

## Increased Deformability of Red Blood Cells is Associated with a Deletion Polymorphism of the Angiotensin-Converting Enzyme Gene

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BOR-KUCUKATAY, M., TURGUT, S., EMMUNGIL, G., TURGUT, G. and KUCUKATAY, V. *Increased Deformability of Red Blood Cells is Associated with a Deletion Polymorphism of the Angiotensin-Converting Enzyme Gene.* Tohoku J. Exp. Med., 2006, **208** (2), 147-155 — Angiotensin-converting enzyme (ACE) plays important roles in the renin-angiotensin system. ACE converts angiotensin I to angiotensin II and also inactivates bradykinin, thereby modulating the vascular tone. A polymorphism of the ACE gene, located on chromosome 17, has been found in intron 16, and is characterized by the presence (insertion [I]) or absence (deletion [D]) of a 287-base-pair Alu repeat. Individuals with the D allele of the ACE gene have higher ACE levels and are at higher risk of cardiovascular events. We aimed to investigate the possible relationship between the I/D polymorphism of the ACE gene and hemorheological parameters, including red blood cell (RBC) deformability. The study was performed on 28 healthy young volunteers (13 women and 15 men, mean age  $24 \pm 2$ ). The prevalence of the I and D alleles was 30.4% and 69.6%, respectively. The I/I genotype (II) was found in 21.4%, I/D genotype (ID) in 17.9%, and D/D genotype (DD) in 60.7% of the subjects tested. No significant relationship between ACE I/D polymorphism and RBC aggregation or whole blood and plasma viscosity was observed. In contrast, RBC deformability was significantly increased in the subjects with the DD genotype compared with the II ( $p < 0.05$ ) or the ID ( $p < 0.01$ ) genotype, and in the subjects with the D allele compared with the I allele ( $p < 0.01$ ). We suggest that RBC deformability of individuals with the D allele, who have higher risk for cardiovascular pathologies, may have been increased by a compensatory mechanism. ——— angiotensin-converting enzyme; polymorphism; hemorheology

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Angiotensin-converting enzyme (ACE) is an important enzyme in the renin-angiotensin system. ACE converts angiotensin I into angiotensin II and inactivates bradykinin. Angiotensin II and bradykinin are involved in modulation of vascular

tone and proliferation of vascular smooth muscle cells (Soubrier et al. 1993). The levels of tissue and circulating ACE activity are under tight genetic control (Holla et al. 1999). ACE gene is located on chromosome 17, and the polymor-

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phism is characterized by the presence of a 287-base-pair (bp) Alu repeat within intron 16 (Prasad et al. 2000). The presence of the extra fragment is associated with lower circulating and tissue ACE activity and this variant of the ACE gene is called insertion (or I) allele. The absence of this fragment (deletion [D] or D allele) is associated with relatively higher ACE activity. The three genotypes include ACE D/D and I/I homozygotes and I/D heterozygotes (Rigat et al. 1990). Studies have shown that the homozygosity of D allele is associated with both an increased risk and a parental history of myocardial infarction compared with that of I allele (Cambien et al. 1992; Tiret et al. 1993). The D allele has also been associated with other cardiovascular pathologies, such as cardiomyopathy, coronary and carotid artery disease, coronary artery spasm, left ventricular hypertrophy, increased vascular smooth muscle tone and hypertension (Cambien et al. 1992; Marian et al. 1993; Raynolds et al. 1993; Tiret et al. 1993; Mattu et al. 1995; Gharavi et al. 1996; Agerholm-Larsen et al. 1997; Montgomery 1997; Prasad et al. 2000).

Evidence from experimental, clinical and epidemiological studies suggest that hemorheological factors might not only play an important role in the evolution of many cardiovascular pathologies, but may also take part in the pathophysiology of them. A close relationship was established between disturbances in hemorheological and hemostatic factors and myocardial infarction (Vaya et al. 2002; Caimi et al. 2003; Sargento et al. 2003). It was demonstrated that patients with dilated cardiomyopathy had significantly higher blood viscosity at all shear rates. Erythrocyte filterability at 5-micron pore size was also significantly lower in those patients (Gustavsson et al. 1994). During hypertension, important alterations in rheological characteristics of erythrocytes and blood flow have been shown. A number of clinical studies have documented increases in hematocrit (Hct), plasma fibrinogen, plasma and whole blood viscosity and erythrocyte aggregability as well as impaired deformability of red blood cell (RBC) in hypertension (Cicco and Pirrelli 1999; Meiselman 1999; Sandhagen 1999).

