Enhanced Expression of iNOS in Human Endothelial Cells during Long-Term Culturing with Extracellular DNA Fragments

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NO synthesis by endothelial cells plays an important role in normal function of the cardiovascular system. This work was designed to evaluate the role of variations in properties of extracellular DNA in the regulation of NO synthesis. We studied the effect of four DNA samples with various base sequences (50 ng/ml) on functional activity of endothelial cells HUVEC during 24-h culturing. Human DNA fragments with high content of CG repeats increased intracellular content of NO and its metabolites (nitrites and nitrates) and accelerated oxidation of nitrites to nitrates. Changes in the content of NO metabolites after 24-h culturing was shown to depend on the expression of gene for inducible, but not for endothelial NO synthase. Increased expression of inducible NO synthase positively correlated with an increase in the content of mRNA for the adapter protein *MyD88*, but did not depend on *TLR9* gene expression that encodes protein receptor for CG-DNA recognition. The intracellular concentration of *MyD88* mRNA did not depend on the content of *TLR9* mRNA. The existence of a variety of DNA-binding receptors apart from TLR9 receptor on the surface of endothelial cells was hypothesized. Activation of these receptors by extracellular DNA fragments stimulates expression of the adapter protein *MyD88*.

Key Words: *endothelium; nitric oxide; eNOS; iNOS; CG-DNA; TLR9; MyD88*

Extracellular DNA (ecDNA) has attracted much interest over the past decade. ecDNA circulates in the blood and other biological fluids of the organism. The properties of ecDNA are modified under pathological conditions [11]. Our previous studies showed that the concentration of ecDNA in the blood increases during cardiovascular diseases. These changes are accompanied by an increase in the amount of CG-rich repeats in ecDNA (CG-DNA) [1]. Since vascular endothelial cells produce protein receptors (TLR9) for CG-DNA, it can be hypothesized that endogenous DNA fragments activate the intracellular signal pathway associated with activation of the expression of *TLR9* gene and adapter protein *MyD88* [9]. Binding of CG-DNA to TLR9 results in the synthesis of cellular cytokines, reactive oxygen species, and nitrogen [10]. TLR9 activation in some cells is accompanied by stimulation of NO synthesis due to an increase in the expression of inducible NO synthase (iNOS) [7]. It was hypothesized that functional activity of TLR receptors plays a role in the development of atherosclerosis [5].

This work was designed to evaluate whether the properties of ecDNA (*e.g.*, variations in CG-DNA content) may affect the endothelial function via activation of TLR9 expression. We studied the long-term effect

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of DNA fragments with various base sequences on cultured endothelial cells (HUVEC).

MATERIALS AND METHODS

Endothelial cells (HUVEC) were cultured in 25-cm^2 culture flasks (Corning-Costar) with medium 199 (PanEco) containing 20% FBS (PAA), 50 U/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml gentamicin (PanEco), and 20 μl HEPES (PanEco) at 37°C. The study was performed with passage 2-4 cells (subconfluent culture). The cells were counted in a Goryaev chamber.

We used a fragment from the rDNA transcribed region (TR) (from -515 to 5321 according to HSU13369, GeneBank) inserted into a vector pBR322 (p(TRrDNA). The satellite III fragment 1.77 (p(satIII) region 1q12 of chromosome 1) served as AT-rich DNA. DNA was purified from LPS by successive treatment with Triton X-114 [4] and gel filtration on the carrier HW-85. ecDNA were isolated from blood plasma of a healthy donor (ecDNA(HD) and a tumor patient (ecDNA(P). DNA was isolated by the method of phenol extraction. DNA concentration was measured fluorometrically. The content of CG-DNA (transcribed region of the ribosomal repeat) was estimated by quantitative hybridization [2].

NO concentration was measured as described elsewhere [8]. CuFL was used as a NO-specific reagent. The study was conducted in 96-well plates. Fluorescence was recorded on a Victor device (PerkinElmer). Nitrite concentration in the medium was measured colorimetrically with standard Griess reagent [6]. Nitrates were reduced into nitrites (zinc in the presence of complex copper salts). Nitrite concentration was calculated as the difference between nitrite concentrations before and after reduction of nitrates into nitrites. In each experiment, the concentration of NO metabolites in the medium was corrected to the number of cells.

