Effect of cell cycle phase on the sensitivity of SAS cells to sonodynamic therapy using low-intensity ultrasound combined with 5-aminolevulinic acid *in vitro*

NAN LI^{1,2*}, MIAO SUN^{3*}, YAO WANG⁴, YANHONG LV⁴, ZHENG HU², WENWU CAO^{2,5}, JINHUA ZHENG^{2,4,5} and XIAOHUI JIAO¹

 ¹Department of Oral Maxillofacial Surgery, School of Stomatology, Harbin Medical University, Harbin, Heilongjiang 150001;
²Laboratory of Sono- and Photo-theranostic Technologies, Harbin Institute of Technology, Harbin, Heilongjiang 150080;
³Department of Stomatology, The Fourth Clinical College, Harbin Medical University, Harbin, Heilongjiang 150001;
⁴Department of Anatomy, Basic Medical Science College, Harbin Medical University, Harbin, Heilongjiang 150081, P.R. China; ⁵Materials Research Institute, Pennsylvania State University, University Park, PA 16802, USA

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Abstract. Sonodynamic therapy (SDT) with 5-aminolevulinic acid (5-ALA) can effectively inhibit various types of tumor in vitro and in vivo. However, the association between the efficacy of SDT and the phase of the cell cycle remains to be elucidated. 5-ALA may generate different quantities of sonosensitizer, protoporphyrin IX (PpIX), in different phases of the cell cycle, which may result in differences in sensitivity to 5-ALA-induced SDT. The present study aimed to investigate the effect of the cell cycle on the susceptibility of SAS cells to SDT following synchronization to different cell cycle phases. These results indicates that the rates of cell death and apoptosis of the SAS cells in the S and G₂/M phases were significantly higher following SDT, compared with those in the G1-phase cells and unsynchronized cells, with a corresponding increase in PpIX in the S and G₂/M cells. In addition, the expression of caspase-3 increased, while that of B-cell lymphoma (Bcl)-2 decreased markedly in theS and G₂/M cells following SDT. Cyclin A was also expressed at higher levels in the S and G₂/M cells, compared with the G1-phase cells. SDT also caused a significant upregulation of cyclin A in all phases of the cell

*Contributed equally

cycle, however this was most marked in the S and G_2/M cells. It was hypothesized that high expression levels of cyclin A in the S and G_2/M cells may promote the induction of caspase-3 and reduce the induction of Bcl-2 by SDT and, therefore, enhance apoptosis. Taken together, these data demonstrated that cells in The S and G_2/M phases generate more intracellular PpIX, have higher levels of cyclin A and are, therefore, more sensitive to SDT-induced cytotoxicity. These findings indicate the potential novel approach to preventing the onset of cancer by combining cell-cycle regulators with SDT. This sequential combination therapy may be a simple and cost-effective way of enhancing the effects of SDT in clinical settings.

Introduction

Sonodynamic therapy (SDT), using low-intensity ultrasound combined with a sonosensitizer, is a promising approach to cancer therapy, which has rapid progressed in previous years (1,2). In vitro and in vivo experiments have demonstrated that SDT can effectively inhibit several types of cancer cells (3-7). 5-Aminolevulinic acid (5-ALA) itself is not a sonosensitizer, however, it is the prodrug of protoporphyrin IX (PpIX). PpIX is a sonosensitizer, which preferentially accumulates in tumor cells, but not in normal tissues (8) due to an imbalance of porphobilinogen deaminase activity and thus ferrochelatase activity in neoplastic tissues (9,10). Schick et al (11) and Wyld et al (12) reported that, compared with resting cells, proliferating cells generate more PpIX following incubation with 5-ALA. Thus, 5-ALA may produce different quantities of PpIX in different cell cycle phases, leading to differential sensitivity to 5-ALA-SDT. Therefore, the presents study hypothesized that susceptibility to SDT is likely to be associated with certain phases of the cell cycle of tumor cells.

