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ORIGINAL ARTICLE

Modified Genomic Self-DNA Influences In Vitro Survival of HT29 Tumor Cells via TLR9- and Autophagy Signaling

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Abstract

In relation of immunobiology, the consequence of the crosstalk between TLR9-signaling and autophagy is poorly documented in HT29 cancer cells. To assess the TLR9-mediated biologic effects of modified self-DNA sequences on cell kinetics and autophagy response HT29 cells were incubated separately with intact genomic (g), hypermethylated (m), fragmented (f), and hypermethylated/fragmented (m/f) self-DNAs. Cell viability, apoptosis, cell proliferation, colonosphere-formation were determined. Moreover, the relation of TLR9-signaling to autophagy response was assayed by real-time RT-PCR, immunocytochemistry and transmission electron microscopy (TEM). After incubation with g-, m-, and m/f-DNAs cell viability and proliferation decreased, while apoptosis increased. F-DNA treatment resulted in an increase of cell survival. Methylation of self-DNA resulted in decrease of TLR9 expression, while it did not influence the positive effect of DNA fragmentation on MyD88 and TRAF6 overexpression, and TNFα downregulation. Fragmentation of DNA abrogated the positive effect of methylation on IRAK2, NFκB and IL-8 mRNA upregulations. In case of the autophagy genes and proteins, g- and f-DNAs caused significant upregulation of Beclin1, Atg16L1, and LC3B. According to TEM analyses, autophagy was present in each group of tumor cells, but to a varying degree. Incubation with m-DNA suppressed tumor cell survival by inducing features of apoptotic cell death, and activated mitophagy. F-DNA treatment enhanced cell survival, and activated macroautophagy and lipophagy. Colonospheres were only present after m-DNA incubation. Our data provided evidence for a close existing interplay between TLR9-signaling and the autophagy response with remarkable influences on cell survival in HT29 cells subjected to modified self-DNA treatments.

Keywords HT29 · Colon cancer · TLR9 · Autophagy · Colonosphere · Self-DNA

Introduction

Cancer represents a major health problem worldwide, and colorectal cancer is the third most diagnosed malignancy. The multi-step, complex process of tumorigenesis resulting uncontrolled proliferation and spreading of malignantly transformed cell clones depends mainly on the interaction of cancer cell intrinsic factors and the host's immunosurveillance, along with the active contribution of an inflammatory microenvironment [1].

Toll-like receptors (TLRs) are usually expressed in innate immune cells, though several types of cancer cell lines and cancer tissues have also been reported to display them [2]. Nucleic acid components detected by TLRs include dsRNA by TLR3 [3], ssRNA by TLR7 and TLR8 [4], and oligodeoxynucleotide (ODN)-containing cytosine–guanine (CpG) motifs of dsDNA by TLR9 [5, 6]. Almost all these structures are represented in host-derived nucleic acids.

Eukaryotic cells are continually exposed to self-DNA, so to distinguish host and foreign DNA is essential. It is still unclear, how TLR9 discriminates, but this complex process in part could be attributed to biochemichal characteristics of DNA-sequences [6, 7]. TLR9-associated recognition of DNA takes place in the endolysosomes [8], and this localization ultimately determines TLR9 activation [9]. The activation might evoke inflammatory signals [10], nonetheless, TLRs and related downstream pathways regulate cell proliferation

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and survival, as well [11]. The final biological outcome depends on the cell compartment where TLR9 binds the ligand and the assembly of protein complexes driving various signaling cascades which finally lead to chromatin remodeling and activation of transcription factors [12]. HT29 cells spontaneuosly express not only TLR9 mRNA, but also TLR9 protein, and display a clear response to CpG-ODN stimulation. Moreover, HT29 cells in a unique way are able to distinguish between CpG-ODNs mimicking bacterial DNA and non-CpG or backbone variants [13].

In cancer patients generally increased amount of circulating cell-free (cf) DNA can be detected, and further, cf-DNA might even reflect tumor severity [14, 15]. The integrity, including the size and structure of cf-DNA of tumor origin is highly vary from that of healthy subjects. Consequently, cancerous DNA is frequently more fragmented and aberrantly methylated [16, 17].

Because host-derived DNA from dying cells has been shown to induce TLR9-mediated inflammation in live cells [18, 19], it is assumable that DNA released by apoptotic or necrotic cancer cells or shed by viable tumor cells could serve as an endogenous TLR9-ligand in surviving cancer cells with apparent biological consequences [20, 21]. However, according to this field not too much previous information was presented, since most studies focused on the immunotherapeutic applications of synthetic CpG-DNAs by modulating the host's immune system [22, 23]. In our recent work using the HT29 cancer cell line upon incubation of tumor cells with modified self-DNAs different effects on TLR9-signaling and cell differentiation were detected [24].

Regarding cell biology, autophagy is intensely involved in the regulation of various functions such as cell survival, development, senescence, differentiation, or immune responses [25–29]. Inadequacy of basal autophagy may lead to genomic instability, while changes of induced autophagy could result in decreased cell survival [28, 29]. In tumorigenesis a Janus-faced aspect of autophagy has been suggested. The direction of autophagy to pro- or anticancer activities is microenvironment- and context-dependent [30–32], though its exact connection to tumor networks is not fully understood. With regard to cell fate, the involvement of autophagy in different cell death types (e.g.: apoptotic, non-apoptotic or necrotic), and their potential interplay are fairly complicated [33].

The interaction between TLRs and autophagy networks results in the induction and activation of innate immunity [34–38]. Autophagy basically exerts a repressive effect on TLR-related pathways, but this impact can be reversed [39]. In cancer pathophysiology, however, the interrelated role of TLR9 and autophagy in non-immune cells, particularly in tumor cells has not yet been clarified, and only few data have been published so far [40–44]. Local tumor growth especially depends on the proliferative and invasive capacity of cancer cells. Presumably it could be influenced by DNA structures internalized by viable cells of the same tumor via TLR9- and autophagy-mediated invasion-promoting or suppressive cellular mechanisms. HT29 cells, a human colorectal adenocarcinoma cell line with epithelial morphology represent a useful model for studying epithelial cells within malignancy. The purpose of the present study was to asses how cancer cell-derived DNAs affect the TLR9-dependent autophagy response and consequental cancer cell survival in HT29 cells. Therefore HT29 cancer cell cultures were exposed to intact (genomic) or modified (hypermethylated, fragmented) self-DNA forms. Various assay systems were used to evaluate the potential responses. This in vitro model lacks both the tumor microenvironment and the immune system of the tumor-bearing host, thus it permits to analyze the pathobiological consequences of self-DNA administration in HT29 colon cancer cells *per se.*

Materials and Methods

Cell Culture

HT29 undifferentiated colon adenocarcinoma cell line was purchased from the 1st Department of Pathology and Experimental Oncology, Semmelweis University, Budapest, Hungary. The cells were maintained in RPMI 1640 (Sigma-Aldrich, USA) medium supplemented with 10% (v/v) fetal bovine serum (FBS; Standard Quality; PAA Laboratories GmbH, Austria), 160 μg/ml gentamycin (Sandoz, Sandoz GmbH, Austria), and 125 μg/ml amphotericin B (Sigma-Aldrich, USA). The medium was replaced every second day.

