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Research article

High concentration of extracellular nucleotides suppresses cell growth via delayed cell cycle progression in cancer and noncancer cell lines

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ABSTRACT

Tumor necrosis frequently occurs in malignant tumors, showing rapid growth and invasion. This phenomenon is generally regarded as simple ischemic necrosis due to insufficient tumor vessels and blood supply. However, the necrotic tissue contains high amount of nuclear substances, DNA, and nucleoproteins that may affect the surrounding tumor cells by promoting or suppressing the tumor cell growth *in vivo*. This study focused on the effects of an externally administered water-soluble nuclear crude extract (SNE) containing nuclear protein and oligonucleotides on several human cancer and noncancer cell lines. The results demonstrated that the SNE suppressed cell growth in cancer and noncancer cells *in vitro*. Through the flow cytometry analysis of the nuclear DNA content, it was observed that the SNE increased and decreased cell proportion in the S and G2/M phases, respectively, thereby suggesting that the cell growth inhibition was due to cell cycle delay, and not due to apoptosis. These studies suggest that the high-concentration of extracellular nucleotides generated as a result of tumor necrosis and/or released from infiltrated neutrophils could suppress the growth of surrounding cancer and intrinsic cells, which provides us some insights into an alternative anticancer strategy for patients with highly malignant necrotic tumor.

1. Introduction

Unignorable amounts of extracellular nucleotides, alias cell-free DNA (cfDNA) were discovered in the human circulatory system in 1948 [1, 2]. These nucleotides are derived from dead cells induced by necrosis, pyroptosis [3], mitotic catastrophe [4], and neutrophil extracellular traps (NETosis) [5]. Viral, bacterial, and fungal infections also induce cell death in the infected tissue and provide the nucleotides from the dead cells derived from both host and infected organisms. The extracellular nucleotide has many functions; (i) unmethylated DNA from microorganisms and virus are recognized by toll-like receptor 9 (TLR9) to induce innate immunity; (ii) double and single ribonucleic acid (RNA) from virus, bacteria, and dead cells, is recognized by TLR3, 7, and 8 to induce innate immunity; and (iii) miRNA enclosed in exosome are captured by endocytosis to play essential roles in intercellular communication [6, 7, 8]. Furthermore, (iv) digested nucleotides contribute to cellular metabolism by de novo synthesis [9, 10], and (v) extracellular DNA is incorporated into recipient cells to allow genetic evolution by natural genetic transformation (horizontal gene transfer) [11].

Today, some extracellular DNA is clinically applied for the prevention and treatment of several diseases. Bacillus Calmette Guérin (BCG), a nucleotide containing *Tubercle bacillus* extract, has successfully promoted tumor remission in bladder cancer [12]. CpG DNA has also been reported to have therapeutic effects on bacterial and viral infections, adjuvant vaccination, and cancer treatments [6]. Although these effects are mainly due to enhanced innate immunity via the TLR system recognizing unmethylated DNA, a direct effect of the nucleotides administrated for the treatments has not been investigated so far. On the other hand, it is

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still unclear how self-DNA, i.e., methylated DNA, is recognized, consumed, and functions after releasing from dead cells. Nucleic acids taken orally from food are expected to be found in low concentrations in the body; however, long-term ingestion may be effective. Some researchers have demonstrated the effects on immunity improvement [13], neuroprotection [14], and protection against alcoholic hepatitis disorders [15]. By contrast, nucleic acids released from necrotic cells are highly concentrated locally, and there is still considerable uncertainty regarding their effects.

This study focused on the effect of methylated DNA on the cell growth phase of tumor cell lines. Tumor necrosis frequently occurs in malignant tumors and leaves its nuclear substrates, including methylated DNA and nucleoproteins, on the surrounding tumor cells, affecting the remaining tumor cells' growth [16].

In order to investigate this hypothesis, the present study was aimed at determining the following: (a) whether nuclear substrates inhibit or promote tumor growth; (b) which components of the nucleotides contribute to the effects on tumor growth; and (c) whether nuclear substrates could affect cell cycle or apoptosis. Our studies demonstrated that highly methylated DNA could disturb the cell cycle progression into the G2/M phase, accumulate in the S phase, and possibly suppress cell growth.