On the other hand, it was shown that blood pressure reduction with an ACE inhibitor, enalapril, leads to improvement of blood rheology in hypertensive patients with renal disease (Shand et al. 1995).

Impaired hemorheology has not only been demonstrated in cardiovascular patients, but also in healthy individuals with increased cardiovascular risk factors. Increased RBC aggregation and plasma viscosity were found in a group of children with familial hypercholesterolemia but without vascular atherosclerosis, whose parents also suffer from familial hypercholesterolemia, suggesting that rheological alterations may appear prior to the development of the cardiovascular disease (Vaya et al. 1996). In the light of the above findings, we aimed to evaluate the possible relationship between the I/D polymorphism of the ACE gene and hemorheological parameters, such as RBC deformability, RBC aggregation, and plasma and whole blood viscosity in healthy young subjects. Healthy young subjects were chosen as the study group to investigate the presence of hemorheological alterations in individuals with the D/D genotype (DD), who are under increased risk of cardiovascular diseases.

## MATERIALS AND METHODS

### *Subjects and blood sampling*

This study was performed on 28 healthy young volunteers (13 women and 15 men, mean age  $24 \pm 2$ ). Blood pressure of each subject was measured prior to blood sampling and it was confirmed that there was no statistically significant difference. Anticoagulated blood samples were obtained from the antecubital vein of subjects and all analyses were performed within 4 hours after blood sampling. RBC and white blood cell counts, hemoglobin (Hb), Hct, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) of each subject were determined using an electronic hematology analyzer (ACT 10, Beckman Coulter; Fullerton, CA, USA), all of which were in normal limits.

The experimental procedures were explained to all subjects, and their written informed consent was obtained. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the institution's human

research committee. Additionally, the study was approved by the Ethics Committee of Pamukkale University.

#### *Genotyping*

DNA was extracted from peripheral blood samples by standard phenol/chloroform extraction methods (Poncz et al.1982). Polymerase chain reaction (PCR) was used to detect the presence of the I and D alleles in intron 16 of the ACE gene according to the method described by Tiret et al. (1992) using upstream primer 5'- CTGGAGACCACTCCCATCCTTCT- 3' and the downstream primer 5' - GATGTGGCCATCACATTCGT CAGAT - 3'. Amplification was performed for 35 cycles with denaturation, extension and annealing temperatures of 94°C, 60°C and 72°C, respectively. The sizes of amplified fragments were determined by 2% agarose gel electrophoresis and identified by UVI Gel Documentation system (Cambridge, UK).

#### *Measurements of RBC deformability*

RBC deformability (i.e., the ability of the entire cell to adopt a new configuration when subjected to applied mechanical forces) was determined at various fluid shear stresses by laser diffraction analysis using an ektacytometer (LORCA, RR Mechatronics; Hoorn, The Netherlands). The system has been described elsewhere in detail (Hardeman et al. 1994). Briefly, a low hematocrit suspension of RBC in an isotonic viscous medium (4% polyvinylpyrrolidone 360 solution; MW 360 kD, Sigma P 5288, St. Louis, IL, USA) was sheared in a Couette system composed of a glass cup and a precisely fitting bob, with a gap of 0.3 mm between the cylinders. A laser beam was directed through the sheared sample, and the diffraction pattern produced by the deformed cells was analyzed by a microcomputer. On the basis of the geometry of the elliptical diffraction pattern, an elongation index (EI) was calculated for shear rates between 0.3 and 30 Pascal (Pa) as:  $EI = (L - W)/(L + W)$ , where  $L$  and  $W$  are the length and width of the diffraction pattern, respectively. An increased EI at a given shear stress indicates greater cell deformation and hence greater RBC deformability. All measurements were carried out at 37°C.

