Extracellular DNA Affects NO Content in Human Endothelial Cells

L. V. Efremova, A. Yu. Alekseeva, M. S. Konkova, S. V. Kostyuk, E. S. Ershova, T. D. Smirnova, I. L. Konorova*, and N. N. Veiko

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 149, No. 2, pp. 156-160, February, 2010
Original article submitted August 13, 2009

Fragments of extracellular DNA are permanently released into the blood flow due to cell apoptosis and possible *de novo* DNA synthesis. To find out whether extracellular DNA can affect the synthesis of nitric oxide (NO), one of key vascular tone regulators, we studied *in vitro* effects of three artificial DNA probes with different sequences and 10 samples of extracellular DNA (obtained from healthy people and patients with hypertension and atherosclerosis) on NO synthesis in endothelial cell culture (HUVEC). For detection of NO in live cells and culture medium, we used a NO-specific agent CuFL penetrating into the cells and forming a fluorescent product FL-NO upon interaction with NO. Human genome DNA fragments affected the content of NO in endothelial cells; this effect depended on both the base sequence and concentration of DNA fragments. Addition of artificial DNA and extracellular DNA from healthy people into the cell culture in a low concentration (5 ng/ml) increased the detected NO concentration by 4-fold at most. Cytosine-guanine (CG)-rich fragment of the transcribed sequence of ribosomal repeat was the most powerful NO-inductor. The effect of DNA fragments on NO synthesis was comparable with that of low doses of oxidizing agents, H_2O_2 and 17β-estradiol. Extracellular DNA samples obtained from patients with hypertension and atherosclerosis decreased NO content in cells and medium by 1.3-28 times compared to the control; the effect correlated with the content of CG-rich sequences.

Key Words: *endothelium; nitric oxide; CG-DNA*

Cell death is associated with the appearance of extracellular DNA (ecDNA) fragments in blood plasma. ecDNA can also enter the blood flow as a result of *de novo* synthesis in cells (metabolic DNA) [6,14]. ecDNA differ from DNA in cell nucleus: it contains single- and double-strand breaks, accumulates CG-enriched genome fragments (CG-DNA), and loses AT-enriched fragments [1,3]. Accumulation of CG-DNA fragments in ecDNA is not indifferent for the cells, because they act as ligands interacting with TLR family proteins (TLR9) [3]. We previously showed that accumulation in ecDNA of one of repeating CG-DNA of human genome, transcribed region of ribosomal repeat (TRrDNA), containing binding sites for TLR9 can result in significant lymphocyte activation accompanied by synthesis of proinflammatory cytokines [2]. In *in vitro* experiments, endogenous CG-DNAs accumulated in the blood of patients with cardiovascular diseases [1] reduced the rate of contraction of rat cardiomiocytes [7]. Human vascular endothelial cells express known proteins of the TLR family [9]. Activation of TLR-receptors with the corresponding ligands plays the key role in the development of endothelial dysfunction, which results in atherosclerosis [8]. We can hypothesize that endogenous CG-DNAs in ecDNA would interact with TLR of endothelial cells, modulating functional activity of these cells. NO synthesis is an important function of the endothelium normally

Medical Genetics Research Center, Russian Academy of Medical Sciences; * Research Center of Neurology, Russian Academy of Medical Sciences, Russia. ***Address for correspondence:*** ribgene@rambler.ru. N. N. Veiko

0007-4888/10/1492-0196

realized by endothelial NO-syntase (eNOS). This mediator is of critical importance for the maintenance of normal vascular tone. Changes in eNOS-dependent NO synthesis lead to the development of hypertension. On the other hand, excessive stimulation of NO synthesis under conditions of hyperexpression on inducible NOS (iNOS) is associated with the formation of active oxygen and nitrogen compounds and development of oxidative stress.

Here we compared the effects of short-term (3 h) exposure of human endothelial cell culture (HUVEC) to human genome DNA fragments with different base sequences, bacterial DNA, and samples of ecDNA obtained from patients with atherosclerosis and hypertension and from healthy individuals on NO content in these cells.