RNA was isolated using RNeasy Mini kit (Qiagen) and treated with DNase I. The reverse transcription reaction was performed with a Sileks kit. Real-time PCR (TaqMan) was conducted on a Rotor Gene 300 device (Corbett Research). The *AKTB* gene served as an internal standard. Expression was analyzed in several independent experiments with the device software. The error was 2%. We used the following primers: ***TLR9*** (F: CCA CAA CAA CAT CCA CAG CCA AGT, R: TCA GGC CTT GGA AGA AGT GCA GAT, P: FAM – TTC AGC GGC AAT GCA CTG GGC CAT AT – BQH1); ***MyD88*** (F: TCC ACA GTG ATG CCT ACT GAT GCT, R: ATG CAG ATG AGA GGT GGA CCC ATT P: FAM – ACA CCG CCC ATT CCA CTT CCT CCT T – BQH1; ***AKTB*** (F: ACC AAC TGG GAC GAC ATG GAG AAA, R: TAG CAC AGC CTG GAT AGC AAC GTA, P: ROX – ACC ACA CCT TCT ACA ATG AGC TGC GT-3' – BQH2); ***eNOS*** (F: TGG CTG GTA CAT GAG CAC TGA GAT, R: CAC GTT GAT TTC CAC TGC TGC CTT, P: FAM – TAC AAC ATC CTG GAG GAT GTG GCT GT – BHQ1); and ***iNOS*** (F: GCG TTA CTC CAC CAA CAA TGG CAA, R: ATA GCG GAT GAG CTG AGC ATT CCA, P: FAM – AGC GGA GTG ATG GCA AGC ACG ACT T – BHQ1).

RESULTS

We compared the effects of two model samples of DNA on NO synthesis by endothelial cells. These samples contained the same number of TLR9-binding sites, but different amount of inhibitory sites for TLR9. The p(TRrDNA) plasmid has 8 binding sites to human TLR9 (GTCGTT) [4]. It should be emphasized that 1 ligand site corresponds to 15 sites of effective inhibitors of TLR9 (repeats with a common formula of Gn) [3]. The p(satIII) plasmid includes an AT-rich sequence of the 1q12 region of human chromosome 1, which does not have the binding sites or inhibitors of TLR9. The vector pBR322 has 7 ligand sites. Each of these sites corresponds to only 3 inhibitory sites. DNA model samples (1-50 ng/ml) were added to the subconfluent culture of endothelial cells (HUVEC). The concentration of NO was estimated from an increase in fluorescence in the presence of a staining agent CuFL for 24 h (Fig. 1). NO concentration in endothelial cells was elevated in the presence of DNA samples. This effect was particularly pronounced at a p(TRrDNA) concentration of 1 ng/ml. The amount of NO decreased in the presence of p(TRrDNA) at a concentration of 50 ng/ml, but remained above the control level. The most significant differences (as compared to the control) were observed over the first 15 min after addition of DNA samples.

Endothelial NO concentration at a certain time depends on the following two processes: NO synthesis by enzymes (NO synthases); and NO oxidation to nitrites/nitrates and/or peroxynitrites. To study these processes, the concentration of nitrates/nitrites in the medium and amount of intracellular mRNA for NO synthases (iNOS and eNOS) were measured after 24-h culturing of cells in the presence of DNA samples at a concentration of 50 ng/ml. We used two model samples of ecDNA(HD) and ecDNA(P). The sample of ecDNA(P) was enriched with CG-DNA. The content of TRrDNA (marker of CG-DNA) was 11.1 pg/ng, which exceeded that in the genome by several times (1.8±0.4 pg/ng). ecDNA(HD) contained a relatively low amount of CG-DNA (2 pg/ng TRrDNA; Figs. 2 and 3). The total concentration of NO metabolites in

