Abstract&Methods on «***The extracellular DNA containing easily oxidizable Gc-Rich motifs of human ribosomal DNA stimulates ROS generation in MCF7 cancer cell, is oxidized, penetrates the cell and can be expressed***» researching

Abstract

OBJECTIVE. During a series of diseases, human circulating cell free DNA (ccfDNA) becomes enriched with a fraction of easily oxidizable GC-rich fragments (GC-ccfDNA). The human oxidized DNA is known to penetrate easily the MCF7 breast cancer cells and significantly changes the physiology of the cells. Therefore, the purpose of this work was to assess whether the transfection rate and the expression level of extracellular easily oxidizable GC-DNA is increased in the cancer cells. METHODS. MCF7 cells were cultured in the presence of two types of GC-DNA probes: (1) vectors pBR322 and pEGFP, and (2) plasmids carrying inserted human rDNA (pBR322–rDNA and pEGFP-rDNA). pEGFP and pEGFP-rDNA contained CMV promoter and a fluorescent protein gene EGFP. ROS generation rate, the level of accumulation of the DNA probes in MCF7, 8-oxodG content, expression of EGFP, NOX4, and localization of EGFP, NOX4 and 8-oxodG in MCF7 were evaluated. Applied methods were: qPCR, fluorescent microscopy, immunoassay, flow cytometry. RESULTS. GC-DNA probes interact with MCF7 and induce a transient burst of ROS generation. The ROS oxidize the GC-DNA. Oxidized DNA penetrate the cells. Insertions of rDNA significantly increase the GC-rDNA oxidation degree as well as the rate of plasmid transfection into the cells and the EGFP expression level. In the nucleus, the oxidized GC-rDNA fragments are localized within the nucleolus. At the same time, the cellular rRNA content is decreased. CONCLUSIONS. GC-rich ccfDNA fragments that are prone to oxidation can easily penetrate the cancer cells and be expressed. The ccfDNA should become a therapeutic target for the tumor care.

Methods

Cell culture

ER/PR-positive MCF7 breast cancer cells were purchased at ATCC, Manassas, USA (Cat: HTB-22). MCF7 cells were cultured in DMEM medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL of streptomycin. Cells were grown in a humidified atmosphere with 5% CO2 in air at 37°C. Before treatment with GC DNA probes, cells were grown for 48 h in slide flasks.

Model GC-DNA

Plasmid pEGFP-C1 (pEGFP) that contains the EGFP gene (http://www.bdbiosciences.com, GenBank accession number U55763) was used as vector (Fig.1B). The DNA fragment to be inserted was synthesized and consisted of 420 base pairs flanked with BamHI restriction sites and containing the rDNA. Cloned rDNA fragment covers positions from 601 to 1021 b of human rDNA (Fig.1A). The vector pEGFP-C1 was treated with BamHI and added to the DNA fragment with subsequent ligation with T4 DNA ligase. Competent E. coli (strain JM110) were then transformed and grown on LB with agarose and kanamycin (50 μg/ml). The clones were analyzed by PCR. Selected clones were grown in liquid medium and plasmids were isolated. After confirmation of the designed DNA sequence by sequencing, the plasmids were extracted using an Invisorb Plasmid Maxi Kit (http://www.invitek.de).

Plasmid pBR322 is the commercial product (Sigma-Aldrich). pBR322-rDNA: plasmid DNA contains rDNA sequences cloned into EcoRI site of pBR322 vector. Cloned rDNA fragment covers positions from −515 to 5321 of human rDNA (Fig.1A).

All the GC-DNA samples were subjected to the purification procedure removing lipopolysaccharides; this included sequential treatment with Triton X114 (Merck, Germany) followed by gel filtration on the HW 85 [19] or the use of Endotoxin Extractor (Sileks, Russia).

Flow cytometry

Cells were washed in Versene solution, than treated with 0.25 % trypsin under control of light microscopic observation. Cells were washed with the culture media, then centrifuged and suspended in PBS.