MATERIALS AND METHODS

Human umbilical vein endothelial cells (HUVEC) were incubated in 25-cm^2 culture flasks (Corning-Costar) in medium 199 (PanEco) containing 20% FCS (PAA), penicillin 50 U/ml, streptomycin 50 μg/ml, gentamicin 10 μg/ml (PanEco), HEPES 20 μl (PanEco) at 37°C. Cells of 2-4 passages were used. The incubation was carried out in culture flasks (Corning-Costar) during passages 1 and in slide flasks (Nunclon) and 96-well plates (Corning-Costar) during passages 2-4. Subconfluent cultures were used in the experiments. Inoculation density was 500 thousand cells per 25 cm^2 flask and 7 thousand cells per plate. The cells were counted using a Goryaev chamber.

CG-rich fragment of TRrDNA (from -515 to 5321 in accordance with HSU13369, GeneBank) incorporated into vector pBR322 (p(TRrDNA) was used as CG-DNA of human genome. Fragment 1,77 of satellite III (p(satIII) 1q12 region, chromosome 1) was used as a AT-rich DNA. *E. coli* DNA was obtained from strain MG 1655 by phenol extraction. All DNA samples were additionally purified from LPS: successive treatment with Triton X-114 [5] and gel filtration chromatography on carrier HW-85. Human ecDNA was obtained from peripheral blood plasma [1,3]. TRrDNA content in ecDNA samples was established using quantitative hybridization [1,3]. ecDNA was separated from blood plasma, obtained from patients with atherosclerosis and arterial hypertension II grade (1-4) or arterial hypertension without signs of atherosclerosis (5).

The content of NO was measured using CuFL reagent enabling NO detection in cells [10] by fluorescent microscopy (Axioplan microscope, Opton; RETIGA 2000*R* digital camera, Imaging) and in culture medium [11] by fluorescence (LS 55 spectrofluorometer or Victor plate reader, PerkinElmer). DNA fragments were added to cells, CuFL in concentration 1 μM was added after 20 min, incubation was performed for 15 min – 24 h, and then the fluorescence was measured in plates (495 nm excitation wavelength, 526 nm emission wavelength). The following assumption was made for calculation of the absolute NO concentration. Since in the main experiment we measured the fluorescence in the wells without separating the cells and the medium, the recorded signal was a sum of signals from FL-NO (derivative of NO and CuFL) absorbed in cells and on the surface of the cells and signal from FL-NO dissolved in culture medium. We neglected possible differences in the quantum yields of FL-NO in cells and in the medium and used the dependence of CuFL fluorescence in solution on NO concentration (inorganic origin) in the culture medium for endothelial cells as a calibration curve.

RESULTS

In the presence of CuFL, green fluorescent coloration appeared at sites of NO formation in cell cytoplasm (Fig. 1). When NO synthesis was activated, the cell edges looked blurred, since NO had enough time to diffuse into the medium before it interacted with the dye with the formation a compound, which can be only partially sorbed on the cell surface (Fig. 1, *c-e*). The formed fluorescent compound partially remained dissolved in the culture medium. Using known stimulators of endothelial NO synthesis H_2O_2 [13] and 17β-estradiol we showed that stimulation of NO synthesis in the presence of CuFL in the culture medium is associated with intensification of fluorescence of both cells on the surface of a solid carrier and culture medium.

The choice of DNA sequences for evaluation of their effect on the endothelium was determined by their capacity to act as TLR9 ligands. TLR9 are known to interact with 6-8-moiety DNA fragments with nonmethylated CG-sequence in the centre. The effects of three DNA samples were investigated. p(TRrDNA) plasmid contains CG-rich insert, a 5836 b.p. fragment TRrDNA. Previous studies showed several-fold increased content of this sequence in blood ecDNA from patients with cardiovascular diseases and rheumatic arthritis and from irradiated patients compared to that in healthy individuals [1,3,4]. The fragment includes binding sites for TLR9-receptors [3]. Moreover, pBR322 vector also includes CG-repeats and binding sites for TLR9. Some sites of TRrDNA insert contain TLR9 suppressor sequences (short repeats with general formula (G)n), which can block TLR9-binding sites of p(TRrDNA) sequence. Plasmid p(satIII) contains AT-rich sites (area 1q12 of chromosome 1), which do not include neither ligands, nor suppressors of TLR9,

