osaka, Japan).

The 500 bp DNA target of bacteriophage lambda DNA was amplified by polymerase chain reaction (PCR) as described elsewhere.[11] PCR was performed on a thermal cycler (Techne, PHC-3, Princeton, NJ, U.S.A.) with 25 cycles of amplification (1 min at 94 °C, 1 min at 37 °C, and 1 min at 72 °C).

Human genomic DNA was prepared from leukocytes of four Japanese subjects. The oligonucleotide primers were 20 nucleotides in length. The sequences of primers were chosen such that they flanked the targeted region of the genome on the 3′-side of the APOB gene. The sequence of the 5′-PCR primer was 5′-ATGGAAACGGAGAAATTATG-3′. The sequence of the 3′-PCR primer was 5′-CCTTCTCACTTGGCAAATAC-3′. The PCR was carried out in a final volume of 20 μl, containing 0.2 μg genomic DNA, 0.5 μM oligonucleotide primers, 200 μM of the four deoxyribonucleotide triphosphates (dNTPs), and 0.5 U thermostable Taq Polymerase. Annealing and extension were carried out for 6 min at 58 °C, and denaturing was carried out for 1 min at 94 °C.

Sample solution was electrophoretically introduced into the capillary at a negative polarity of 7.4 kV for 20 s and was developed with the running buffer solution described above at a negative polarity of 8.6 kV (150 V/cm, 8.9—9.6 μA) at 30 °C. DNA fragments were detected at 560 nm (Ex.; 488 nm). Prior to electrophoretic analysis of the samples, the capillary was washed with methanol (50 v/v %) for 5 min and then with running buffer solution for 5 min.

Slab gel electrophoresis was carried out as described by Boerwinkle.[1]

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Results and Discussion

Analysis of 100 bp DNA Ladder Figure 1 shows an electropherogram of a mixture of the 500 bp PCR product and the 100 bp DNA ladder. The 100 bp ladder is a mixture of 100—1000 bp DNA fragments which differ by 100 bp. The 500 bp DNA target corresponding to the 7131—7630 nucleotides of bacteriophage lambda DNA was amplified by PCR as described elsewhere.[11] The separation conditions were optimized in several preliminary experiments with different methylcellulose concentrations (0.5—1.0%), electric fields (50—300 V/cm), capillary temperatures (25—35 °C), and capillary sizes (27—67 cm). As can be seen from Fig. 1, DNA fragments ranging from 100 to 1000 bp are baseline resolved and the large peak at around 27 min was that of the 500 bp PCR product overlapping 500 bp in the 100 bp DNA ladder. It is clear that the size of the PCR product was similar to that of the 500 bp in the DNA ladder. The 100 bp DNA ladder up to 1000 bp was completely separated within 35 min. In the following experiments, therefore, the 500 bp PCR product was used as the internal standard.

We next examined the repeatability of the analysis of the 100 bp DNA ladder by CE-LIF. Firstly, the separation of the DNA ladder mixed with 500 bp PCR product was carried out 9 times, and the average migration time, mobility, and relative mobility for the 500 bp PCR product of each DNA in the 100 bp DNA ladder were calculated. The mobility (μ) is expressed by Eq. 1.

$$\mu = l/tE \quad (1)$$

Where l is the effective length of capillary (cm), E the electric field (V/cm), and t the migration time (s).

The repeatability of the migration time and the mobility of DNA fragments in Fig. 1 was about 0.6% relative standard deviation (R.S.D., $n=9$), but that of the relative mobility for the 500 bp PCR product was less than 0.1% R.S.D. (Table 1). However, the mobility was influenced by adsorption of YO-PRO-1 on the inner surface of the capillary. Therefore, washing the capillary with methanol resulted in excellent repeatability in the separation of the 100 bp DNA ladder. This is because washing removed the fluorescent dye, YO-PRO-1, adsorbed to the inner surface of the capillary. Thus, use of the internal standard and rinsing of the capillary with methanol are recommended for the analysis of the DNA ladder by CE-LIF. The human APOB gene sizes were determined using the relative mobility of the 500 bp PCR product with good results (Table 1).

The relative mobilities of DNA fragments in Fig. 1 were calculated and plotted against the DNA fragment size (bp) as shown in Fig. 2. A linear relationship was observed for a range of DNA fragment sizes up to 600 bp, indicating an Ogston dependence.[12] The transition from the Ogston region to the reputation mode[13] occurred at around 600 bp. DNA fragment size can be easily determined by measuring the relative mobility for the 500 bp PCR product from Fig. 2. However, the resolving power decreased with increasing DNA fragment size.