2. Materials and methods

2.1. Materials

A water-soluble nuclear crude extract (SNE) was prepared from salmon soft roe by following an industrial procedure using nuclease and protease. The primary components and the nucleotide and amino acid composition of the SNE are shown in Table 1. The salmon soft roe contains protamine as a protein; however, because SNE is treated enzymatically, it no longer contains protamine as a protein. SNE were provided by the Life Science Institute Co. Ltd. (Tokyo, Japan) and FORDAYS Co. Ltd. (Tokyo, Japan). Nuclear protein contains 26.6% nucleic acids (of which 40% are monomers and 60% oligomers less than 10 bases), 47.12% amino acids (of which 15.3% is Arginine), and others including nitrogen compounds, lipids, and minerals. Protamine sulfate (P3369) was purchased from Sigma-Aldrich, and enzymatically digested protamine was prepared by incubating with 2.5% trypsin in phosphate buffer (pH 7.8) at 37 °C for overnight.

2.2. Cell culture

The human cell lines HeLa, MCF7, MCF10A, HepG2, A549, HSC3, LNCap, Caco2 were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT/AMED, Japan. The human cell lines HUH7 and HLE were provided from Health Science Research Resource Bank, Japan. All these cell lines except MCF10A were cultured in Dulbecco's modified Eagle's medium (DMEM; Corning, USA) containing 10% fetal bovine serum and streptomycin at 37 °C under 5% CO_2. MCF10A was cultured, adding a further 100 ng/ml cholera toxin. Purified oligo DNA fraction was prepared from SNE by ethanol precipitation and measured the nucleic acid concentration by a spectrophotometer (NanoDrop, Wilmington, DE).

2.3. Cell viability assay

CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, USA) was used to assess cell viability. Cells were seeded in 96-well plates and treated with various concentrations (0–8 mg/ml) of SNE. After 72 h, cells were washed with DMEM (without phenol red) three times, 100ul of DMEM (without phenol red), and 15ul of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) reagent was added. After incubation at 37 °C for 4 h, 100ul of stop reagent was added, optical density was measured by a multi-plate reader (FLUOsar Omega, BMG LABTECH, Germany) at a wavelength of 570nm.

2.4. Time-lapse phenotypic assay

Time-lapse images were obtained using a Biostation CT (Nikon, Japan) incubator equipped with a video imaging camera. 6×10^4 MCF7 cells were loaded in each well of a six-chamber system, and then SNE was added. Phase-contrast microscopic image was acquired every 6 h for 96 h. Five locations were measured per well, and the number of cells at five locations was totaled to calculate the number of cells per 1 mm^2 ($n = 4$). The images were analyzed using the image analysis software CL-Quant Ver. 3.4 (Nikon, Japan).

2.5. Immunocytochemistry to detect apoptosis

2×10^5 MCF7 cells were seeded on glass coverslips in a 60 mm dish, and after 5 h, SNE was added to the medium. On day three of culture, the cells were fixed in 4% paraformaldehyde at room temperature for 10 min, washed once with PBS (Phosphate-Buffered Saline), and then treated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase and preincubated with normal horse serum for 20 min to eliminate background staining. The specimens were subsequently washed once in PBS for 5 min and incubated with the rabbit anti-phosphorylated Histone H3-pSer^{10} (Sigma Cat# H0412, RRID; AB_477043, diluted in 1:500) in a humid chamber at 4 °C overnight. The slides were washed with PBS at room temperature and then incubated with goat biotinylated anti-rabbit IgG (Santa Cruz Cat#16114, RRID; AB_2534787, diluted in 1:200) for 2 h. The reaction was visualized using an avidin/biotin complex with diaminobenzidine (DAB; Vector

Table 1. Composition of water-soluble nuclear crude extract (SNE) from salmon soft roe.

Components	Amount (g/100g)
Water	3.9
Nitrogen	15.5
Lipid	<0.1
Minerals (Ash)	17.8
Sodium	4.8
Nucleotides	
5′-dCMP	5.46
5′-dAMP	7.15
5′-dTMP	8.73
5′-dGMP	5.02
Total	23.36
Amino Acids	
Arg	15.3
Lys	3.31
His	0.74
Phe	0.97
Tyr	1.01
Leu	2.24
Ile	1.34
Met	0.64
Val	2.21
Ala	2.28
Gly	3.82
Pro	2.56
Glu	3.89
Ser	2.41
Thr	1.49
Asp	2.51
Trp	0.20
Cys	0.20
Total	47.12

Laboratories) as a chromogen. Subsequently, hematoxylin staining was performed to detect nuclei. Six arbitrary positions were photographed in each group, and the number of H3-pSer^{10} positive cells was counted. Furthermore, image processing was performed with *ImageJ* [17] to determine the total number of cells, and the ratio of H3-pSer^{10} positive cells to the total number of cells was calculated.