#### *Measurements of RBC aggregation*

RBC aggregation was also determined by LORCA as described elsewhere (Hardeman et al. 2001). The measurement is based on the detection of laser back-

scattering from the sheared (disaggregated), then unsheared (aggregating) blood, performed in a computer-assisted system at 37°C. Back-scattering data are evaluated by the computer and the aggregation index (AI), aggregation half time ( $t_{1/2}$ ) which shows the kinetics of aggregation and the threshold shear rate ( $\gamma_{thr}$ ) which is a measure for the tendency to aggregate and aggregate stability are calculated on the basis that there is less light back-scattered from aggregating red cells. The Hct of the samples used for aggregation measurements was adjusted to 40% and blood was fully oxygenated.

#### *Determination of whole blood and plasma viscosity*

Whole blood viscosities (WBV) were determined with a Wells-Brookfield cone-plate rotational viscometer (model DV-II + Pro, Brookfield engineering Labs, Middleboro, MA, USA) at shear rates between 75-375  $s^{-1}$  at 37°C at both native Hct and standard Hct (40%). The Hct of blood samples was adjusted to 0.4 L/L by adding or removing a calculated amount of autologous plasma obtained by centrifugation at 1,400  $\times g$  for 6 minute. Plasma viscosity (PV) was determined using the same viscometer at 375  $s^{-1}$  at 37°C.

#### *Statistical analysis*

Results are expressed as means  $\pm$  S.E. Statistical comparisons between and among groups were done by Mann Whitney's U and Kruskal Wallis tests, respectively. Genotype and allele distribution according to gender was evaluated by Chi square test.  $P$  values  $< 0.05$  were accepted as statistically significant. All analyses were carried out with the computerized SPSS 10.0 program (Statistical Package for Social Sciences, SPSS Inc.).

## **RESULTS**

ACE polymorphisms were determined, as judged by the lengths of the PCR products in 2% agarose gel electrophoresis. The lengths of the amplified fragments were 190 bp for the DD genotype, 490 bp for the I/I genotype, and 190 bp and 490 bp the I/D genotype (Fig. 1). The genotype and allele frequencies of 28 subjects (13 women and 15 men) are shown in Table 1. The I/I genotype (II) was found in 6 subjects (21.4%), the ID genotype in 5 subjects (17.9%), and the DD genotype in 17 subjects (60.7%). The frequencies of the I and D alleles are 30.4% and 69.6%, respectively (Table 1). There was no statistically significant difference in the distribution of ACE geno-

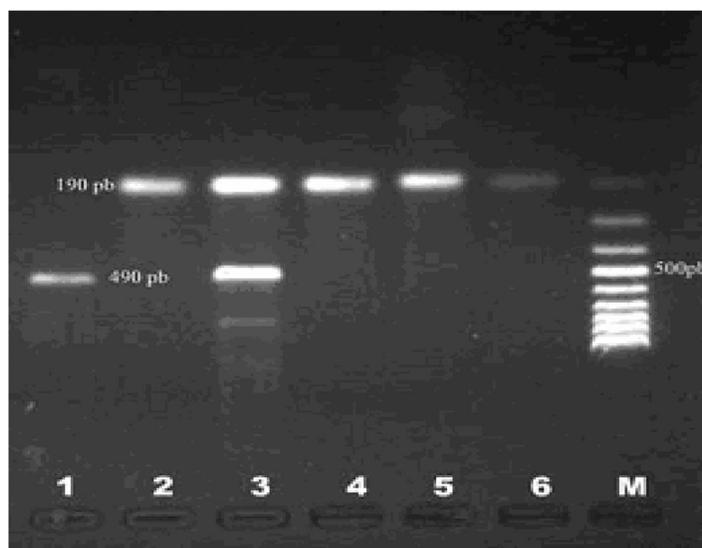


Fig. 1. Angiotensin-converting enzyme (ACE) genotypes. Polymerase chain reaction (PCR) amplification of ACE alleles insertion (I) and deletion (D) showed the presence of 490 bp and 190 bp products on 2% agarose gel electrophoresis. Line 1: II polymorphism; Lines 2, 4, 5, 6: DD polymorphism; Line 3: ID polymorphism; M: 100 bp DNA marker.