Isolation, Methylation and Fragmentation of Genomic DNA for HT29 Cell Incubation

Intact (genomic) DNA was isolated from $5x10^7$ steady state, proliferating HT29 cells cultured in normal growth medium. DNA isolation was performed by using High Pure PCR template preparation kit containing proteinase K (Roche GmbH, Germany). The DNA samples were treated with 5 μl RNase A/T1 Mix (Thermo Scientific, Germany). DNA concentration was determined by Nanodrop (Thermo Scientific, Germany).

Genomic DNA was divided into four equal shares: the first one was neither hypermethylated nor fragmented (genomic-DNA: g-DNA). The second share was methylated (methylated-DNA: m-DNA) using CpG methyltransferase M.SssI (New England Biolabs Ipswich, USA). The third one was fragmented (fragmented-DNA: f-DNA) by ultrasonic fragmentation for 2 min. The fourth share was both methylated and fragmented (methylated/fragmented-DNA: m/f-DNA).

Length of the fragmented DNA shares was determined by agarose gel electrophoresis (data not shown).

For incubation with the DNA samples $0.5x10^6$ HT29 cells were placed into a 12-well-plate in RPMI 1640, supplemented with gentamycin, amphotericin B and FBS, as described above. After 24 h the starting medium was changed to RPMI 1640 with gentamycin but without FBS. Aliquots of 15 μg of each modified g-DNA types were separately dissolved in 200 μl sterile phosphate buffered saline (PBS). According to MALDI-TOF mass spectometry measurements, the DNA samples were free of protein, RNA or lipopolysaccharide (LPS) contamination (data not shown).

HT29 cells were incubated with the different DNA samples at 37°C in a humidified atmosphere of 5% CO2 and 95% O2. For the control cells only 200 μl sterile PBS was added. After 72 h cells were washed twice with 5 ml of sterile PBS and resuspended in a final volume of 5 ml PBS. In case of TLR9-inhibited controls, HT29 cells were pretreated with TLR9 antagonist (ODN2088; Invivogen, CA, USA) for 1 hour before treatments with DNAs.

Isolation of Total RNA

Half of the cells was used for total RNA isolation, (and the remnant for immunocytochemistry). Total RNA from HT29 cells was extracted with RNeasy Mini Kit (Qiagen, USA) according to the prescription of the manufacturer.

MTT (3-/4,5-Dimethylthiazol-2-yl/-2,5-Diphenyltetrazolium Bromide) Assay

To detect the effect of the DNA on HT29 cell viability $5x10^3$ cells/well in 100 μl medium were seeded into 6-well plates, and treated with g-, f-, m- and m/f-DNAs. After 72 h incubation 25 μl of 5 mg/ml MTT solution in PBS was added to the cells and incubated for an additional 4 h at 37°C. Then the medium was removed and 100 μl dimethylsulfoxide (DMSO) was added to each well. The formazan salts were quantified by reading the absorbance at a test wavelength of 570 nm and a reference wavelength of 690 nm. Wells containing HT29 cells only served as the blanks.

TUNEL (TdT-Mediated dUTP Nick-End Labeling) Assay

To quantify the presence of apoptosis in HT29 cells TUNEL assay (DeadEnd Fluorometric TUNEL System; Promega, WI, USA) was performed. After 72 h incubation with the different DNAs HT29 cells were smeared onto sterile glass slides, washed twice with ice-cold PBS, fixed in 4% methanol-free formaldehyde in PBS for 25 min. at 4 °C, and permeabilized by 0.1% Triton X-100 (Sigma-Aldrich, MO, USA) in PBS for 5 min. After washing with PBS cells were covered with 100 μL Equilibration Buffer for 10 min. at room temperature (RT) and treated with 50 μl recombinant terminal deoxynucleotidyl transferase (rTdT) incubation buffer at 37°C for 60 min. in a humidified chamber. The tailing reaction was terminated by immersing the slides in 2X saline-sodium citrate buffer for 15 min. at RT. Unincorporated fluorescein-12-dUTP was removed by rinsing with PBS, and cells were stained with 1 μg/mL propidium iodide (PI) in PBS for 15 min. at RT in a dark humidified chamber. After washing with PBS slides were covered with the antifading VectaShield (Vector Laboratories Inc., CA, USA) and coverslips. Cells were visualized and counted by using FluoView Laser Scanning confocal microscope (Olympus, Center Valley, PA, USA).

Immunocytochemistry for Ki-67, Atg16l1, Beclin1, LC3 and CD133

To analyze the proliferation of HT29 cells Ki-67 immunocytochemistry was performed. After 72 h incubation HT29 cells were smeared onto sterile glass slides, washed twice with ice-cold PBS, and fixed in 4% methanol-free formaldehyde in PBS for 25 min. at 4 °C. Slides were incubated in blocking solution (PBS with 1% BSA and 0.05% Tween-20) for 30 min. just before the admixture of the rabbit anti Ki-67 antibody (ab15580, Abcam). The primary antibody (1/500) was incubated for 60 min. at RT in a dark humidified chamber. After washing twice with PBS goat anti-rabbit IgG (Alexa Fluor 647, ab150079, Abcam), a secondary antibody (1/500) was incubated for 45 min. at RT. After washing with PBS nuclear 4',6-diamidino-2-phenylindole (DAPI) (1 μg/mL) co-staining was performed. After PBS rinsing slides were covered with the antifading VectaShield (Vector Laboratories Inc., CA, USA) and coverslips. Cells were visualized and counted by using FluoView Laser Scanning confocal microscope (Olympus, Center Valley, PA, USA).