EGFP. Non-fixed cells were analyzed at 488 nm. To quantify the background fluorescence, the control cells were analyzed.

8-oxodG, NOX4. Staining of the cells with antibodies was performed as described below [18]. Briefly, to fix the cells, the paraformaldehyde (Sigma) was added at a final concentration of 2 % at 37°C for 10 min. Cells (~ 50 x 103) were washed three times with 0.5% BSA-PBS and permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 15 min at 4°C. Cells were washed with 0.5% BSA-PBS and stained with 1 µg/mL 8-oxodG or NOX4 antibody (Abcam) for 24 h at 4°C, then again washed thrice with 0.5% BSA-PBS. After washing with 0.01% Triton X-100 in PBS MCF-7 cells were incubated for 2 h at room temperature with the FITC or PE goat anti-mouse IgG and washed with PBS. To quantify the background fluorescence, we stained a portion of the cells with secondary FITC (PE)-conjugated antibodies only.
Cells were analyzed at CyFlow Space (Partec, Germany).

Quantification of mRNA Levels
Total mRNA was isolated using RNeasy Mini kits (Qiagen, Germany), treated with DNAse I, and reverse transcribed by a Reverse Transcriptase kit (Sileks, Russia). The expression profiles were obtained using qRT-PCR with SYBR Green PCR Master Mix (Applied Biosystems). The mRNA levels were analyzed using the StepOnePlus (Applied Biosystems); the technical error was approximately 2%. The following primers were used (Sintol, Russia):
EGFP (F TACGGCAAGCTGACCCTGAAG; R TGAAGCACTGCACGCCGTAGG);
NOX4 (F:TTGGGGCTAGGATTGTGTCTA; R:GAGTGTTCGGCACATGGGTA);
TBP (reference gene) (F: GCCCGAAACGCCGAATAT; R: CCGTGGTTCGTGGCTCTCT).

Quantification of pEGFP and pEGFP-rDNA in the cells and medium
The cells. After incubation medium removal by centrifugation at 460 g, cells were mixed with the solution (1 mL) containing 0.2 % sodium lauryl sarcosylate, 0.002 M EDTA, and 75 µg/mL RNAse A (Sigma, USA) and incubated for 45 min, then treated at 37°C with proteinase K (200 µg/mL, Promega, USA) for 24 h. After two cycles of the purification with saturated phenolic solution, DNA fragments were precipitated by adding two volumes of ethanol in the presence of 2 M ammonium acetate. The precipitate was then washed with 75% ethanol twice, dried and dissolved in water. The concentration of DNA was determined by measuring fluorescence intensity after DNA staining with the PicoGreen (Molecular Probes/Invitrogen, CA, USA). The contents of pEGFP and pEGFP-rDNA were obtained using qPCR with SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used (Sintol, Russia):
EGFP (F TACGGCAAGCTGACCCTGAAG; R TGAAGCACTGCACGCCGTAGG);
Human B2M (reference gene, accession number M17987):
F GCTGGGTAGCTCTAAACAATGTATTCA; R CATGTACTAACAAATGTCTAAAATGG
Culture medium. For the isolation of DNA from the cell culture medium, a procedure similar to that described above for the cells was used. Electrophoresis of DNA was carried out in an 2% agarose gel stained with ethidium bromide.