Analysis of Human APOB VNTR Locus Next, capil-

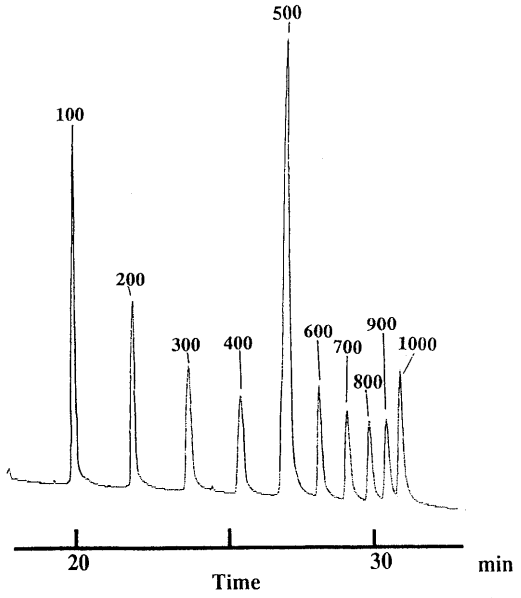

Fig. 1. CE Separation of a Mixture of the 500 bp PCR Product and the 100 bp DNA Ladder

DB-17 capillary: 100 μm i.d., 360 μm o.d., total length, 57 cm; effective length, 50 cm. Running buffer: 50 mM Tris–borate, 0.5% methylcellulose, and 0.1 μM YO-PRO-1. Field: 150 V/cm; current, 9.0 μA. Injection, 7.4 kV for 20 s. Temperature, 30 °C. Detection: Ex., 488 nm; Em., 560 nm. Resolved fragments are shown by the size of the base pairs.

Table 1. Repeatability ($n=9$) of Migration Time, Mobility, and Relative Mobility of DNA Fragments in 100 bp DNA Ladder

DNA size (bp)	Migration time (min)	R.S.D. (%)	Mobility ($cm^2 V^{-1} s^{-1}$)	R.S.D. (%)	Relative mobility	R.S.D. (%)
100	19.48	0.53	3.21×10^{-4}	0.53	1.402	0.145
200	21.72	0.56	2.88×10^{-4}	0.56	1.259	0.108
300	23.84	0.61	2.62×10^{-4}	0.61	1.146	0.057
400	25.76	0.62	2.43×10^{-4}	0.60	1.060	0.042
500	27.30	0.62	2.29×10^{-4}	0.62	1.000	—
600	28.67	0.61	2.18×10^{-4}	0.61	0.953	0.057
700	29.62	0.58	2.11×10^{-4}	0.58	0.922	0.057
800	30.33	0.56	2.06×10^{-4}	0.56	0.900	0.069
900	30.85	0.57	2.03×10^{-4}	0.56	0.885	0.066
1000	31.19	0.56	2.00×10^{-4}	0.56	0.875	0.068

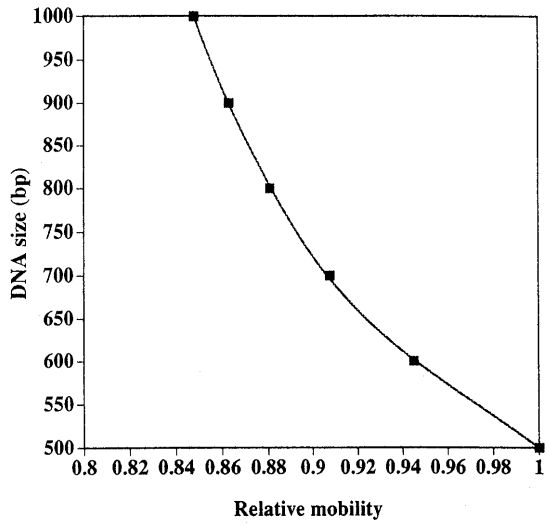

Fig. 2. Relationship between the DNA Sizes of the 100 bp DNA Ladder and the Relative Mobility for the 500 bp PCR Product

Conditions as in Fig. 1.