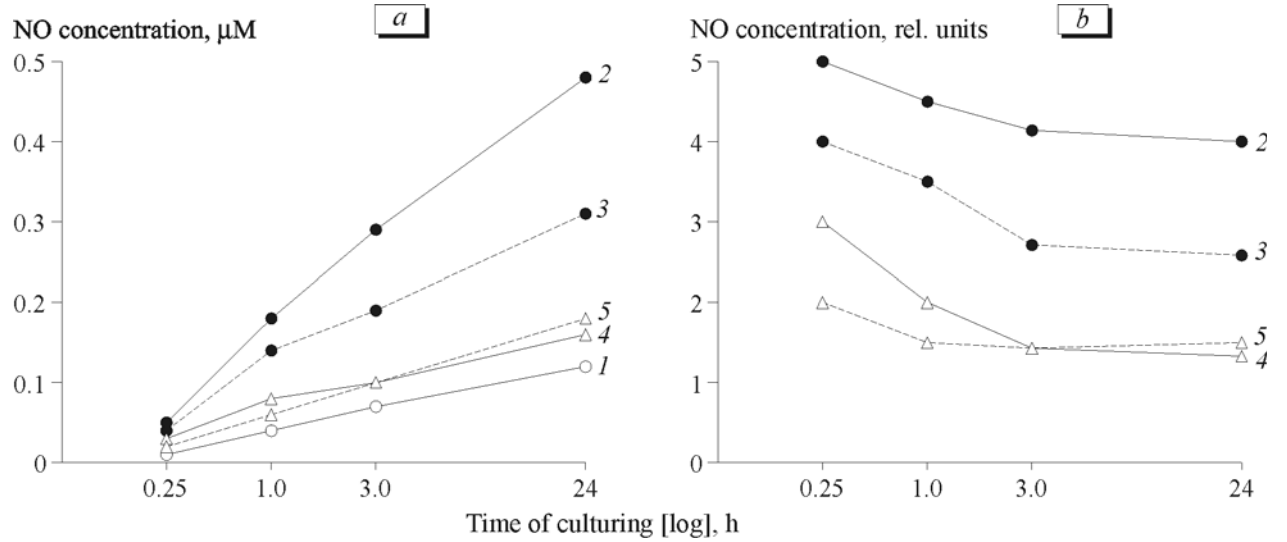

Fig. 1. Absolute (*a*) and relative content of NO (*b*) after treatment of HUVEC cells with DNA samples. Control (*1*); sample p(TRrDNA) (*2*, *3*); p(satIII) (*4*, *5*). Solid line: DNA concentration of 1 ng/ml; dotted line: 50 ng/ml. *SE*=10±3%.