2.6. Analysis of cell cycle distribution

MCF7 cells were harvested by trypsin-treatment after 3 days incubated with 1 mg/ml SNE or 3 mg/ml cyclophosphamide (CPA) as positive control for apoptosis. The cells were then fixed in cold 70% ethanol overnight and subsequently stained with a 50ug/ml propidium iodide solution (eBioscience) containing 100ug/ml RNase A (Sigma) for 30 min at 37 °C and applied to a flow cytometer (Gallios, Beckman Coulter). The percentage of cells in each phase of the cell cycle and apoptosis was analyzed by Kaluza and FlowJo (ver. 7.65) software (Becton Dickinson).

2.7. Statistical analysis

Triplicate measurements were made for each treatment. The data were analyzed by one-way analysis of variance by using Microsoft Excel. Values are expressed as mean ± standard deviation. The t-test was used to calculate statistical significance, and a $P < 0.05$ was considered statistically significant.

3. Results

3.1. SNE suppresses cell growth in a cell type-specific manner

To explore the phenomenon occurring in necrotic tumors, we first examined the cell viability by the MTT assay to evaluate the effect of SNE on various cancer and noncancer cell lines. The culture period was set to 4 days after examining several conditions. Furthermore, because nucleotides released from dead cells were immediately digested by DNase and spread out, it is unlikely that the state of the highly concentrated nucleic acid cannot be maintained for a long period. As shown in Figure 1 A, cell growth inhibition occurred significantly in some cancer cell lines initially at about 0.14 mg/ml, especially in MCF7 and LNCap. In contrast, other cell lines like HepG2 and A549 did not decrease cell viability so much under the same conditions. To confirm the specific effect of SNE on breast cancer cell proliferation, we investigated the effects of HCC1937, derived from breast cancer cells, and MCF10A from noncancer breast cells on cell proliferation. HCC1937, and MCF10A also showed cell growth inhibition in the same manner as MCF7 (Figure 1 B). These data suggested that SNE suppressed both cancer and noncancer cells growth in a cell type-specific manner.

3.2. SNE does not induce apoptosis but delays cell cycle progression by accumulating in the S phase

Next, we assessed the changes in growth rate and morphology of MCF7 affected by SNE to elucidate whether the mechanism of cell toxicity was observed, even under conditions higher than Figure 1 (1.5 mg/ml). Biostation CT (NIKON); a cell culture observation device that allows time-lapse analysis revealed that MCF7 proliferated slowly without dying even under 5 mg/ml SNE concentrations although showing slight cellular damage morphologically (Figure 2A-C). To investigate further whether this suppression of cell proliferation was due to apoptosis or cell cycle delay, we conducted immunocytochemistry to detect phosphorylated histone H3 on Ser^{10} (H3-pSer^{10}); a mitotic marker (Figure 2A). In addition to the prolonged cell growth caused by SNE, the ratio of the H3-pSer^{10} positive cells was also decreased in SNE concentration-dependent manner (7%→3%) (Figure 2D). These results suggested a possibility that cell proliferation was inhibited due to cell cycle delay.

Next, a flow-cytometric analysis was conducted to assess the phase of the cell cycle in each stage. The analysis was performed by quantifying the amount of DNA in each cell: Go/G1 (2N DNA), S phase (2N∼4N DNA), and G2/Mitosis (4N DNA) stained with propidium iodide (PI) [18]. As shown in Figure 3A, in comparison with the control (S phase of 36.35%, G2/M phase of 14.32%), treatment with 1 mg/ml SNE for 3 days increased the S phase proportion to 58.06% and decreased the G2/M phase to 7.44%. Furthermore, fragmented DNA were not detected when the cells were treated with 1 mg/ml SNE for 3 days, although cyclophosphamide induced DNA fragmentation in MCF7 cells (Figure 3B). Figure 3C shows the graphical representation of the percentage of apoptotic cells with DNA fragmentation. DNA fragmentation was not detected even after 4 days. Thus, it is speculated that the SNE inhibited cell proliferation by accumulating in the S phase and thereby causing cell cycle delay, and not by cellular apoptosis.