TABLE 1. Distribution of angiotensin converting enzyme genotype and allele in healthy young subjects according to gender

	Subjects (n = 28)	Female (n = 13)	Male (n = 15)
<i>Genotypes</i>			
II	6 (21.4%)	3 (23.1%)	3 (20%)
ID	5 (17.9%)	3 (23.1%)	2 (13.3%)
DD	17 (60.7%)	7 (53.9%)	10 (66.7%)
<i>Alleles</i>			
I	17 (30.4%)	9 (34.6%)	8 (26.7%)
D	39 (69.6%)	17 (65.4%)	22 (73.3%)

types and alleles between women and men.

RBC deformability (i.e., the elongation index EI) was determined at a shear stress of 0.53 Pa in individuals with the II, ID or DD genotype (Fig. 2) and in those with the I or D allele (Fig. 3). RBC deformability was found to be significantly increased in individuals with the DD genotype or the D allele, compared to those with the II ( $p < 0.05$ ) or ID ( $p < 0.01$ ) genotype and those with the I allele ( $p < 0.01$ ).

The aggregation index (AI), aggregation half time ( $t_{1/2}$ ), and threshold shear rate ( $\gamma_{thr}$ ) were measured in subjects with the II, ID or DD genotype (Table 2) and those with the I or D allele (Table 3). There were no significant alterations in aggregation parameters among the groups. Likewise, there were no significant differences in whole blood and plasma viscosities (Tables 2 and 3).

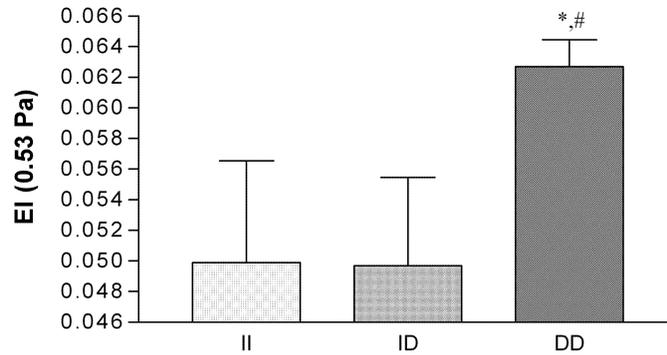


Fig. 2. RBC elongation index (EI) values of subjects with the II, ID and DD genotypes measured at 0.53 Pascal shear stress. Values are expressed as means  $\pm$  s.e. \*  $p < 0.05$  difference from II genotype, #  $p < 0.01$  difference from ID genotype.

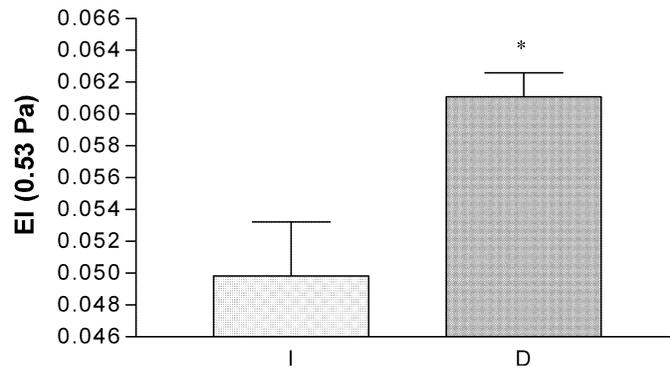


Fig. 3. RBC elongation index (EI) values of subjects according to the I and D alleles measured at a shear stress of 0.53 Pascal. Values are expressed as means  $\pm$  s.e. \*  $p < 0.01$  difference from I allele.