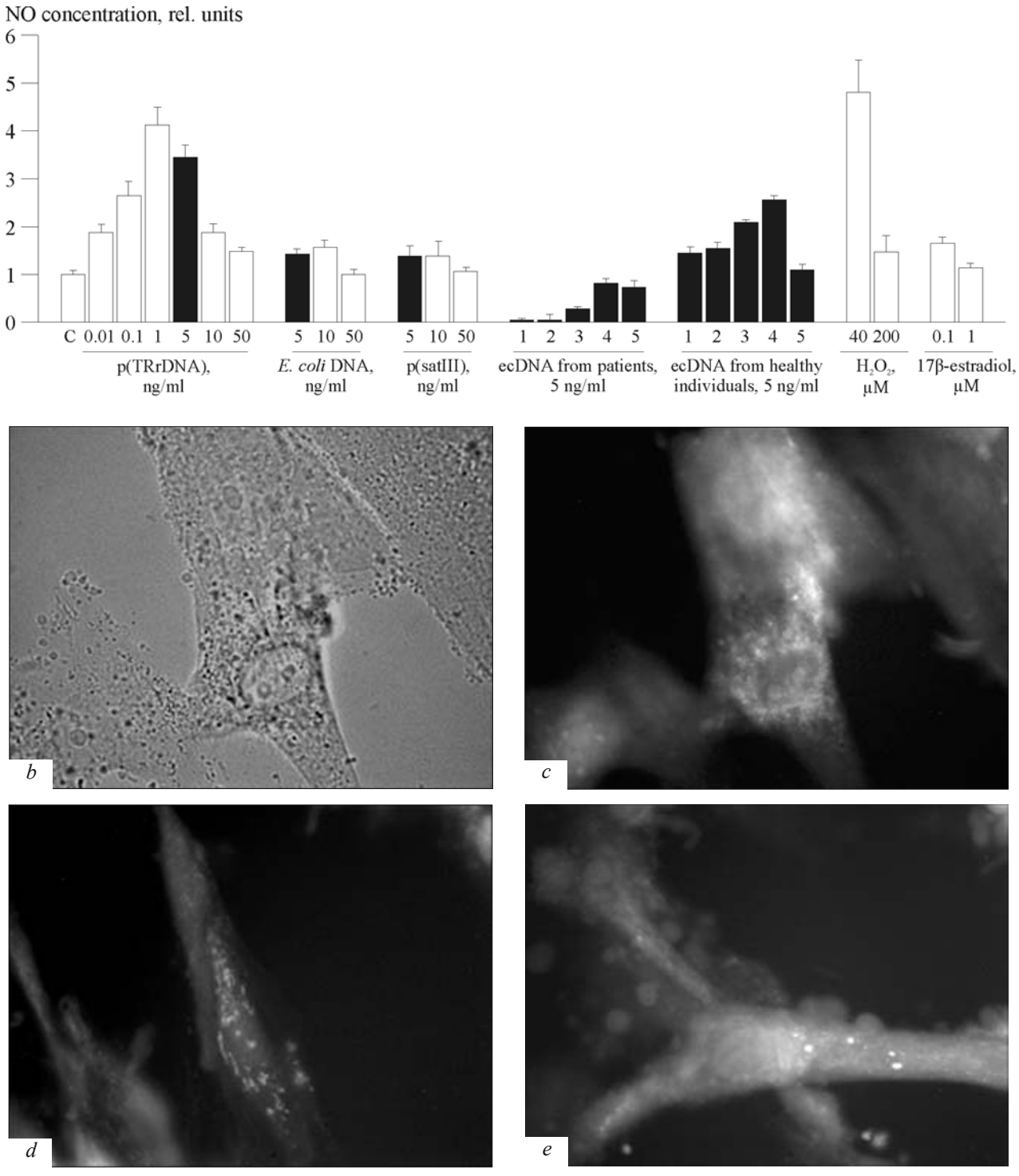

Fig. 1. DNA fragment effects on NO synthesis in endothelial cells. *a*) correlation of relative NO concentration with sequence and concentration of DNA samples, ecDNA of healthy people and patients, H_2O_2, and 17β-estradiol. Dark bars: data for different DNAs at the same concentration of the fragment in the medium (5 ng/ml). Results of three experiments are pooled. *b-e*: NO synthesis in endothelial cells detected microscopically using CuFL reagent (×100). *b* and *c*: the same cells (incubated for 3 h with 40 μM H_2O_2) captured in visible (*b*) and UV light (*c*); *d*) control cells; *e*) simulation with 1 ng/ml p(TRrDNA).

and CG-rich DNA fragments (vector pBR322). *E. coli* DNA is a classical TLR9 ligand [12].