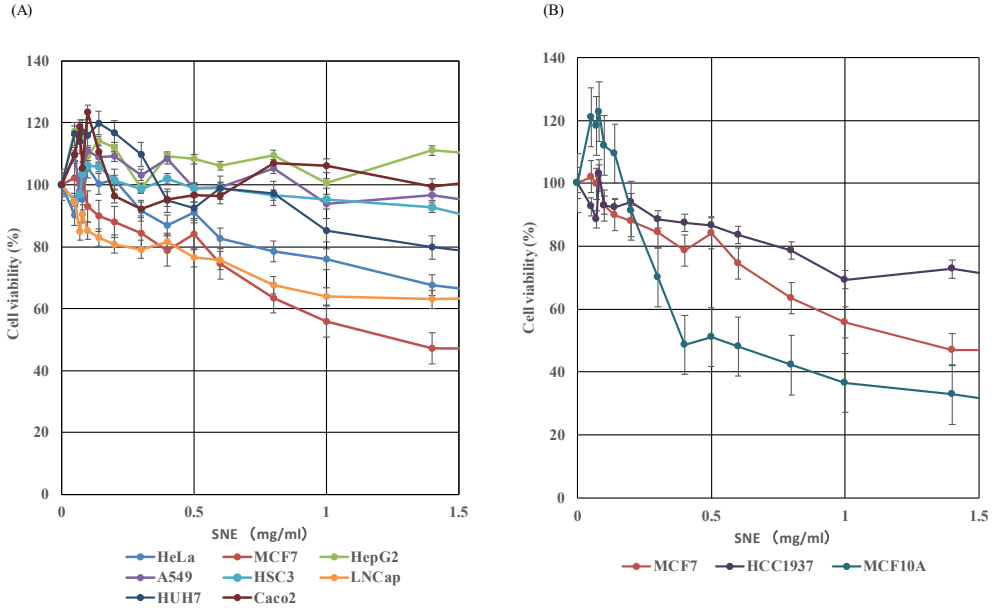
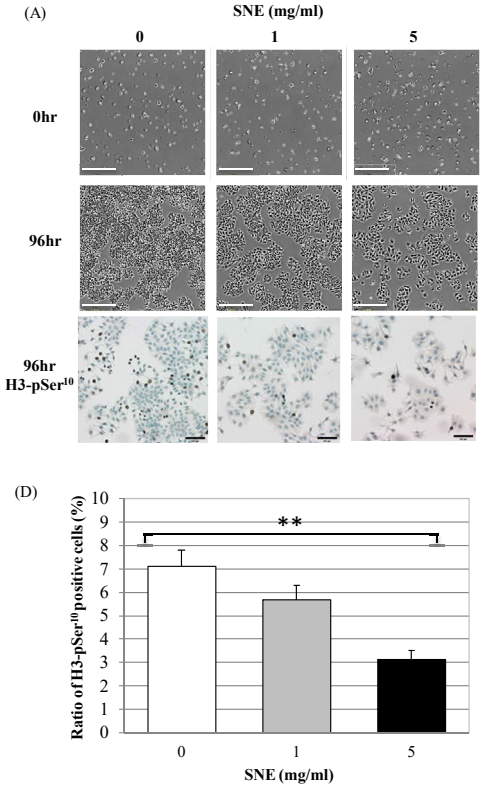


Figure 1. The water-soluble nuclear crude extract (SNE) suppresses some cancer and noncancer cell growth *in vitro*. Cells were treated with indicated doses (0–1.5 mg/ml) for 4 days. The viability was assessed by MTT assay. (A) HeLa (uterine cervix), MCF7 (breast), HepG2 (liver), A549 (lung), HSC3 (tongue), LNcap (prostate), HUH7 (liver), Caco2 (colon), HLE (liver). (B) MCF7 and HCC1937 from breast cancer, and MCF10A from noncancer breast cells. Values are mean ± SD of three independent experiments, each dosage in triplicate. All the used doses are statistically significant as determined by the Student-t-test (**$P < 0.05$). For the sake of simplicity, asterisks (**) are not reported in the graph.