For detection of autophagy-associated Atg16l1, Beclin1, LC3, and the CD133 protein expressions HT29 cell smears were incubated with anti-ATG16L1-, anti-BECN1-, and anti-MAP1LC3B antibody (1:200, Antibody Verify, LA, USA), and anti-CD133/1-biotin antibody (1:100, Miltenyi) at 4 °C overnight. After rinsing 3 times with PBS cell smears were finally treated with an anti-rabbit EnVision polymerHRP conjugate kit (K4003, DAKO) for 40 min. Secondary immunodetection was performed with EnVision System Labelled Polymer–HRP K4001 (Anti-Mouse 1/1; DAKO), as described in the manual. Signal conversion was carried out with Liquid DAB+ Substrate Chromogen System (DAKO). After rinsing in PBS hematoxylin co-staining was performed. Cell smears were then digitalized using high-resolution MIRAX DESK

instrument (Zeiss, Gottingen, Germany), and analyzed with the MIRAX TMA Module software (Zeiss).

Reverse Transcription and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Selected genes of the TLR9-pathway (*TLR9, MyD88, IRAK2, TRAF6, NFκB, TNFα*), autophagy (*Beclin1, Atg16l1, LC3*), and interleukin-8 (*IL-8*) were selected, followed by oligonucleotide primers' design. After quantitative (Nanodrop) and qualitative analysis (Bioanalyzer Pico 600 chip kit RNA program; in all cases RIN >8), reverse transcription was performed using 1μg total RNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, USA). qRT-PCR was performed using Probes Master and SYBR green (Roche GmbH, Germany). Gene expression levels for each individual sample were normalized to 18S expression. Mean relative gene expression was determined and differences were calculated using the 2-ΔC(t) method. The list of oligonucleotide primers used is detailed in Table 1.

Inverted Microscopy

After 72 h incubation with the different DNAs the number of colonospheres in the wells was counted to quantify the frequency of sphere formation. Images were captured at 200x magnification.

Table 1 List of the oligonucleotide primers used

Toll-like receptor-9(TLR9)-related genes	
TLR9	F: CAATGTCACCAGCCTTTCCT R: CAGGTGGGCAAAGTCAGAAT
MYD88	F: CTCCTCCACATCCTCCCTTC R: CGCACGTTCAAGAACAGAGA
IRAK2	F: CTTGGAGTGGGCTTTCTGAG R: AGGCTGGAATTGTCAACGTC
TRAF6	F: CTTTGGCAAATGTCATCTGTG R: CTGAATGTGCATGGAATTGG
NFκB	F: TATGTGGGACCAGCAAAGGT R: GCAGATCCCATCCTCACAGT
TNFα	F: ATCTGGGACGGTGCTGTAAC R: CAGGGCAGACATACACTGTCA
Epithelial cell-derived proinflammatory chemokine: IL-8	
IL-8	F: GTGCAGTTTTGCCAAGGAGT R: AAATTTGGGGTGGAAAGGTT
Autophagy-related genes	
BECLIN1	F: GGGAAGTCGCTGAAGACAGA R: GTCACCCAAGTCCGGTCTAC
ATG16L1	F: TCGAGGAGATCATCCTGCAATAA R: GTTCCTCTTGGTGCTTAATCCT
LC3	F: GTCACCGGGCGAGTTACC R: CTCGCGCTCAAGGGCTC

Transmission Electron Microscopy

HT29 cells in the wells were fixed in 2% glutaraldehyde (0.1M Millonig buffer, pH 7.4) for 60 min. After washing 3 times for 5 min. with 0.1 M of phosphate buffer, and 3 times for 5 min. with 0.1 M pH 7.2 sodium cacodylate buffer. The samples then were post-fixed with 1% osmium tetroxide in 0.1 M sodium-cacodylate buffer for 60 min. at 4°C in the dark. After washing 3 times for 5 min. with sodium-cacodylate buffer (pH 7.4) cells were pelleted by centrifugation and embedded into 10 % gelatine in phosphate buffer (pH 7.4). After dehydration in graded series of alcohol the samples were embedded into Poly/Bed epoxy resin. Ultrathin sections (70-80 nm) were contrast stained with uranyl acetate and lead citrate, respectively. Ultrastructural analyses were perfomed by using Hitachi H-9500 Transmission Electron Microscope.

Statistical Analysis

At least three independent experiments were conducted. Data of cell viability, survival, and apoptosis were expressed as means ± SD. For a possible and better comparison of cell kinetic parameters and gene expressions, the percentage of target/control values were determined and visualized in Fig. 1. Chi2-test and Student's t-test were used for statistical analyses. $P < 0.05$ was considered as statistically significant.

Results

Effect of DNA Incubation on Viability and Survival of HT29 Cells

According to the MTT assay 72 h incubation with g-DNA resulted in an average cell viability of 81.34%±1.96% as compared to control, nontreated cells. Following incubation with structurally modified DNAs cell viability values changed as the follows: 73.02%±1.04% (m-DNA), 101.47%±2.96% (f-DNA), and 85.24%±1.17% (m/f-DNA), respectively (Fig. 1.). In case of TLR9-inhibited controls, the viability results showed no significant alteration as compared to control, PBS-treated cells (data not shown).

The percentage of Ki-67-immunopositive HT29 cells indicative for proliferation varied like 26.55%±3.12% (g-DNA), 19.59%±3.53% (m-DNA), 89.25%±6.57% (f-DNA), and 33.58%±4.54% (m/f-DNA), respectively (Fig. 1.).

The percentage of TUNEL-positive apoptotic HT29 cells was the following: 69.53%±5.23% (g-DNA), 78.22%±7.48% (m-DNA), 8.44%±3.45% (f-DNA), and 64.55%±7.88% (m/f-DNA), respectively (Fig. 1.). In case of TLR9-inhibition, the percentage of proliferative or apoptotic HT29 cells was similar to control, PBS-treated ones (data not shown).