Previous studies have suggested that the antitumor effects of several cancer therapeutic approaches are associated with the phase of the cell cycle (13-16). Certain appropriate chemotherapeutic agents, which induce cell cycle arrest at

Correspondence to: Professor Xiaohui Jiao, Department of Oral Maxillofacial Surgery, School of Stomatology, Harbin Medical University, 143 YiMan Road, Harbin, Heilongjiang 150001, P.R. China E-mail: jxhln@hotmail.com

Professor Jinhua Zheng, Department of Anatomy, Basic Medical Science College, Harbin Medical University, 157 Baojian Road, Harbin, Heilongjiang 150081, P.R. China E-mail: jhzheng@ems.hrbmu.edu.cn

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the S phase or G_2/M phase increase the overall viral replication and then potentiate viral oncolysis (15,17,18). In human myeloma cell lines, the cytotoxicity induced by bortezomib, is markedly amplified in synchronous S phase entry and progression (16). Thus, the phase of the cell cycle can affect tumor sensitivity to anticancer treatments.

At present, the mechanisms underlying SDT-induced cancer cell death remain to be fully elucidated. Few studies have investigated the association between cell cycle phase and the effect of SDT on tumor cells. Our previous investigations demonstrated the effects of 5-ALA-induced SDT on human tongue squamous carcinoma (19,20). The present study aimed to investigate the production of PpIX in different phases of the cell cycle and the effects of these phases on the susceptibility of the cells to SDT-induced cell death. The differential expression of apoptosis-associated factors and enrichment of cyclins in certain cell cycle phases were also examined. Determination of the likely underlying mechanism may provide a theoretical basis for optimizing the application of SDT in oncology.

Materials and methods

Cell culture. The human tongue cancer SAS cell line was obtained from the Human Science Research Resources Bank (Osaka, Japan). The SAS cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (GE Healthcare, Logan, UT, USA) at 37°C and 5% CO₂. The RPMI1640 medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS; GE Healthcare), 100 U/ml penicillin and 100 μ g/ml streptomycin (GE Healthcare).

Cell cycle synchronization. To synchronize the cells to the G_0/G_1 phase, the SAS cells were arrested by serum starvation (21). The exponentially growing cells (1x10⁶ cells) were plated onto dishes containing RPMI-1640 without FBS, and incubated at 37°C for 48 h prior to harvesting. The SAS cells were synchronized to the S phase using Banfalvi's double thymidine block method (22). The exponentially growing cells were incubated at 37°C with 2 mM thymidine (Sigma-Aldrich, St. Louis, MO, USA) for 21 h, washed with PBS (Hyclone, Logan City, UT, USA) and placed in fresh RPMI-1640 medium with 10% FBS for 18 h. Subsequently, the cells were retreated with 2 mM thymidine for 21 h. Following release from the second inhibition at 37°C for 2 h, the cells were synchronized to the S phase. To arrest the cells at the G₂/M junction, the SAS cells were incubated at 37°C with 100 ng/ml nocodazole (Sigma-Aldrich) for 20 h, washed twice with PBS, resuspended in fresh RPMI-1640 medium with 10% FBS for 1 h and harvested. Following synchronization to the G_1 , S and G_2/M phases, the cells from each phase were allowed to grow in RPMI-1640 medium with 10% FBS. The present study used cells that were normal cycling (N), G1-phase (G1), S-phase (S) and G2/M-phase (G2/M) cells. The cells were sampled after 0, 1, 2, 3 and 4 h, following which flow cytometry analysis was performed to assess cell cycle duration.

Flow cytometric analysis of DNA content. Following cell cycle synchronization, the cells $(1x10^6)$ were harvested by trypsinization (0.25% trypsin; Hyclone) and washed twice in cold phosphate-buffered saline (PBS). The cells were

fixed in 70% ethanol (Luck Mouse, Changzhou, China) and stored at 4°C overnight. The fixed cells were resuspended in PBS containing 2.5 mg/ml RNase A (Sigma-Aldrich) and 1 mg/ml propidium iodide (Sigma-Aldrich), and incubated for 30 min at 37°C. Following filtration through a nylon mesh (300 mesh; Yuexing, Guangzhou, China), the cells were evaluated using flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA), and the results were analyzed using ModFit LT version 4.0 software (Verity Software House, Topsham, ME, USA).