Addition of DNA fragments to the culture medium was associated with quantitative changes in NO synthesis by endothelial cells (Fig. 1). In low concentrations (5 ng/ml), all artificial DNA samples and ecDNA from healthy individuals stimulated NO accumulation. The maximum increase in NO concentration (4-fold in

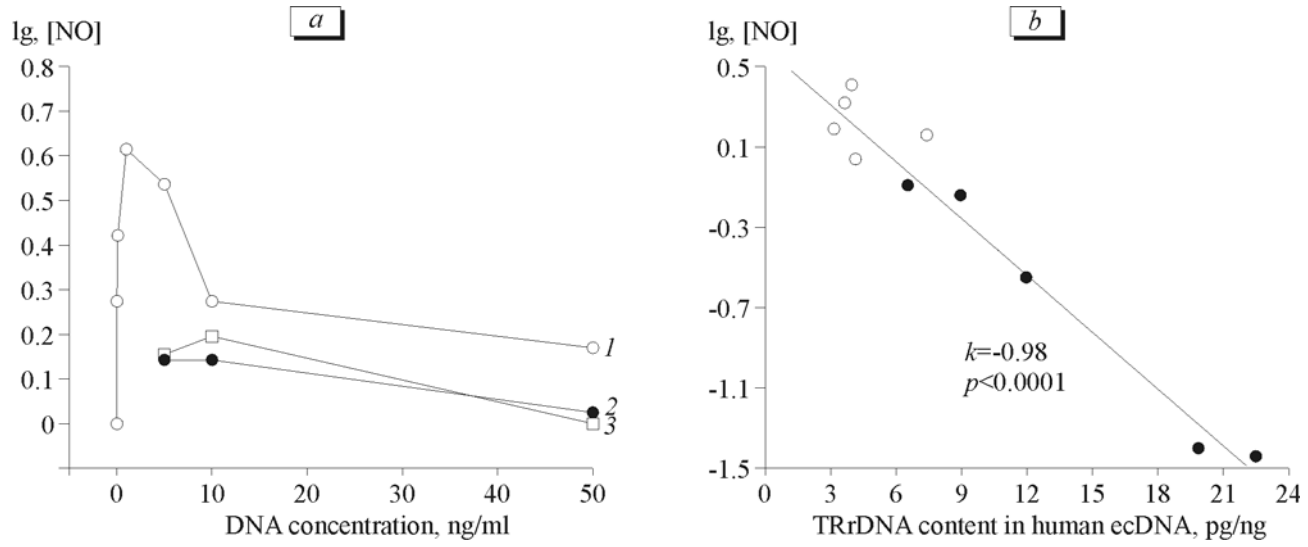

Fig. 2. Correlation of NO concentration decimal logarithm with artificial DNA probe concentration (*a*) and with marker CG-DNA (TRrDNA) content in human ecDNA (*b*). *a*: *1*) p(TRrDNA); *2*) p(satIII); *3*) *E. coli* DNA. *b*: dark and light circles correspond to ecDNA from patients and healthy individuals, respectively.

comparison with the control) was observed in the presence of 1 and 5 ng/ml p(TRrDNA). The effect of this DNA sample is comparable with the effect of small amounts H_2O_2 (40 μM). When high concentrations of artificial DNA (50 ng/ml) were used, the stimulation effect on NO synthesis was little or absent. The absolute NO amounts (calculated with assumptions mentioned in the experimental part), varied from 0.14 to 0.58 nmol/million cells. Addition of ecDNA samples from patients with hypertension and/or atherosclerosis in a concentration 5 ng/ml reduced NO concentration in both cells and culture medium by 1.3-28 times.