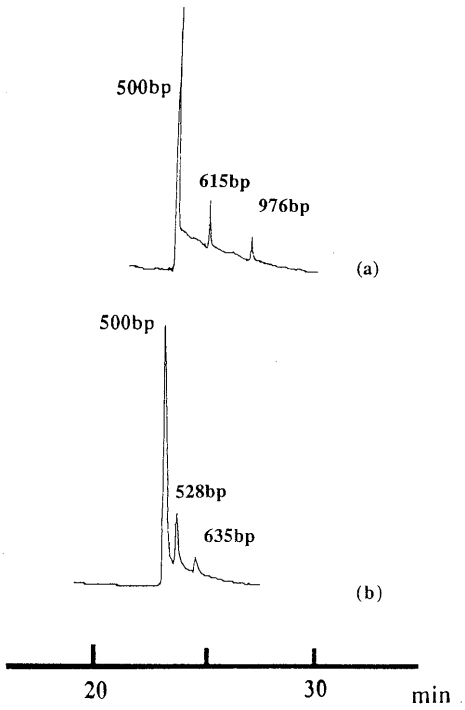

Fig. 3. CE Separation of the Mixture of PCR-Amplified DNA Fragments for the APOB VNTR Alleles and 500 bp PCR Product

(a), 615 bp and 976 bp; (b), 528 bp and 635 bp. Conditions as in Fig. 1.

lary electrophoresis was applied to the analysis of a PCR-amplified human apolipoprotein B(APOB) VNTR locus. Figure 3 shows the capillary electropherogram of the mixture of PCR-amplified APOB DNA fragments from two different individuals and 500 bp PCR product under the optimum conditions listed in Fig. 1. Two peaks corresponding to APOB fragments were observed at around 24—27 min in addition to the large peak corresponding to the 500 bp PCR product at around 23 min. This electropherogram clearly illustrates that the PCR-amplified APOB DNA fragments are produced from heterozygous individuals. Two individuals had heterozygous alleles: two bands were observed for the PCR-amplification products from each individual. In DNA typing applications it is important to differentiate heterozygous from homozygous individuals.

The DNA sizes of the peaks in Fig. 3 were estimated from the relative mobility in Fig. 2. Based on relative mobilities for the 500 bp PCR product, the numbers of base pairs in the upper electropherogram were estimated to be 615 and 976 bp and those in the lower one 528 and 635 bp, respectively. The DNA fragments in electropherogram (a) were amplified from a heterozygous individual with alleles 361 bp (23 repeat units) apart, and those in electropherogram (b) from a heterozygous individual with alleles 107 bp (7 repeat units) apart. It can be seen from Fig. 3 that large DNA fragments such as 976 bp (Fig. 3a) are satisfactorily resolved within 30 min and that APOB VNTR alleles differing by only one repeat unit are distinguishable from one another because the 500- and 528-bp DNA fragments (Fig. 3b) can be completely separated by capillary electrophoresis.

Table 2 shows the repeatability in migration time, mobility, and relative mobility of DNA fragments amplified from human APOB VNTR loci, with the 500 bp PCR product as an internal standard. Each allele of three heterozygous individuals was expressed as A1 and A2, B1 and B2, and E1 and E2, respectively. The reproducibility of the migration time and mobility of each DNA fragment ranged from 0.30—0.55% R.S.D., while the relative mobility ranged from 0.08—0.27% R.S.D., showing that CE-LIF allows one to accurately determine the number of repeat units in APOB VNTR alleles using the 500 bp PCR product as an internal standard. The repeat units in Table 2 were calculated from Fig. 2.

Table 2. Repeatability ($n=9$) of Migration Time, Mobility, and Relative Mobility of DNA Fragments Amplified from Human APOB VNTR Loci

Allele	Migration time (min)	R.S.D. (%)	Mobility ($cm^2 V^{-1} s^{-1}$)	R.S.D. (%)	Relative mobility	R.S.D. (%)	Repeat units
A1	25.77	0.47	2.16×10^{-4}	0.47	0.914	0.13	35.6
A2	27.65	0.55	2.01×10^{-4}	0.55	0.852	0.27	53.5
B1	30.50	0.46	1.82×10^{-4}	0.46	0.905	0.09	35.6
B2	31.99	0.48	1.74×10^{-4}	0.48	0.863	0.08	48.6
E1	25.95	0.35	2.14×10^{-4}	0.35	0.916	0.08	34.5
E2	26.96	0.31	2.06×10^{-4}	0.30	0.881	0.12	44.8

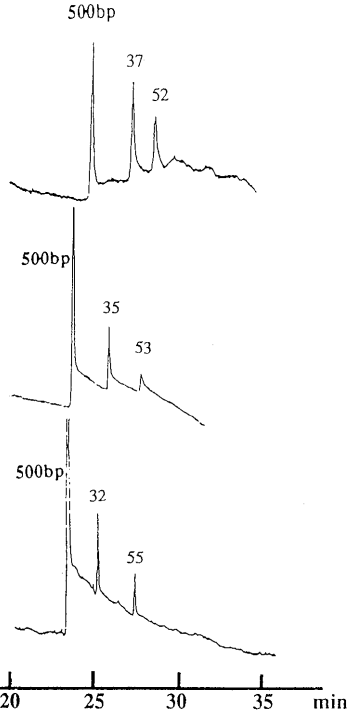

Fig. 4. CE Separation of PCR-Amplified DNA Fragments for the APOB VNTR Alleles of Three Heterozygous Individuals

(a), 37 (693 bp) and 52 (935 bp) Repeat Units; (b), 35 (665 bp) and 53 (955 bp) repeat units; (c), 32 (615 bp) and 55 (976 bp) repeat units. Conditions as in Fig. 1.