PpIX determination. The SpectraMax 5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) was selected to detect the production of 5-ALA-induced PpIX in the SAS cells (excitation, 405 nm; emission, 590 nm). The correlation between the fluorescence intensity of the samples and the exogenous PpIX concentrations were assessed, following which a standard curve of PpIX was constructed using SPSS 13.0 softward (SPSS, Inc., Chicago, IL, USA). The SAS cells were seeded into a 96-well plate (2x10⁴ cells/well) and cultivated for 24 h in RPMI-1640 medium. At the time-points corresponding to each phase of the cell cycle following synchronization, as described above, the cells were incubated with 1 mM 5-ALA in RPMI-1640 medium for 2 h in the dark at 37°C. The cells were then washed three times with PBS and the concentrations of PpIX in the cells in each cell cycle phase were determined using the microplate reader. The fluorescence intensity indicated the level of intracellular PpIX.

Ultrasonic device. Following synchronization, the cells treated with SDT (Harbin Institute of Technology, Harbin, China) were incubated with 1 mM 5-ALA in the dark for 2 h at 37°C. The ultrasound treatment system used in the present study, as shown in Fig.1, was designed and manufactured by the Harbin Institute of Technology (Harbin, China). This ultrasonic device has been described in a previous publication (23). A 3.5 cm petri dish containing the cultured cells was placed in center of the transducer. The cells were exposed to ultrasound (1.0 MHz; 0.05 W/cm²; 10% duty cycle) for varying durations (1, 2 and 3 min), in the dark. Following treatment, the cells were either harvested or incubated continuously for subsequent analyses.

Cell survival assays. Following treatment with SDT, the cells were harvested and reseeded into 96-well plates at a density of 1x10⁴ cells/well for 24 h. The cell viability was subsequently determined using a Cell Counting kit (CCK)-8 (Beyotime Institute of Biotechnology, Nantong, China), according to the manufacturer's instructions. The absorbance value (AV) was measured at 450 nm using a SpectraMax 5 microplate reader. The absorbance data were expressed as the percentage survival, which were corrected for background and compared with the controls using the following formula: AV of test well / AV of control well x 100%.

Analysis of cell apoptosis. The cells of all the groups were harvested by trypsinization without EDTA and were washed three times with pre-cooled PBS. Apoptosis was detected using an Annexin V-Propidium Iodide (PI) Apoptosis Detection kit (Biosea Biotechnology, Beijing, China), according to the



Figure 1. Schematic diagram of the ultrasonic device used for synchronization of the SAS cells.

manufacturer's instructions. The cells were re-suspended in 200 μ l binding buffer (Biosea Biotechnology, Beijing, China) and stained with annexin V (10 μ l) and PI (5 μ l) sequentially. Following incubation at 4°C for 30 min in the dark, the cells were counted using flow cytometry on a FACSCalibur flow cytometer.

Immunoblotting. The cells were lysed in radioimmunoprecipitation buffer for protein extraction. Equal concentrations $(50 \mu g)$ of protein from each sample were resolved on 10% polyacrylamide-sodium dodecyl sulfate gels (Beyotime Institute of Biotechnology Co., Ltd., Nantong, China) and electrophoretically transferred to polyvinylidene difluoride membranes (Beyotime Institute of Biotechnology Co., Ltd.). The membranes were blocked using non-fat dried milk (Wandashan, Harbin, China) for 1 h; incubated overnight at 4°C with antibodies against human cyclin A (cat.no. sc-751), B-cell lymphoma (Bcl)-2 (cat.no. sc-492), caspase-3 (cat.no. sc-7148) and β-actin (cat.no. sc-130619; all rabbit polyclonal antibodies used at 1:200 from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); and subsequently incubated for 2 h at 4°C with an horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:5,000; cat. no. ZDR-5306; ZSGB-BIO, Beijing, China). The immunoreactive proteins were visualized, and the protein levels were normalized with respect to the band density of β -actin as an internal control. The protein bands were detected using an image analyzer (Quantity One; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and an enzymatic chemilluminescence kit (Beyotime Institute of Biotechnology, Nantong, China).