To find out why ecDNA from patients and healthy people produced different effects on endothelial cells, we studied the correlations of NO concentrations with concentrations of artificial DNA in the medium (Fig. 2, *a*) and with the content of marker CG-rich sequence TRrDNA in ecDNA samples (Fig. 2, *b*). NO content increased with increasing p(TRrDNA) concentration in the medium from 0.01 to 1 ng/ml, further increase in DNA concentration to 50 ng/ml resulted in 3-fold decrease in NO content. The increase in the concentration of two other DNA samples to 50 ng/ml also led to a decrease in NO content. We found an exponential relationship between NO amount in cells and TRrDNA content in ecDNA samples. TRrDNA we regard as the marker reflecting changes in ecDNA properties: accumulation of CG-rich sequences. Undoubtedly, other CG-DNA accumulate in ecDNA in parallel with TRrDNA. For example, a correlation between TRrDNA content and another CG-DNA (telomere repeat) was found for the studied ecDNA samples (k=0.65, $p<0.05$, $n=10$). Significant NO reduction in endothelial cells in the presence of ecDNA samples can be explained by the increase in total amount of CG-DNA in ecDNA obtained from the patients.

Thus, fragments of human genome DNA affect NO synthesis by the endothelium and this effect depends on both base sequence and concentration. We previously showed that ecDNA in healthy individuals contains increased amounts of CG-rich TRrDNA fragment compared to cell DNA, but TRrDNA concentration in blood plasma does not exceed 1 ng/ml. These amounts of p(TRrDNA) stimulate NO synthesis *in vitro* (Fig. 1). Circulating ecDNA permanently released and eliminated from the blood flow can be a relevant factors, regulating endothelial NO synthesis in mammals. Changes in the concentration and content of ecDNA fragment accompanying cardiovascular and other pathologies [1] and resulting from ionizing radiation exposure [4] can disturb this function of the endothelium.

The mechanism of regulation of NO concentration in endothelial cells in the presence of ecDNA remains unclear. NO concentration depends on the amount and functional activity of enzymes, NO-synthases, and on the rate of molecule NO metabolism, particularly on its oxidation to nitrites/nitrates and formation of peroxynitrite toxic for cells. Thus, it is necessary to investigate the expression of genes of the two main endothelial NO-synthases (eNOS and iNOS) and to evaluate the effect of DNA samples on the level of reactive oxygen species. In addition, it is important to find out which DNA-binding receptors are activated in endothelial cells in response to changes in ecDNA properties. This will allow using ecDNA properties as a potential target for the therapy of vascular disease.

REFERENCES

1. N. N. Veiko, N. V. Bulicheva, O. A. Roginko, *et al.*, *Biomed Khimiya*, **54**, No. 1, 78-93 (2008).
2. N. N. Veiko, E. A. Kalashnikova, S. N. Kokarovtseva, *et al.*, *Byull. Eksp. Biol. Med*, **142**, No. 10, 410-414 (2006).
3. N. N. Veiko, N. O. Shubaeva, S. M. Ivanova, *et al.*, *Ibid*, No. 9, 282-285 (2006).
4. S. V. Kostyuk, I. A. Zamulaeva, R. K. Agapova, *et al.*, *Radiats. Biol. Radioekol.*, **48**, 5-13 (2008).
5. Y. Aida and M. J. Pabst, *J. Immunol. Methods*, **132**, No. 2, 191-195 (1990).
6. P. Anker, J. Lyautey, C. Lederrey, and M. Stroun, *Clin. Chim. Acta*, **313**, Nos. 1-2, 143-146 (2001).
7. N. Bulicheva, O. Fidelina, N. Mkrtumova, *et al.*, *Ann. N. Y. Acad. Sci.*, **1137**, 273-277 (2008).
8. L. K. Curtiss and P. S. Tobias, *J. Lipid Res.*, **50**, Suppl. S340-S345 (2009).
9. N. Fitzner, S. Clauberg, F. Essmann, *et al.*, *Clin. Vaccine Immunol.*, **15**, No. 1, 138-146 (2008).
10. M. H. Lim, *Nat. Protoc.*, **2**, No. 2, 408-415 (2007).
11. M. H. Lim, B. A.Wong, W. H. Pitcock Jr., *et al.*, *J. Am. Chem. Soc.*, **128**, No. 44, 14,364-14,373 (2006).
12. M. Magnusson, R. Tobes, J. Sancho, and E. Pareja, *J. Immunol.*, **179**, No. 1, 31-35 (2007).
13. H. Shimokawa and K. Morikawa, *J. Mol. Cell Cardiol.*, **39**, No. 5, 725-732 (2005).
14. M. van der Vaart and P. J. Pretorius, *Ann. N. Y. Acad. Sci.*, **1137**, 18-26 (2008).