Analysis of the APOB PCR-amplified DNA fragments from three individuals is shown in Fig. 4. Using the CE-LIF technique, we distinguished three allelic variations within 30 min. All individuals had heterozygous alleles, as shown by the two peaks for the PCR-amplification products. Based on the relative mobility with respect to the 500 bp PCR product as an internal standard, the observed numbers of repeat units for the three alleles were estimated

Table 3. Base Pair (bp) and Repeat Number for APOB VNTR Alleles of Nine Heterozygous Individuals by CE-LIF and Slab Gel Electrophoresis

Sample No.	Base pair (bp)	Repeat number		Difference
		CE	Slab gel	
1	528	27	27	0
	635	34	35	1
2	591	31	31	0
	640	34	35	1
3	648	34	35	1
	710	38	39	1
4	675	36	35	1
	808	44	45	1
5	642	34	35	1
	869	48	47	1
6	734	39	37	2
	860	48	49	1
7	693	37	37	0
	935	52	51	1
8	665	35	35	0
	955	53	53	0
9	615	32	33	1
	976	55	57	2

and indicated on the peaks in Fig. 4. The products from the individual shown in the top electropherogram have two bands of 37 and 52 repeat units. The other electropherograms also show heterozygous alleles containing 35 and 53 repeat units (b), and 32 and 55 repeat units (c), respectively. The results of slab gel electrophoresis for three different individuals show heterozygous alleles of 37 and 51 repeat units (a), 35 and 53 repeat units (b), and 33 and 57 repeat units (c). The numbers of repeat units obtained by CE-LIF were very similar to those obtained by slab gel electrophoresis in the three individuals. Thus, these results demonstrate that CE-LIF accurately and rapidly determines the number of repeat units. A genetic linkage study of the APOB locus revealed that large alleles containing more than 35 repeat units are significantly associated with coronary heart disease. Thus, all the individuals in Fig. 4 are thought to be at risk of coronary heart disease. The smaller DNA fragments (32, 35, and 37 repeat units), which differ by only two or three repeat units, were resolved completely. Further, larger DNA fragments of 52, 53, and 55 repeat units, could be analyzed within 30 min. APOB VNTR alleles differing in length by only one repeat unit (16 bp) were accurately analyzed by the highly sensitive CE-LIF.

Table 3 shows the results of CE-LIF and slab gel electrophoresis for the APOB PCR-amplified DNA fragments from nine individuals with heterozygous alleles. The base pair (bp) in Table 3 was calculated from Fig. 2. Nine individuals had 27—55 repeats. The base pair in Table 3 contains 99 bp, consisting of the primer (20 bp) and other nucleotides (79 bp) bound to APOB, in addition to APOB VNTR loci. As shown in Table 3, there was a difference of 1 or 2 repeats, a difference of less than 32 bp, between the results of CE-LIF and slab gel electrophoresis. This is because that the number of base pairs making up the repeat units differs in the APOB VNTR loci of the nine individuals and the mobility decreases with increasing DNA size (reputation mode in Fig. 2). However, APOB VNTR loci can be speedily analyzed by CE-LIF with high accuracy. CE-LIF is very effective because of its high-speed and resolving power.

While conventional analysis distinguishes alleles that differ by 2 or more repeat units, it cannot distinguish alleles differing by only one unit.[1-3] Figure 4 and Table 3 clearly demonstrate that the typing of APOB VNTR loci can be completely resolved by CE-LIF. Thus, CE-LIF can been applied to the rapid diagnosis of coronary heart disease.

Conclusion

Using CE-LIF, we determined APOB VNTR size with high accuracy, resolution, and speed. Furthermore, the results obtained by CE-LIF agreed well with those obtained by slab gel electrophoresis. CE-LIF is superior to conventional slab gel electrophoresis in terms of the speed of analysis and resolving power, and allows rapid analysis of PCR products and DNA diagnosis with high precision.

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