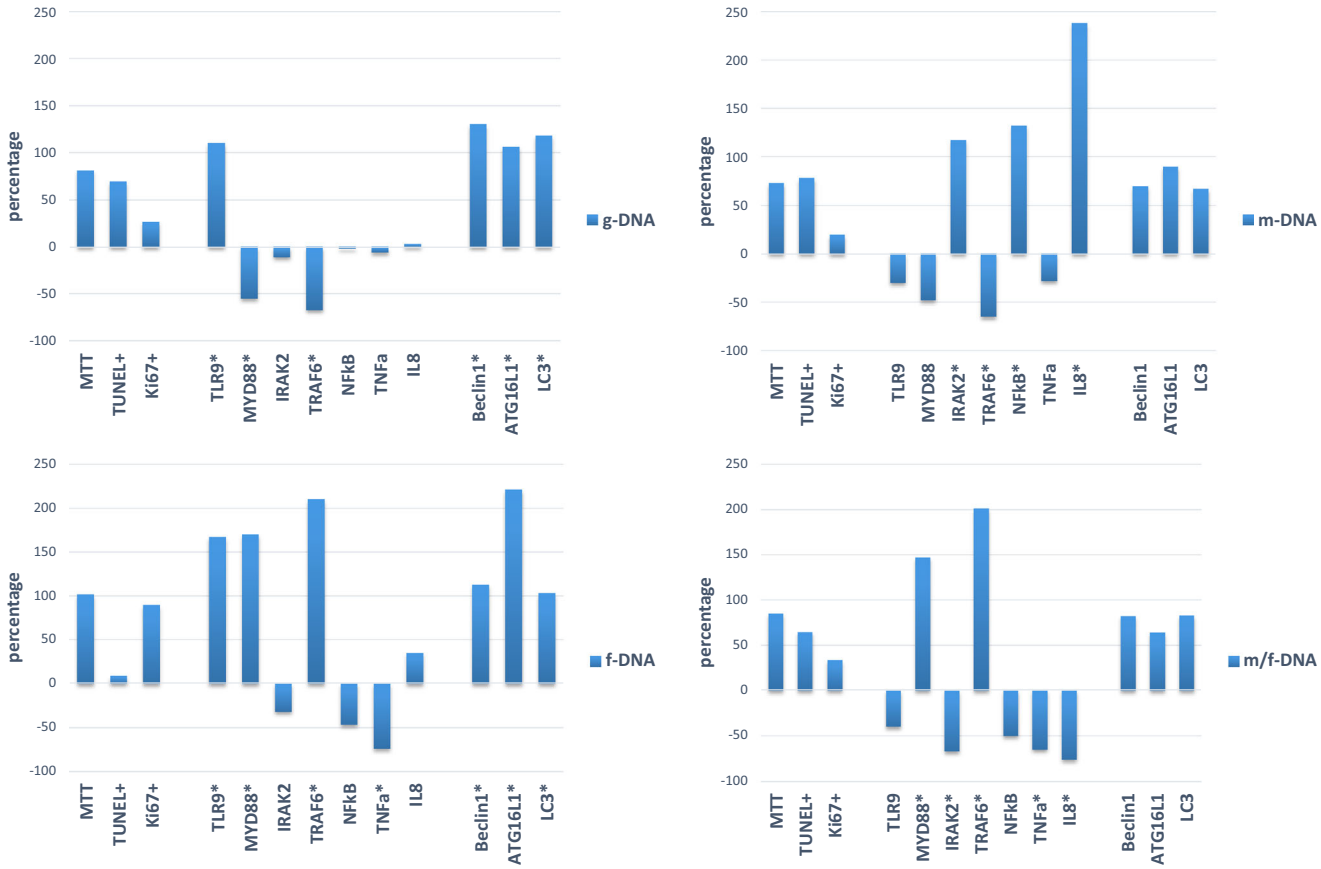

Fig. 1 Comparison of the mean percentage of HT29 cell survival parameters with relative expressions of selected TLR9- and autophagy-related genes. *represents significant gene overexpression or downregulation as compared to contol ($p<0.05$). In case of all represented parameters, ±SD <2.5%

Per these results, following incubation with g-, m-, and m/f-DNAs viability and proliferation rate of HT29 tumor cells decreased, while the percentage of apoptotic cells increased. The most efficient tumor cell suppression was related to m-DNA treatment. On the contrary, incubation with f-DNA resulted in an appreciably increase of tumor cell survival.

Effect of DNA Incubation on Gene Expressions of HT29 Cells

Regarding the TLR9-pathway-related genes incubation of cells with g-DNA resulted in significant ($p<0.01$) overexpression of TLR9, and a significant ($p<0.01$) downregulation of MyD88 and TRAF6 mRNAs (Fig. 1.). After incubation with m-DNA expression of TLR9 and MyD88 slightly, while TRAF6 significantly ($p<0.01$) decreased, but that of IRAK2, NFκB, and IL-8 became significantly ($p<0.01$) increased (Fig. 1.). Treatment with f-DNA resulted in significant ($p<0.01$) upregulation of TLR9, MyD88, and TRAF6 along with significant ($p<0.01$) downexpression of TNFα, and a decline in IRAK2 and NFκB mRNA (Fig. 1.). Following incubation with m/f-DNA expression of MyD88 and TRAF6 was significantly ($p<0.01$) upregulated, while that of IRAK2, TNFα, and IL-8 mRNAs were significantly ($p<0.01$) decreased. Expression of TLR9 and NFκB was also downregulated (Fig. 1.).

Overall, methylation of self-DNA (like m- and m/f-DNAs) resulted in decrease of TLR9 expression, while it did not influence the positive effect of DNA fragmentation on MyD88 and TRAF6 overexpression, and TNFα downregulation. However, fragmentation of DNA (such as f- and m/f-DNA) abrogated the positive effect of methylation on IRAK2, NFκB and IL-8 mRNA upregulations.

In case of the autophagy genes g- and f-DNAs caused significant ($p<0.01$) upregulation of Beclin1, Atg16L1, and LC3 mRNAs (Fig. 1.). However, treatment with m- and m/f-DNAs resulted in a rather modest expression of the same genes.

In case of TLR9-inhibited cells, expression of TLR9 and MyD88 was slightly decreased, no alteration of autophagy related-gene expressions was seen (data not shown).

Effect of DNA Incubation on Colonosphere-Forming Capacity of HT29 Cells

Under conventional adhesion conditions colonosphere formation in HT29 cells could only be detected after incubation with m-DNA (Fig. 2.), though tumor cell proliferation was the