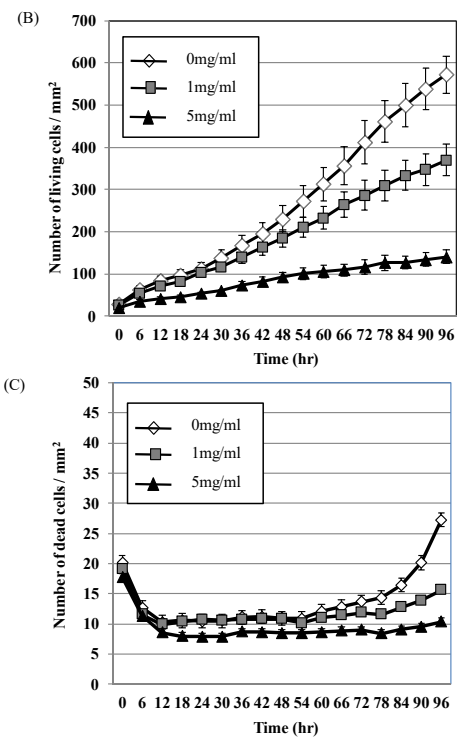
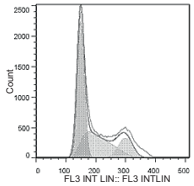
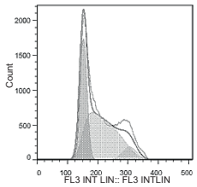


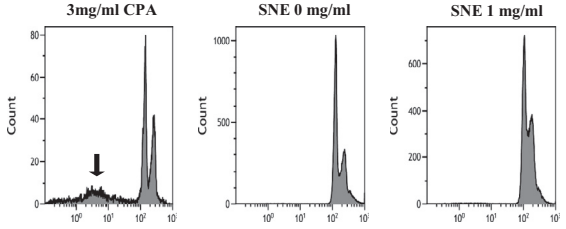
Figure 2. Time-lapse imaging acquisition implies that MCF7 cells grow slowly by cell cycle delay. (A) Phase-contrast image of control and SNE treatment (1, 5 mg/ml) at 0 and 96 h (Scale bars: 500 um, magnification 20x) and representative immunocytochemistry images of MCF7 cells for H3-pSer^{10} after SNE treated 96 h (Scale bars: 100 um, magnification 20x). (B) The number of living cells of each group was represented by mean values ± SD of four independent experiments. (C) The number of dead cells of each group was represented by mean values ± SD of four independent experiments. (D) The graph shows a ratio of H3-pSer^{10} positive cells. The total number of cells is counted by *ImageJ* software (NIH), and H3-pSer^{10} positive cells were counted with eyes. All the used doses were statistically significant as determined by the Student-t-test (**$P < 0.05$).

(A)

		Go/G1	S	G2/M
SNE 0 mg/ml		49.33	36.35	14.32
SNE 1 mg/ml		34.5	58.06	7.44

Values express percentage

(B)



(C)

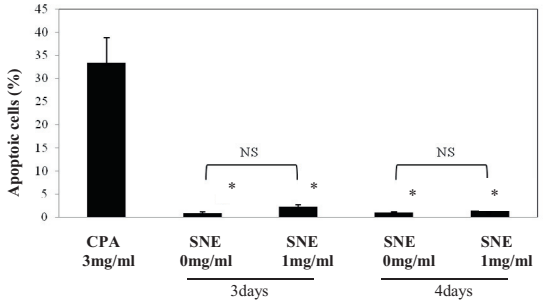


Figure 3. SNE delays cell cycle progression accumulating in the S phase. MCF7 cells were stained with propidium iodide (PI) and conducted by flow cytometry to analyze the percentage of the cells in each phase of cell [with the addition of 1 mg/ml SNE (A) and 3 mg/ml of cytophosphamide; CPA, (B) 0 and 1 mg/ml of SNE for 3 and 4 days]. (C) The percentage of apoptotic cells that showed DNA fragmentation in each group was presented graphically for clarity. (* : $P < 0.05$ vs. Cyclophosphamide; Mann-Whitney test) .