8-oxodG levels in pEGFP and pEGFP-rDNA
MCF7 1h (Fig.5C). MCF7 were cultured in the presence of plasmids for one hour. The RNA fraction contained fragments of plasmid DNA was isolated using YellowSolve (Sileks, Russia). RNA was digested (1h, 37°, 75 µg/mL RNAse A), DNA was precipitated with 75% ethanol. The contents of pEGFP and pEGFP-rDNA were obtained using qPCR.
UV/H2O2 (Fig.5C). The method for DNA oxidation was specified previously [18]. Briefly, plasmids pEGFP and pEGFP-Gn (100ng/µL) were oxidized in 0.1% H2O2 solution with UV irradiation (λ> 312 nm) for 3 minutes at 25°C. Modified DNA was precipitated with 2 volumes of ethanol in the presence of 2 M ammonium acetate. The precipitate was washed twice with 75% ethanol, then dried and dissolved in water. Resulting DNA concentrations were assessed by the analysis of UV spectra.
The method for 8-oxodG quantitation was specified in details previously [20]. Briefly, the DNA samples were applied to a prepared filter (Optitran BA-S85, GE healthcare). 3 dots (10 ng/dot) were applied per each sample. Four standard samples of the oxidized genomic DNA (10 ng/dot) with a known content of 8-oxodG (was determined by ESI-MS/MS using AB SCIEX 3200 Qtrap machine [18]) were applied to the same filter, in order to plot a calibration curve for the dependence of the signal intensity on the number of 8-oxodG copies in a particular sample. The filter was heated at 80 °C in vacuum for 1.5 h. 8-oxodG

antibody conjugated with alkaline phosphatase was used. Then the filter was placed into a solution of substrates for alkaline phosphatase NBT and BCIP. Upon the completion of reaction, the filter was washed with water and dried in the darkness. The dried filter was scanned. For the quantitative analysis of the dots, special software was used (Images6, RCMG, Moscow). Signals from several dots for the same sample are averaged. The 8-oxodG content in a studied sample is calculated using the calibration curve equation. Relative standard error was 15 ± 5%.

Fluorescence microscopy

Immunocytochemistry. MCF7 cells were fixed in 3% formaldehyde (4°C) for 20 min, washed with PBS and then permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature, followed by blocking with 0.5% BSA in PBS for 1 h and incubated overnight at 4°C with the NOX4 and 8-oxodG antibody. After washing with 0.01% Triton X-100 in PBS MCF7 cells were incubated for 2 h at room temperature with the FITC/PE goat anti-mouse IgG, washed with PBS and then stained with DAPI.
Intracelullar localization of labeled GC-DNA fragments. Labeling of pBRR322 and pBR322-rDNA was performed by nick translation using CGH Nick Translation Kit (Abbott Molecular) under manufacturer's protocol. Labeled pBR322green and pBR322-rDNA green or pBR322-rDNA red were added to the cultivation media for 30 min. Cells were washed three times with PBS, fixed in 3% paraformaldehyde (4°C) for 10 min, washed with PBS and stained with 2 μg/mL DAPI.
EGFP. Non – fixed cells were analyzed at 488 nm. To quantify the background fluorescence, the control cells were analyzed.
Mitochondria. The cells were stained with 30 nM TMRM (tetramethylrhodamine methyl ester) (Molecular Probes) for 20 min at 37°C.

ROS assay

The cells were analyzed using total fluorescence assay in the 96-well plate format at λex = 488 nm and λem = 528 nm (EnSpire equipment, Finland). MCF7 were treated with 5 μM H2DCFH-DA (Molecular Probes/Invitrogen, CA, USA) at 37˚C. 16 (8×2) repeated measurements were provided for each GC-DNA concentration and 24 for the control. The mean absolute intensities were divided by the average value of the intensity corresponding to t = 0, obtaining the values of I0. The graphs are presented in the coordinates I−time. The obtained data were approximated by linear dependence; the value of the tangent of the slope (index ki) together with the error of determination was calculated. k0 – reaction rate constant for DCF formation in control cells; Δk = ki−k0.

Statistics

All reported results for qPCR, PT-qPCR, immunoassay and FCA were reproduced at least three times as independent biological replicates. The significance of the observed differences was analyzed using non-parametric Mann- Whitney U-tests. The data were analyzed with StatPlus2007 Professional software (http://www.analystsoft.com/). All p-values considered statistically significant at $p < 0.05$. The software for 'Imager 6' was designed by R.Veiko (RCMG, Moscow).