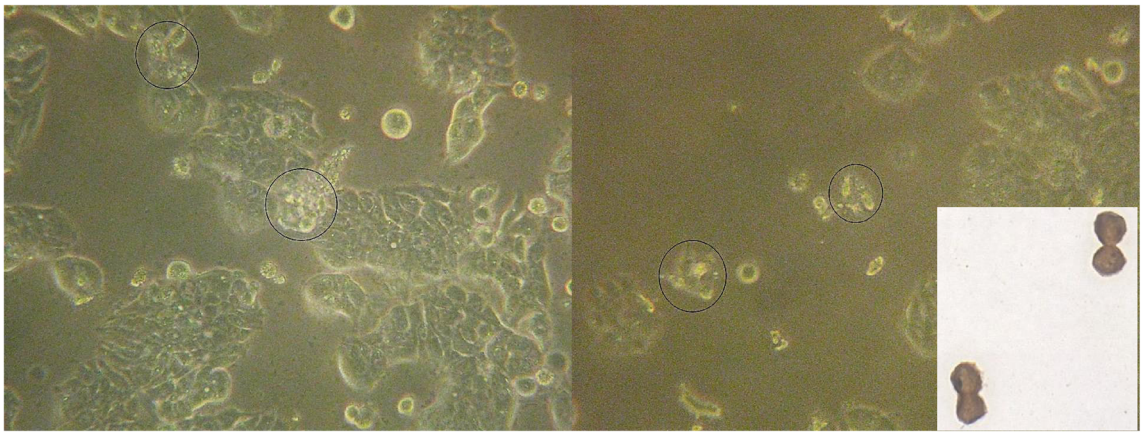

Fig. 2 Results of inverted microscopic analysis. Colonospheres (circles) in wells of m-DNA-treated monolayer of HT29 cancer cells (200x magnification). Insert: CD133-immunopositive HT29 cells from the spheroids

lowest in this group. Isolated and separated cells of the spheres displayed CD133 immunoreactivity (Fig. 2., insert).

Effect of DNA Incubation on Ultrastructure of HT29 Cells

Control, nontreated, metabolically active HT29 cells - similarly to TLR9-inhibited controls /data not shown/- displayed autophagic vacuoles in the cytoplasm indicating macroautophagy (Fig. 3a, b). Incubation with g-DNA resulted in the appearance of a more intense macroautophagy, and sometimes that of dense vacuoles, more probably indicative for late endosomes or autolysosomes (Fig. 3c). Following incubation with m-DNA the cell structure became fully desorganized along with chromatin condensation, blebbing, and nuclear fragmentation/desintegration corresponding to dying apoptotic cells. Several degrading mitochondria were also detected suggesting apoptosis, and enhanced degradation via mitophagy (Fig. 3d, e). In case of f-DNA treatment living, metabolically active cells exhibited macroautophagy, and lipophagy, i.e. another selective form of autophagy (Fig. 3f, g). Upon treatment with m/f-DNA surviving, metabolically active cells presented with autophagic vacuoles, and also degraded mitochondria in the cytoplasm (Fig. 3h, i).

Thus, the presence of autophagy was observed in each group of tumor cells, but to a varying degree. Further, incubation with m-DNA definitely suppressed tumor cell survival by inducing features of apoptotic cell death. In addition, m-DNA activated selective autophagy targeting mitochondria, i.e. mitophagy, as well. Treatment of cells with f-DNA, however enhanced cell survival, and the activated macroautophagy apparently contributed to maintain cellular fitness.

Effect of DNA Incubation on Autophagy- and CD133 Protein Expressions of HT29 Cells

Control, nontreated HT29 cells - similarly to TLR9-inhibited controls /data not shown/- exhibited mild immunoreaction for Atg16L1, Beclin1 and LC3 antibodies (Fig. 4a, f, k). After g- and f-DNA treatment tumor cells showed Atg16L1, Beclin1 and LC3 with strong stainings (Fig. 4b, g, l, and d, i, n). Incubation with m-DNA resulted in mild Atg16L1 and Beclin1, and no LC3 immunoreactivity of cells (Fig. 4c, h, m). Following m/f-DNA incubation mild-to-moderate cytosolic immunoreactions for Atg16L1, Beclin1 and LC3 were observed (Fig. 4e, j, o).

Isolated HT29 cells from colonospheres after incubation with m-DNA displayed strong cytoplasmatic CD133 immuoreactivity (Fig. 2., insert).

Discussion

Accumulating evidence indicates that as TLRs, as the autophagy process are involved in carcinogenesis [33, 45]. However, both pathways may exert tumor suppressor mechanisms as well, in a cell-specific and context-dependent manner. Furthermore, TLRs and autophagy are interrelated in response to PAMPs/DAMPS, representative for a bidirectional regulatory cross-talk [34].

TLR9 expressed both by innate immune cells and several types of cancer cell lines and tissues [2] recognizes not only CpG motifs, but the DNA itself, and the receptor engagement initiates signaling specifically in a sequence- and methylation-dependent manner [24]. Self-DNA fragments released by apoptotic or necrotic cancer cells or shed by viable tumor cell could subsequently be internalized by viable cells of the same tumor, and serve as endogenous TLR9-ligands [20, 21]. Upon cell death, presumably altered DNA structures are formed mainly via the action of cell death-activated DNAses [46]. Modifications of DNA affecting the secundary/tertiary structure and molecular stability may be critical for the TLR9-mediated biological activity [47], thus it can elicit diverse, agonistic or suppressive effects requirering the use of differential adaptor protein for the signaling [48]. Nonetheless,