3.3. Cell growth inhibitory effect was mainly due to the oligo DNA

Next, we investigated the factors contributing to the cell growth inhibition caused by SNE using MCF7, one of the most sensitive cell lines. SNE is thought to be composed mainly of nucleic acids and amino acids derived from protamine degradation (Table 1), but may also contain trace amounts of lipids and hormones, which might contribute to cell growth suppression. In this study, we compared two major components of SNE, purified oligo DNA, and protamine. Protamine is widely used for anti-microbial action due to its nature [19], but the protamine contained in the materials in this study is enzymatically digested. Figure 4A shows a comparison of the cell growth inhibition induced by purified oligo DNA, protamine, and trypsin-digested protamine at SNE equivalent concentration (based on Table 1). This result showed that the purified oligo DNA inhibited cell growth at the concentration corresponding to DNA in the SNE. As for protamine, the un-digested one strongly inhibits cell growth, but the trypsin-digested one did not. Furthermore, the cytotoxicity of four dNTPs was compared using commercial polymerase chain reaction grade dNTPs, respectively. All four nucleotides inhibited cell growth, and no difference was observed (Figure 4B). Therefore, these findings suggested that the oligo DNA mainly induced cell growth inhibition.

4. Discussion

Unignorable amounts of extracellular nucleotides are circulating in serum, and daily foods are one of their resources. In healthy individuals, DNase 1 rapidly degrades cell-free DNA (cfDNA) within minutes. A low dose of water-SNE has been reported to work in immunity improvement [13, 20, 21, 22], neuron protection [14, 23], protective effects against ethanol-induced liver injury [15], and amelioration of symptoms in rheumatoid arthritis [24]. These results suggest that SNE might be a dietary nutrient and supplement ameliorating cellular metabolism. Studies using *C.elegans* have shown that SNE promotes the movement, stress tolerance, and lifespan of *C. elegans* [25]. To confirm this hypothesis, we needed to investigate the pharmacokinetics of SNE after oral ingestion, but it is difficult to perform because the dietary SNE is easily absorbed and spread out in serum and tissue and excreted outside of the body. Meanwhile, through investigating the function of SNE, we found that SNE has a cell growth inhibition effect in certain cancer and non-cancer cell lines such as MCF7, MCF10A, and LNCap at relatively low-dose condition (0.1–0.14 mg/ml) (Figure 1 A, B). Next, we compared the effects of nucleotides and amino acids, which are the main components of SNE, on cell growth inhibition. Protamine is abundant in salmon soft roe and rich in arginine with a strong positive charge. and due to its nature, it is widely used for anti-microbial action [19]. In this study, we revealed that nucleotides contribute to cell growth inhibition, and their concentration is about 5 mM, calculatedly equivalent to 7 mg/ml SNE. There was a calculated difference between the results when using SNE and dNTPs. The reason is unknown, but presumed to be the error of the nucleic acid concentration in SNE or the effect of nucleotide oligomers contained in SNE. These results suggested that the SNE worked in a limited area where they were present in high concentration. In the case of serum, the cfDNA (cell-free DNA) typically remains below 40 ng/ml in healthy individuals, whereas they can be significantly increased at 300–600 ng/ml in some cancer patients [26], although the level is still low compared to that used in the experiment to suppress cell proliferation.

To interpret this phenomenon, we built a hypothesis that the concentration of cfDNA levels may be much higher in the necrotic part of the tissue than in serum, especially in the inflammatory lesions induced by bacterial and fungal infections and malignant neoplastic lesions with ischemic damages [27]. Besides the necrotic tissue, many proteins have anti-microbial activity like myeloperoxidase generating hypochlorite and mitochondrial nicotinamide adenine dinucleotide phosphate oxidase, and its nucleic acids are released from dying neutrophils. This sequential process is called NETosis [5]. Although the mechanisms of NETosis have been studied extensively by many researchers [28, 29], little is known on what happens to released nucleotides after being immediately digested by DNase I in extracellular fluid. DNase I immediately digested released nucleotides from necrotic cells and neutrophils in extracellular fluid [28]. Rapid cfDNA clearance is critical to prevent the inflammation and potential development of autoimmunity toward DNA, as seen in systemic lupus erythematosus (SLE). In SLE patients with low DNase I activity, a high concentration of cfDNA was found in serum [30]. Therefore, it is currently thought that there is a correlation between DNase levels and cfDNA. Inflammatory reactions also occur in non-infections, such as ischemic infarction, chemical irritation, radiation, burns, and even in neoplastic diseases under cachexia. About 30%–80% of cancer patients suffer from cachexia with weight loss, dehydration, malnutrition, and general exhaustion progresses severely [31, 32]. In cachexia, inflammatory cytokines are increased in serum and promote inflammation in various organs. Unlike starvation, cancer patients with cachexia have