TABLE 2. Hemorheological parameters of healthy young subjects possessing the ACE II, ID and DD genotypes

	AI (%)	t 1/2 (s)	$\gamma_{thr}$ ( $s^{-1}$ )	WBV at native Hct (mPa.s)	WBV at standard Hct (40%) (mPa.s)	PV (mPa.s)
II	72.88 $\pm$ 1.61	1.34 $\pm$ 0.13	224.79 $\pm$ 32.14	4.17 $\pm$ 0.20 (Hct: 38.63)	4.44 $\pm$ 0.10	1.55 $\pm$ 0.02
ID	71.02 $\pm$ 1.64	1.49 $\pm$ 0.13	208.75 $\pm$ 14.03	4.40 $\pm$ 0.27 (Hct: 41.36)	4.28 $\pm$ 0.14	1.47 $\pm$ 0.03
DD	70.80 $\pm$ 1.13	1.54 $\pm$ 0.10	210.78 $\pm$ 18.69	4.40 $\pm$ 0.16 (Hct: 41.48)	4.44 $\pm$ 0.14	1.54 $\pm$ 0.03

AI, aggregation index; t 1/2, aggregation half time;  $\gamma_{thr}$ , threshold shear rate; WBV, whole blood viscosity at a shear rate of 150  $s^{-1}$ ; PV, plasma viscosity measured at 375  $s^{-1}$ .

TABLE 3. Hemorheological parameters of subjects according to the ACE I and D alleles

	AI (%)	t 1/2 (s)	$\gamma_{thr}$ (s <sup>-1</sup> )	WBV at native Hct (mPa.s)	WBV at standard Hct (40%) (mPa.s)	PV (mPa.s)
I	72.34 ± 0.90	1.38 ± 0.07	220.07 ± 15.67	4.24 ± 0.12 (Hct: 39.44)	4.39 ± 0.06	1.53 ± 0.02
D	70.82 ± 0.71	1.54 ± 0.06	210.52 ± 11.44	4.40 ± 0.10 (Hct: 41.46)	4.42 ± 0.09	1.53 ± 0.02

AI, aggregation index; t 1/2, aggregation half time;  $\gamma_{thr}$ , threshold shear rate; WBV, whole blood viscosity at a shear rate of 150 s<sup>-1</sup>; PV, plasma viscosity measured at 375 s<sup>-1</sup>.

### DISCUSSION

The ACE gene I/D polymorphism in healthy young subjects and its relationship to hemorheological parameters, such as RBC deformability, RBC aggregation, and plasma and whole blood viscosity were investigated in this study. Here we have shown that RBC deformability, measured at 0.53 Pa, is significantly increased in the subjects with DD genotype and those with the D allele, compared with the subjects possessing the II or ID genotype and those with the I allele. No significant relationship between the I/D polymorphism and RBC aggregation or whole blood and plasma viscosity was observed.

There are noticeable differences in the reported frequencies of the ACE gene I/D polymorphism in healthy Turkish population. Isbir et al. (1999) have observed the I and D alleles as 50.7% and 49.3%, respectively, in 58 healthy subjects. On the other hand, Akar et al. (1998) reported the presence of I and D alleles as 38.7% and 61.2%, respectively, in 107 subjects. Turgut et al. (2004) found the presence of the I and D alleles as 20.7% and 79.3% in 80 healthy subjects. The latter two reports are in good agreement with the allele frequencies of the present study.

Flow, deformability and aggregability of blood cells are main components of hemorheology. In large blood vessels, a basic component is the flow, since whole blood viscosity depends on hematocrit and plasma viscosity. In microcirculation, where cells must deform to pass through

narrow capillaries, deformability and aggregation of RBCs are the major determinants of resistance to flow. The ability of the entire RBC to deform is of crucial importance for performing its function of oxygen delivery and it is also a determinant of the cell survival time in the circulation (Mohandas et al. 1983; Stuart and Nash 1990). The inability to deform would make it difficult for the red cells to perform their function of oxygen delivery and RBCs with reduced deformability are removed from the circulation by the reticulo-endothelial system. Thus, significant decreases in RBC deformability compromises blood flow through the microcirculation and curtails nutrient support (Mohandas et al. 1979; Chien 1987). Impairments in RBC deformability have been reported in a number of cardiovascular diseases, including myocardial infarction, dilated cardiomyopathy and hypertension (Gustavsson et al. 1994; Cicco and Pirrelli 1999; Meiselman 1999; Sandhagen 1999; Vaya et al. 2002). The current study shows that healthy young subjects with the DD genotype or the D allele of ACE gene have increased RBC deformability, measured at a shear stress of 0.53 Pa, despite that the ACE D allele represents a risk factor for cardiovascular pathologies (Cambien et al. 1992; Marian et al. 1993; Reynolds et al. 1993; Tiret et al. 1993; Mattu et al. 1995; Gharavi et al. 1996; Agerholm-Larsen et al. 1997; Montgomery 1997; Prasad et al. 2000). We therefore suggest that RBC deformability may have been increased in individuals with the ACE D allele by a compensatory mechanism. RBC deformability has been measured at shear rates in