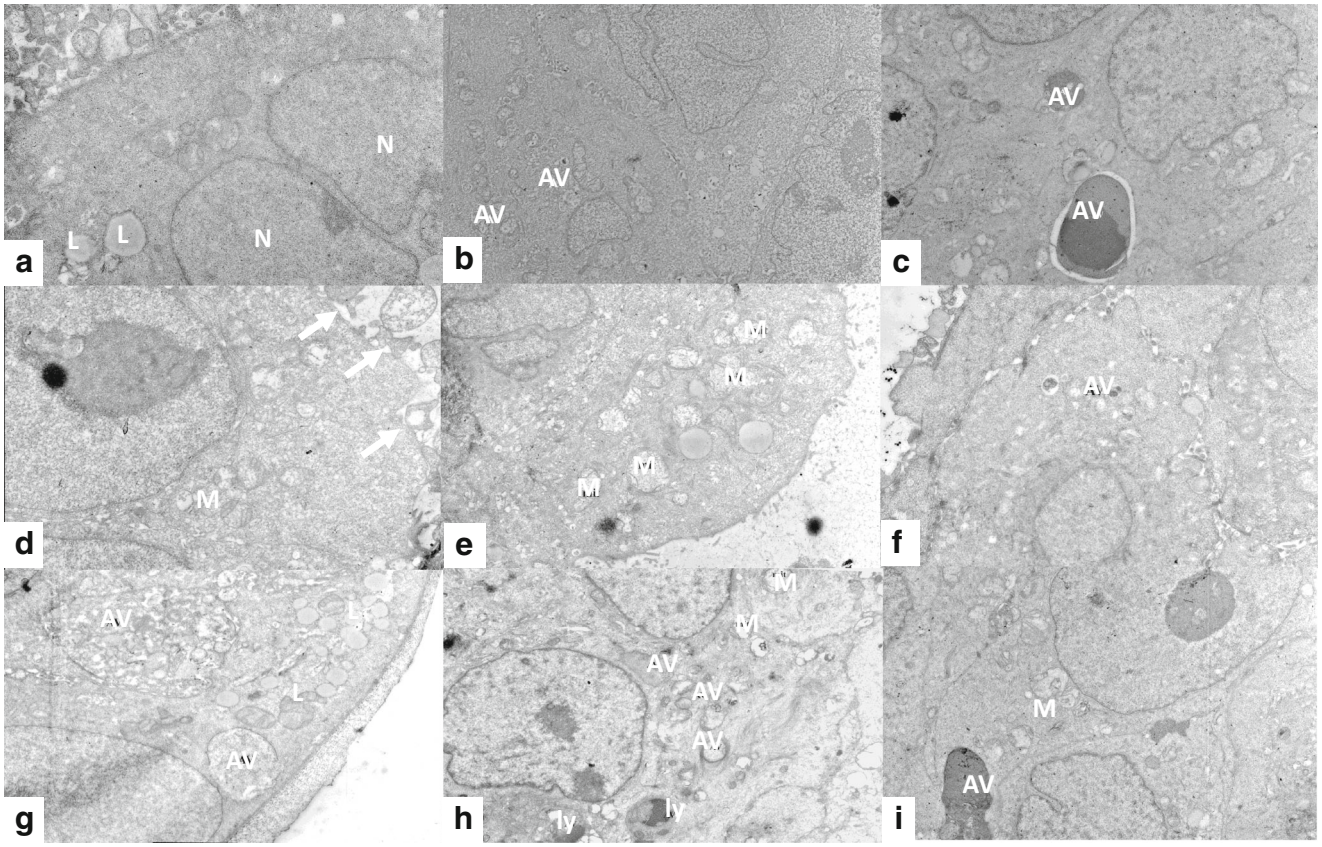

Fig. 3 Results of TEM analysis. a and b. Control HT29 cells (5x). a. Living, metabolically active cells with euchromatic nucleus (N), some lipid droplets (L), and intact nuclear membrane. **b.** HT29 cells with autophagic vacuoles (AV) representing macroautophagy. **c. HT29 cells after g-DNA incubation (6x).** Macroautophagy (AV) with dense vacuoles indicating fusion with lysosomes. **d and e. HT29 cells after m-DNA incubation (10x and 7x). d.** The cell structure is fully desorganized: the chromatin is condensed, the nucleolus is desintegrated. Signs of degrading mitochondria indicative for mitophagy (M). Blebbing is also present on the cell membrane (arrows). **e.** Signs of selective autophagy (M). The mitochondria show signs of apoptosis. **F and G. HT29 cells after f-DNA incubation (8x and 6x). f.** Living, metabolically active cells with autophagic vacuoles (macroautophagy, AV). **g.** Besides AVs lipid droplets are also present (L). Some of the droplets have double mambrane indicating lipophagy. **h and i. HT29 cells after m/f-DNA incubation (5x and 6x). h.** The nucleus is normal, the cell is active with signs of macroautophagy (AV), advanced stage of macroautophagy (i.e. fusion with lysosomes: ly), and mitophagy (M). **i.** A dense autophagic vacuole (AV), and degrading mithochondria (M)

DNA-induced cellular responses may also be mediated through TLR9-independent molecular mechanisms [49].

Studies revealed the constitutive expression of TLR9 mRNA in human colonic epithelial cancer cell lines including HT29 [50]. On a basal level HT29 cells faintly expressed TLR9 mRNA and protein, but after stimulation with CpG-ODN a significant increase was detected [24, 50]. In our previous study upon incubation HT29 cells with modified self-DNAs different effects on TLR9-signaling and cell differentiation were detected [24].

Though exposure of colonic epithelial cells to CpG-ODN induced no significant change on their viability [13], others found that it may lead to apoptosis in HT29 cells via TLR9 in a dose-dependent manner [51]. The latter finding is in accordance with other and our data indicating the potential of TLR9 engagement by DNAs (but not by TLR9 inhibitory ODN2088) to provoke apoptotic death in various tumor cells without involving the immune system [52, 53].

In our present study following incubation of HT29 cells with intact genomic and modified self-DNAs except of f-DNA decreased cell viability and proliferation rate were detected. The most impressive survival decline along with the highest percentage of apoptotic cells was related to hypermethylated DNA (m)-DNA. These data indicate that m-DNA exerted pro-apoptotic and proliferation-suppressive effects on HT29 cells. However, fragmentation of m-DNA (m/f-DNA) reduced the anti-proliferative capacity of methylation. HT29 cells exposed to DNA treatments exhibited yet notable transcriptional alterations in selected TLR9-related genes. Upon treatment with m-DNA expression of TLR9 and MyD88 slightly, while that of TRAF6 significantly decreased, but significant upregulation in IRAK2, NFκB, and IL-8 mRNAs was detected. Consistent with our findings tumor cell apoptosis induced by DNA sequences have been reported for other cancer cell lines, as well. Indeed, in human HCC cell lines TLR9 senzed by modified ODN resulted in