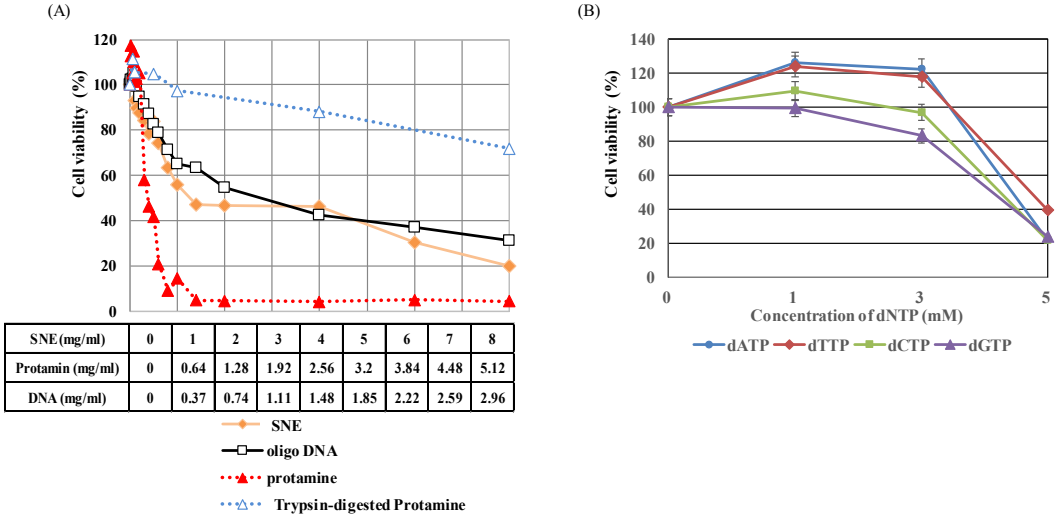


Figure 4. Cell growth inhibitory effect was mainly due to base-independent oligo DNA. (A) MCF7 was treated with SNE and Protamine and purified oligo DNA at the concentrations indicated above for four days. The concentration converted to SNE was also shown based on analysis data (Table 1). (B) Each dNTPs were added to MCF7 at the concentrations indicated above for four days. The viability was assessed by MTT assay. Values are means of three independent experiments, each dosage in triplicate.

Table 2. SNE effects on cell proliferation and cell cycle related genes expression profiles.

Biological Function Categories	Diseases of Functions Annotation	*p*-Value	Molecules (up (↑) or down (↓) Fold)
Cell Cycle	Exit from mitosis of breast cancer cell lines	2.80E-04	CCNE1 (1.49↑), CDKN1A (1.58↑)
DNA Replication, Recombination, and Repair	DNA damage	7.32E-03	ESR1 (0.70↓), RRM2 (1.55↑)
Gene Expression	Binding of estrogen response element	7.32E-03	CXCL12 (0.60↓), ESR1 (0.70↓)
Cell Cycle	senescence of breast cancer cell lines	1.65E-02	CDKN1A (1.58↑), ESR1 (0.70↓)

Altered gene expression profiles in MCF7 cells by adding of 1 mg/ml SNE were performed using Agilent Microarray and Ingenuity Pathway Analysis (IPA). CCNE1; cyclin E1, CDKN1A; cyclin dependent kinase inhibitor 1A, ESR1; estrogen receptor 1, RRM2; ribonucleotide reductase regulatory subunit M2, CXCL12; C-X-C motif chemokine ligand 12.

collapsed even muscle tissue, and they provide many nuclear substrates that flow out from the necrotic muscles. Our data shows that the proliferation of MCF7 was suppressed at 1 mg/ml of SNE (Figure 1), which is a possible concentration in the restricted area where severe inflammation with neutrophil infiltration or tumor necrosis occurred.