a range between 0.3 and 30 Pa. However, increased RBC deformability was detected only at 0.53 Pa in subjects with the DD genotype or with the D allele. The differences observed only in the lower part of the shear rate range but not at the higher shear rates indicate that RBC deformability is mainly related with the cell membrane.

Prasad et al. (2000) have demonstrated that presence of the ACE D allele is associated with increased vascular smooth muscle tone and the enhanced tone appears to be counterbalanced by an increase in basal nitric oxide (NO) activity in patients with atherosclerosis. Additionally, the increase in phenylephrine-induced contraction, by pre-incubation with NG-monomethyl-L-arginine (L-NMMA), was found to be more pronounced in internal mammary artery segments from patients with coronary artery disease who possessed the DD genotype compared with ID and II genotypes, suggesting a higher level of basal nitric oxide release in these patients (Buikema et al. 1996). The mechanisms underlying these observations may be explained by the fact that increased angiotensin II stimulates NO release either via the AT1 or AT2 receptor stimulation (Boulanger et al. 1995; Saito et al. 1996; Siragy and Carey 1997). This raises the possibility that activation of the renin-angiotensin system may also regulate basal NO release so that greater angiotensin II activity in individuals with the ACE D allele would be expected to lead to higher basal NO release. NO synthesized in endothelial cells not only diffuses to the adjacent smooth muscle cells but also to the vascular lumen. In addition to its effects on leukocytes and platelets, NO interacts with RBCs and plays a very important role in preserving or enhancing RBC deformability (Korbut and Gryglewski 1996; Starzyk et al. 1999; Bor-Kucukatay et al. 2003). It is therefore conceivable that NO release may be enhanced as a compensatory mechanism in the current study population of healthy individuals with the ACE D allele, which in turn leads to the increased RBC deformability.

Blood flow in a given vascular bed is determined in part by the network geometry and the fluidity of blood (Schmid-Schönbein 1988). In

turn, blood fluidity is determined by properties of blood cells and plasma, Hct and by shear rate (Chien 1975). WBV has been suggested to be an independent risk factor for cardiovascular diseases (Lowe et al. 1980; Devereux et al. 1984). It was printed that hematocrit, plasma viscosity and RBC aggregation show a significant correlation with the severity of carotid artery stenosis (Szapary et al. 2004). Moreover, a positive correlation between the severity of arterial hypertension and whole blood viscosity was demonstrated. In the present study, we did not find any significant differences in plasma viscosities and WBVs at both native and standard Hct among the individuals possessing the II, ID or DD genotype and those with the I or D allele of the ACE gene.

Erythrocyte aggregation is the reversible adhesion of adjacent RBCs. The physiological importance of erythrocyte aggregation in circulation is its tendency to increase the blood viscosity in low shear flow and to disturb the passage in capillary circulation through the formation of sludge (Chien 1975). RBC aggregation has also been associated with arterial hypertension especially in the severe form of the disease (Bogar 2002). However, in the present study, we show no significant alterations in aggregation parameters among the subjects with the II, ID or DD genotype and those with I or D allele of the ACE gene.

In summary, although the ACE I/D gene polymorphism was found to be associated with alterations in RBC deformability in our present group of healthy young subjects, no significant relationship was detected between this polymorphism and the other hemorheological parameters studied. Based on these observations, we propose that a possible increment in NO release may be responsible for the improvement in RBC deformability. This compensatory mechanism may help to reduce the cardiovascular risks of individuals possessing D allele. Further studies are required to clarify this aspect.

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