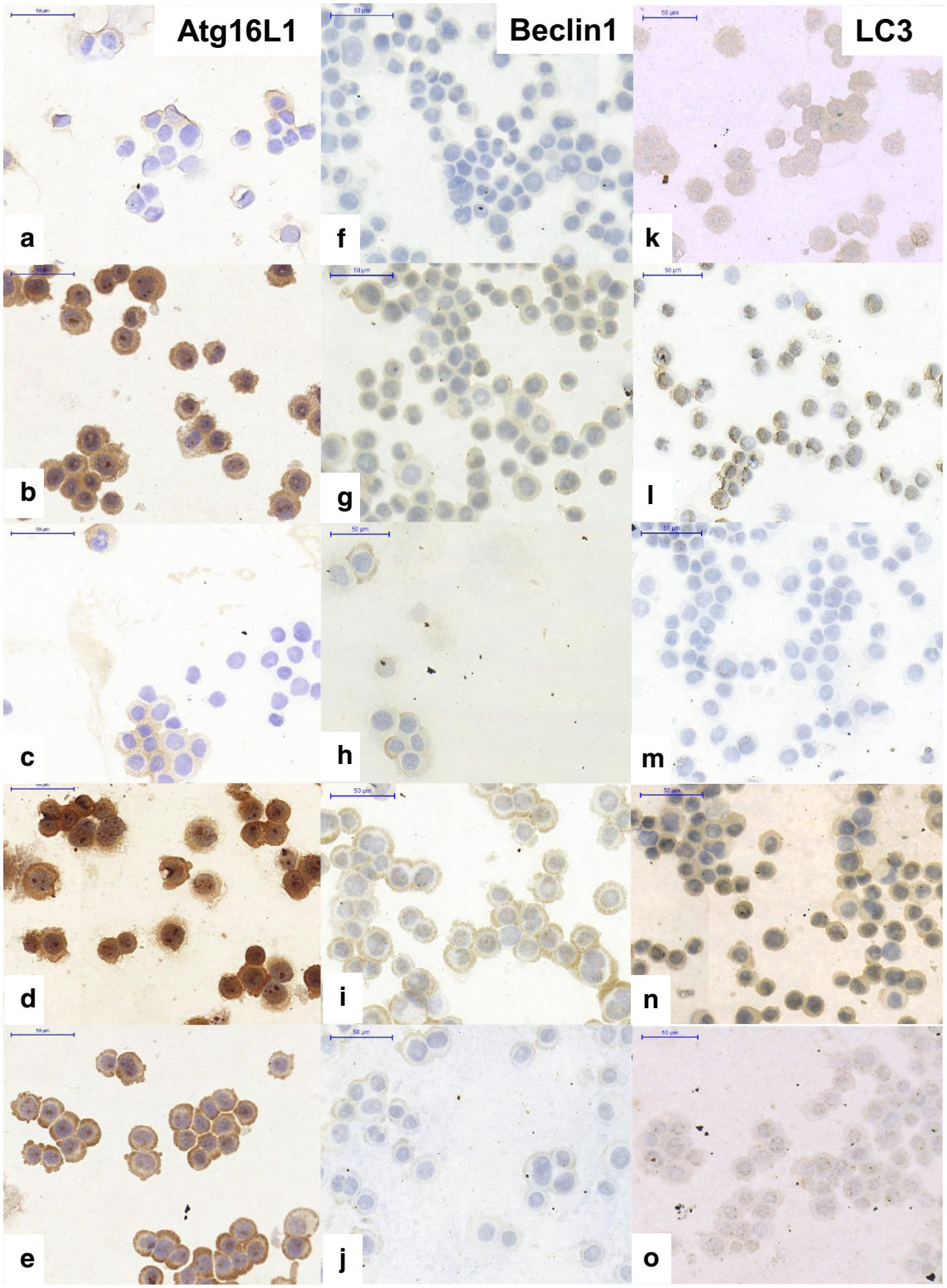

Fig. 4 Results of immunocytochemical analysis. a, **f**, **k**: control HT29 cells with mild cytoplasmatic immunoreactivities. **b**, **g**, **l**: HT29 cells after g-DNA incubation: strong cytoplasmatic immunostainings. **c**, **h**, **m**: HT29 cells after m-DNA incubation: mild cytoplasmatic immunoreactions for Atg16l1 and Beclin1, no immunoreaction for LC3B. **d**, **i**, **n**: f-DNA incubation: strong immunoreactions. **e**, **j**, **o**: m/f-DNA incubation: mild-to-moderate cytosolic immunoreactions. Bars indicate 50 um

receptor upregulation, and inhibition of cell proliferation by inducing apoptosis without affecting cell cycle. However, the apoptosis provoked by different ODNs was independent of TLR9 stimulation [54]. Using a lymphoma B-cell line CpG-

ODN via TLR9 inhibited constitutive proliferation and induced apoptosis of cells by altering the balance between NFκB and c-Myc activation, with downregulation of Bcl-xl, and upregulation of Fas and FasL, but without affecting Bcl-2 [55]. Furthermore, in A549 lung cancer cells CpG-ODN significantly decreased their proliferation [56].

MyD88, a central adaptor required for all TLRs generates a platform for the downstream pathway molecules including IRAKs and TRAF6 [10]. IRAK2, a proximal mediator of IL-1 is required for NFκB activation [11]. It is conceivable, that in our study m-DNA-induced apoptotic cell death might involve a pro-apoptotic signal via IRAK2 with the potential to target the Fas-associated death domain (FADD)/caspase-8 apoptotic pathway [57].

IL-8, a multifunctional chemokine is secreted by a diverse range of cell types including cancer cell [58]. HT29 cells constitutively secretes IL-8, but certain ODNs can inhibit their NFκB-mediated IL-8 production, which was abolished by TLR9 silencing [59]. On the contrary, however, others detected stimulatory activity of CpG-ODNs via TLR9 on IL-8 mRNA by favoring MAPK signaling but not NFκB activation [50], though both pathways might be involved in control of IL-8. Further, mitochondrial dysfunction due to oxidative stress might also be involved in activation of NFκB, and stimulation of IL-8 release from cancer cells.

Unlike m-DNA, in this study incubation of HT29 cells with fragmented self-DNA (f-DNA) led to an appreciably increase of cell viability and proliferation, and a decrease of cell death. These findings suggest that DNA fragmentation may provoke anti-apoptotic and pro-survival signaling. Nonetheless, hypermethylation of DNA sequences (i.e. m/f-DNA) reduced the pro-tumoral effects of fragmentation. Regarding the TLR9-related gene expressions treatment with f-DNA was associated with significant upregulation of TLR9, MyD88, and TRAF6 along with significant downexpression of TNFα, and a decline in IRAK2 and NFκB mRNAs.

TLR-signaling could stimulate proliferation and cell cycle entry in various types of cells, icluding tumor cells [60]. Indeed, in cancer cells synthetic CpG-ODNs irrespectively of the CpG sequences provoked invasion via TIMP-3 suppression and MMP-13 activation mediated by TLR9 and TRAF6, but independently of MyD88 [61]. Moreover, in surviving cancer cells upon uptake of self-DNA fragments from chemotherapy-killed cells of the same tumor TLR9-mediated invasion of live cancer cells was observed [21]. TLR9 ligation by CpG-ODN in lung cancer cells significantly stimulated their prolifation and cell cyle entry by the selective upregulation of CDK2 expression [62]. Moreover, certain CpG-ODNs could stimulate the overexpression of stress-proteins via TLR9/MyD88/PI3K signaling pathway. It was proposed that the enhanced Hsp70 expression may in part be responsible for the CpG-ODN-mediated anti-apoptotic effect via increasing the level of Bcl-XL and inhibiting Bcl-2 translocation without activating caspase-3 [63]. Studies suggest the pro-tumorigenic role of TRAF6 in cancer. Thus, TRAF6 could promote the proliferation and decrease the apoptotic death of various cancer cells, like esophageal cancer, lung adenocarcinoma, glioma and osteosarcoma [64–67]. TRAF6-mediated signaling involves a wide range of inflammatory, apoptotic, and gene regulatory pathways. Though TRAF6 primarily regulates the activity of IKK/NFκB signaling, it modifies more other proteins. TRAF6 can mediate the stimulation of MAPK culminating in activation of AP-1 [68]. TRAF6 could also be involved in mTOR activation through K63 polyubiquitination [69]. Moreover, TRAF6 has the potential to regulate negatively the JAK-STAT signaling [70]. In colon cancer cells TRAF6 was able to force their proliferation via cyclin D1, transcription of which is dependent on the activation of AP-1 and NFκB [71]. Further, TRAF6 affected chemotherapy actions by enhancing drug-resistance of colon cancer cells to agents like 5-fluorouracil (5-FU) or etoposide, as well [71].