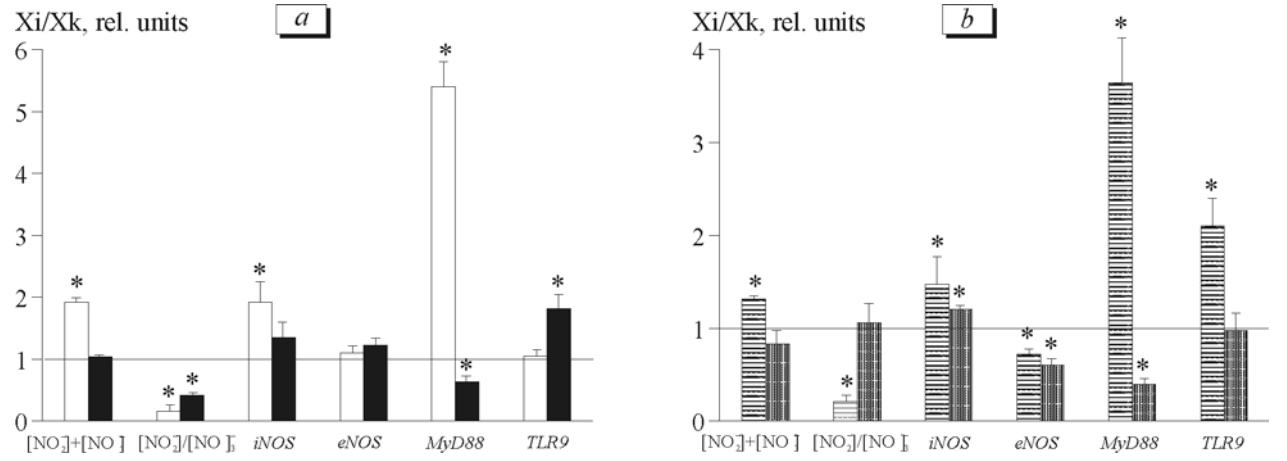

Fig. 2. Changes (relative to the control, Xi/Xk) in the concentration of NO metabolites, nitrite/nitrate ratio, and amount of mRNA for *iNOS*, *eNOS*, *MyD88*, and *TLR9* genes in the presence of DNA model samples (*a*) and ecDNA samples (*b*). Light bars, p(TRrDNA); dark bars, p(satIII); horizontal shading, ecDNA(P); vertical shading, ecDNA(HD). Line, control. **p*<0.001 compared to the control.

the presence of p(TRrDNA) and ecDNA(P) increased by 1.4-2 times compared to the control. The rate of nitrite oxidation to nitrates was higher under these conditions. It was manifested in a significant decrease in the concentration of nitrites and increase in the amount of nitrates (Fig. 3, *a*). The amount of NO metabolites in the medium in the presence of 4 DNA samples depended directly on the content of intracellular mRNA for the *iNOS* gene (but not for the *eNOS* gene; Fig. 3, *d*). The concentration of *eNOS* mNRA did not differ from the control (p(TRrDNA) and p(satIII) or was reduced (ecDNA(P) and ecDNA(HD).

We evaluated the role of DNA-binding TLR9 receptors in activation of NO synthesis, which correlates with increased expression of *iNOS* and rate of nitrite oxidation. The concentration of mRNA for *TLR9* and adapter protein *MyD88* gene (specific for this signal pathway) was measured in cells. The content of NO metabolites depended linearly on the concentration of *MyD88* mRNA (Fig. 3, *b*). Studying the total sample did not reveal the dependence of NO metabolite concentration or nitrite/nitrate ratio on the concentration of *TLR9* mRNA (Fig. 3, *c*). The content of *MyD88* mRNA increased by 5 times in the presence of p(TRrDNA). However, the concentration of *TLR9* mRNA remained unchanged under these conditions. For the other samples of DNA, the rate of nitrite oxidation to nitrates increased with an increase in *TLR9* expression (Fig. 3, *e*).

These data show that addition of the same amounts of DNA samples with various base sequences to the culture medium has different effect on functional ac-