To reveal a mechanism of cell growth inhibition, we performed a DNA microarray analysis on MCF7 cell lines. Therefore, we found several genes, such as cyclin E1 (1.49- fold up), that regulate the G1-S phase transition and cyclin-dependent kinase inhibitor 1A (1.58-fold up) that mediates G1 arrest (Table 2). However, we need further analysis by using other cells, including primary cell culture, to assess the inhibitory effect on cell cycle profile ratio by SNE.

Subsequently, we investigated the components that contributed to the cell growth inhibition by SNE. Interestingly, the suppression was mainly due to nucleic acids among the components of SNE. Unmethylated CpG DNAs in viruses and other pathogens are recognized by TLR-4 and 9 and subsequently promote innate immune mechanism. On the other hand, the nucleic acids used in this study are derived from salmon soft roe and are expected to be highly methylated, the same as those present in humans [8]. In fact, the SNE did not induce TLR-9 signaling (data not shown), which means the CpG DNA of salmon soft roe are methylated and thus cannot be recognized by TLR-4, 9 as well as a self-nucleic acid of mammals, including humans. Therefore, it is a useful model for investigating the function of extracellular nucleotides in humans.

We must also consider the effects of adenosine triphosphate (ATP) and adenosine on cell proliferation because ATP acts as many signal transduction substances, such as pain transmission and inflammation induction, and adenosine acts as anti-inflammatory [33, 34]. Each nucleotide was added, and the cell proliferation inhibitory effect was verified to investigate whether the cell proliferation inhibition this time is ATP or adenosine-specific. Therefore, no significant difference was observed between each nucleotide.

We should pay attention in a future study to the cell type specificity of the growth inhibitory effect of extracellular nucleotide observed in MCF7 (breast cancer) and LNCap (prostate adenocarcinoma). We could not figure out the cause of its specificity, but it might be possible that some specific hormones or receptors like estrogen and androgen are involved. Moreover, we need to investigate the effects on the proliferation or stimulation of normal resident cells such as blood vessel components and inflammatory cells, because extracellular nucleotides are associated with ANCA (anti-neutrophil cytoplasmic antibodies) associated vasculitis and SLE, which are thought to be related with NETosis [35, 36].

In conclusion, the present *in vitro* study indicated a possible hypothesis that high concentrations of extracellular nucleic acids released from necrotic tumors and surrounding neutrophils suppress cancer cell proliferation by delaying the cell cycle. This phenomenon may be associated with the amplifying effect of tumor necrosis, which frequently occurs in highly malignant tumors. Although further clinical and experimental studies are necessary, the present experimental results gave us a novel insight that the local high concentrations of nucleic acids released by tumors and neutrophils may suppress the growth of nearby tumor and normal cells. However, because tumor cells proliferate faster than normal cells, it is speculated that the growth inhibitory effect of high-concentrated extracellular nucleic acids is primarily seen in tumors rather than normal cells.

This study has the following several limitations. First, in this study, we analyzed the cell cycle distribution in MCF7 only, but we also need to verify it in other cells, such as MCF10A and LNCap, and confirm whether it is a phenomenon common to other cells. Second, although we carried out the MTT assay to assess cell viability, it might be better to count live cells by flow-cytometric analysis by using PI staining to exclude the dead cells or carboxyfluorescein diacetate succinimidyl ester (CFSE) staining to detect cell division. Third, we evaluated apoptosis by flow-cytometric analysis, and this time we detected DNA fragmentation; however, it would be better to verify this phenomenon by additional methods, such as Caspase assay to assess apoptosis-related protease and annexin V/PI analysis by flow-cytometric analysis, to measure the loss of plasma membrane asymmetry in apoptosis.

To summarize this study, highly concentrated nucleic acid might suppress excessive cell proliferation in a necrotic tumor environment, thereby giving us a novel idea to use SNE for cancer treatment. Although an effective SNE delivery system must be investigated, it could be a clue to develop an alternative therapy for cancer patients.

Declarations

Author contribution statement

Chika Sawa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sachiko Yofu, Tomomi Saito, Satomi Kishi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Mariko Gunji, Yuriko Inoue: Conceived and designed the experiments.

Masahito Sugi, Seiji Shioda, Keita Sutoh, Keisuke Kiriyama: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Kazuho Honda: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at Showa University Academic Resource repository.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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