Hence, our results suggest that the HT29 tumor cell line in vitro is highly responsive to DNA treatment via TLR9, and depending on the type of DNAs different cancer cell characteristic phenotypes may be exhibited. Nevertleless, it should be noted the far more complexity of TLR9, since its gene has five isoforms [72]. Thus, it is assumable that differential expression of TLR9 isoforms in different types of cells and their engagement by different ligands may radically affect the overall input through TLR9-signaling as in normal as in inflamed tissues [73].

In this study each type of DNA induced autophagy in HT29 cells though to a varying degree. Following incubation with g- and f-DNAs HT29 cells exhibited significantly upregulated Beclin1, Atg16L1, and LC3 mRNA expressions along with strong immunostainings. In both cases TEM indicated live, metabolically active cells with signs of intense macroautophagy, while f-DNA treatment resulted in lipophagy, as well. After incubation with methylated (m)-DNA the cell structure became fully desorganized corresponding to apoptotic death. Moreover, m-DNA was found to induce autophagy targeting mitochondria (mitophagy). RT-PCR studies revealed less significant Beclin1, Atg16L1, and LC3 mRNA expressions. On a protein level mild immunoreactions for Beclin1 and Atg16L1 were detected with no reactivity for LC3. Upon methylation of f-DNA (m/f-DNA) besides macroautophagy signs of microautophagy were also present. Although these cells were still living, the number of degrading mitochondria and other cellular organs became elevated.

The autophagy-lysosome system represents a multi-step process of cellular self-digestion with ubiquitous role in maintaining cellular homeostasis [29, 74–76]. Autophagy and the intrinsic and extrinsic apoptotic pathways can modulate, activate or impede each other through an extended molecular

crosstalk, and cell fate is defined by their actual functional state and interplay [39, 76–78]. The CpG-ODN/TLR9-related and autophagy-associated pathways share numerous similarities. Autophagy can be triggered by CpG-ODNs in tumor cell lines (including colon, breast, and prostate cancers) in a TLR9-dependent manner [43].

Our results suggest that induction of macroautophagy as a cell fitness and survival advantage strategy is mainly present in live HT29 tumor cells. However, m-DNA reduced tumor cell growth that was accompanied by induction of serious mitochondrial damage, apoptosis and mitophagy. Dysfunctional mitochondria generate many stress signals, which finally may result in programmed cell death. Therefore, the recognition and removal of damaged mitochondria via mitophagy provide a survival benefit for energy-depleted cancer cells [79]. Recently, a cell type-dependent mitophagy response has been found to cause apoptosis in tumor cells [80].

In our study colonosphere formation with strong cytoplasmatic CD133 immuoreactivity could only be detected in the m-DNA-treated HT29 cells. Colonospheres from CRC cells exhibit increased expression of putative CRC stem cell markers including CD133 [81]. Autophagy is known to assist stem cell maintenance in several cell types. Studies demonstrated that loss of autophagy decreases the ability of colony and spheroid formations [82]. Upon our experiment, however it is possible that m-DNA-induced mitophagy is not sufficient to rescue HT29 cells from irreversible death but could make some CD133+ stem cell-like cancer cells to survive. Supporting this hypothesis, it was found that curcumin increased proliferation and autophagic survival of colon cancer stem cells [83]. Spheroids were disintegrated by curcumin *in vitro* but reorganized within 30 to 40 days of cisplatin treatment. This finding proposes the survival benefit from autophagy, permitting long-term persistence of colorectal cancer stem cells [83, 84].

Incubation of cells with f-DNA resulted in lipophagy, as well. The increased lipogenesis in cancer cells requires strict accommodation to the intracellular lipid content; a balance between lipid storage and lipid mobilization must be existed [85]. Recently a specific function of autophagy in cellular lipid mobilization has been described [86]. Indeed, in cells lacking an autophagy gene, lipid droplets accumulated in the cytoplasm due to defective catabolism. Lipophagy is achieved by components of the autophagy machinery such as LC3 [87]. In response to cell stress upregulation of lipophagy in cells may contribute to the catabolism of lipid droplets [86]. Lipophagy promotes the release of fatty acids to supply lipids for membrane biogenesis or cell signaling. Moreover, lipophagy has been found to support resistance to cell death by metabolizing toxic free fatty acids [88]. Consistent with these findings the presence of lipophagy in HT29 cells during f-DNA treatment could definitely contribute to cancer cell survival and proliferation.

Since the tumorbiological consequence of the crosstalk between the TLR9 and autophagy pathways is poorly documented, the present study was designed to assess the TLR9-signaling-related biologic effects of modified self-DNA sequences on cell kinetics and autophagy response in HT29 cells *in vitro*. According to our results, we provided evidence for a close existing interplay between TLR9-signaling and the autophagy response with remarkable influences on tumor cell survival in HT29 colon cancer cells subjected to intact or modified self-DNA treatments. Accordingly, unraveling details of the underlying molecular mechanisms and their relation to DNA sequence characteristics may improve not only our understanding of cancer cell destiny, but hopefully may also allow to innovate selective therapeutic approaches.

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Author Contributions Sipos F and Műzes G substantially contributed to the conception and design of the study, acquisition, analysis and interpretation of data. L. Kiss A performed the transmission electronmicroscopic analysis. Constantinnovits M contributed to perform a part of the measurements, Sipos F, Tulassay Z and Műzes G drafted the article and made critical revisions related to the intellectual content of the manuscript, and approved the final version of the article to be published.

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Compliance with Ethical Standards

Conflict of Interests No conflict of interest.

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