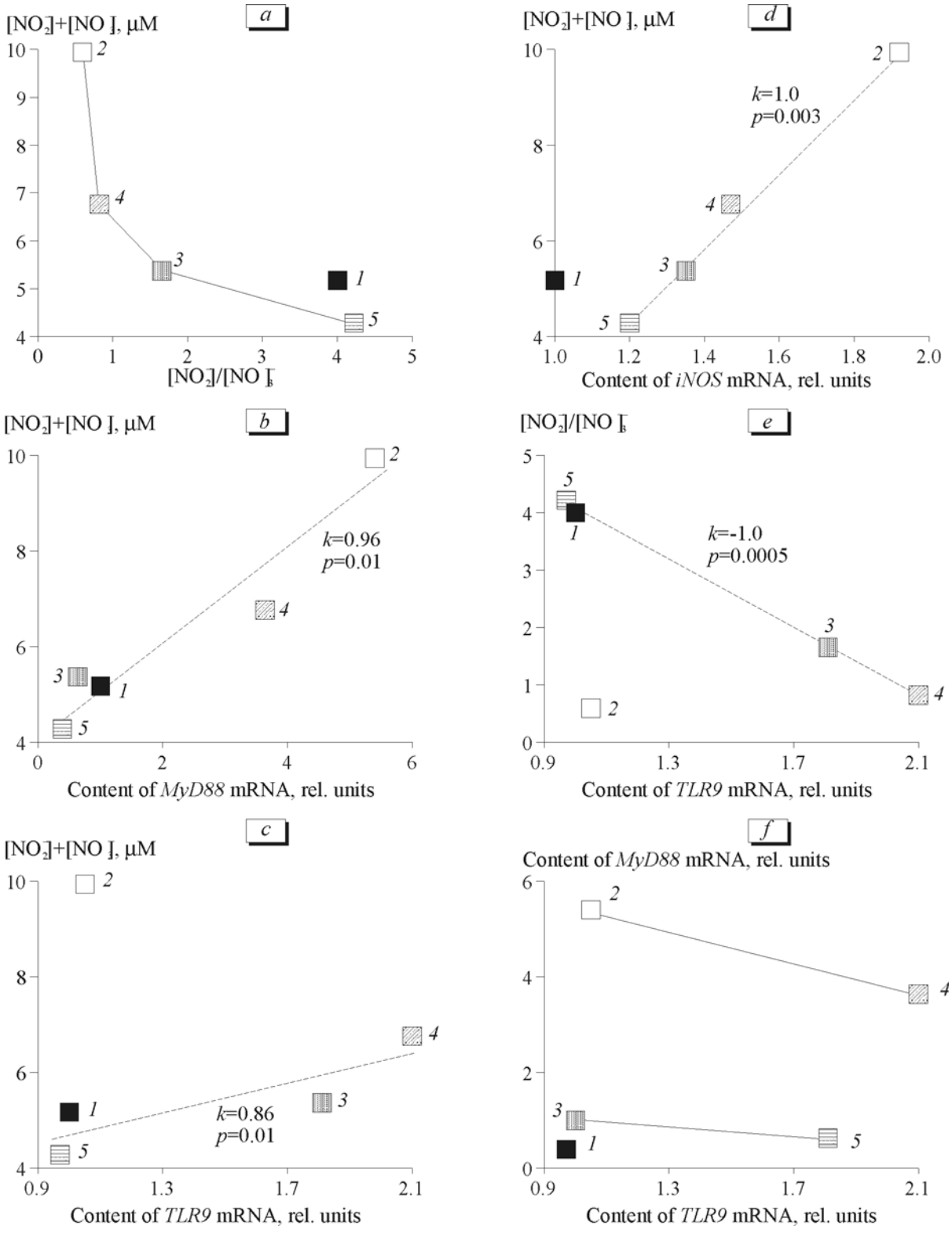

Fig. 3. Dependence of the total concentration of NO metabolites on the nitrite/nitrate ratio (*a*) and amount of mRNA for *MyD88* (*b*), *TLR9* (*c*), and *iNOS* (*d*). (*e*, *f*) Dependence of the nitrite/nitrate ratio and amount of *MyD88* mRNA on the concentration of *TLR9* mRNA. Dotted line, linear regression. Control (*1*); p(TRrDNA) (*2*); p(satIII) (*3*); ecDNA(P) (*4*); ecDNA(HD) (*5*).

tivity of endothelial cells. p(TRrDNA) plasmid DNA containing TLR9-binding sites and numerous inhibitory sites, is most potent in stimulating the synthesis of NO by iNOS. A significant increase in the expression of *MyD88* and slight activation of *TLR9* indicate that the stimulatory effect is realized via DNA-binding sites. These sites differ from TLR9, but interact with the adapter molecule *MyD88*. In whole, DNA samples (50 ng/ml medium) produce a negative effect on endothelial cells during 24-h culturing. This conclusion is derived from the increase in iNOS expression and rate of nitrite oxidation to nitrates. The observed changes reflect the development of oxidative stress. ecDNA(HD) was not enriched with CG-DNA and had little effect on the amount of NO metabolites and rate of nitrite oxidation. However, the concentration of *eNOS* mRNA was reduced in the presence of ecDNA(HD). The content of *eNOS* mRNA was slightly increased under these conditions. The sample ecDNA(P) had a stimulatory effect on the expression of *MyD88* and *TLR9*.

We conclude that ecDNA plays an important role in the regulation of endothelial function in humans. Under normal conditions, the blood contains a small amount of circulating ecDNA fragments. Pathological states are accompanied by cell death, the concentration of ecDNA increases and its composition changes. Blood concentration of fragments interacting with TLR9 and other DNA-recognizing receptors on endothelial cells increases. These changes induce *iNOS* expression and cause oxidative stress. The properties of DNA may be used as a promising target in the therapy of cardiovascular diseases.

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