Statistical analysis. All data are presented as the mean \pm standard deviation. The differences between groups were analyzed using Student's t-test. Statistical differences were evaluated using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Synchronization effect and DNA content in different phases of the cell cycle. Figure 2A shows a histogram of the cell cycle based on flow cytometric analysis of the SAS cells following treatment with various synchronization methods. A single-parameter histogram of DNA enables discrimination of cell populations existing in the G_0/G_1 (2C DNA), S (between 2C and 4C) and G_2/M (4C) phases of the cell cycle. Based on these data, the synchronization performed in the present study was successful. The percentages of cells in the G_0/G_1 , S and G_2/M phases in the normal SAS cells increased between 60.58±2.8, 28.74±1.1, and 10.68±0.9% and 92.48±9.4, 70.59±2.7 and 56.33±1.9%, respectively (P<0.05). The durations of different phases of the cell cycle were calculated 0, 1, 2, 3 and 4 h following release from the different synchronization blocks (Fig. 2B). The percentage of cells in the G1- and S-phases remained high between 0 and 4 h. The percentage of cells in the G_2/M phase decreased 4 h after release. These results indicated that, following incubation with 5-ALA for 2 h following phase synchronization, the cells remained within the limit of that particular phase and did not transit to the next phase of the cell cycle.

Production of PpIX in different cell cycle phases. A standard curve was plotted, according to the fluorescence intensity of a known gradient concentration of exogenous PpIX (Fig. 3A). A positive linear correlation was observed between the fluorescence intensity and the concentrations of PpIX in the liquid phantom (correlation index $R^2=0.9983$). Therefore, the fluorescence intensity was indicative of the level of 5-ALA-induced PpIX in each phase of the cell cycle. Subsequently, the present study investigated the production of PpIX from synchronized cells treated with 5-ALA for 2 h. As shown in Fig. 3B, 5-ALA administration yielded significantly higher PpIX fluorescence intensities in the S and G₂/M phases, compared with the normal cycling cells and G₁ cells (P<0.05). These results suggested that the production of PpIX from exogenous 5-ALA was higher in the S- and G₂/M phase compared with the G₁ phase cells. Thus, it was hypothesized that the increase in cell death was, at least in part, due to an increase in the production of PpIX compared with that of normal cycling cells and G₁-phase cells.

Cell survival and apoptosis in different phases of the cell cycle following SDT. The CCK8 assay revealed that, following SDT, the cell viability was higher in the asynchronous cells and G1 cells compared with that in the S-phase and G_2/M -phase cells (P<0.05; Fig. 4A). The sensitivity of S and G_2/M cells to SDT increased markedly as the duration of sonication increased between 1 and 3 min. The apoptotic rates of the cells were almost identical in the normal cycling cells and G_1 cells following SDT treatment (Fig. 4B). However, the cells in the S and G_2/M phases exhibited a significantly higher apoptotic rate, compared with the other groups (P<0.05). Specifically, there was a 20.91% increase in apoptotic rate in the S-phase cells, compared with the unsynchronized cells.

Effect of cell cycle on apoptotic cytokines and cyclin A in SDT treatment. The results of the western blot analysis (Fig. 5), demonstrated that caspase-3 was significantly increased in the cells in the S and G_2/M phases following SDT treatment (P<0.05), with the opposite change in Bcl-2 (P<0.05). The expression of cyclin A also peaked in the cells in the S and

A



Figure 2. Cell distributions in different phases of the cell cycle were determined using fluorescence-activated cell sorting following synchronization and release for different durations. (A) Representative histograms of DNA content using flow cytometry of normal SAS cells and cells subjected to the different synchronization methods of serum starvation, double thymidine block and nocodazole incubation (n=6). M1, G₀/G₁ (2C DNA); M2, S (between 2C and 4C); M3, G2/M (4C). (B) Percentages of cells in the G0/G1, S and G2/M phases of the cell cycle at different time-points following release from synchronization. The proportion of cells in the G_0/G_1 , S, and G_2/M remained high at 2 h (n=6). Data are presented as the mean \pm standard deviation.



Figure 3. Levels of 5-ALA-derived PpIX differed among the cells in the different phases of the cell cycle. (A) Standard curve of the concentration of PpIX, vs. fluorescence intensity (n=3). (B) Differences in PpIX fluorescence intensity between cells in different phases of the cell cycle following incubation with 5-ALA for 2 h (n=6). Data are presented as the mean ± standard deviation. *P<0.05, vs. N grorup; *P<0.05, vs. G₁-phase group. 5-ALA, 5-aminolevulinic acid; PpIX, protoporphyrin IX; N, normal cycling.



Figure 4. Cell cycle phase affects the sensitivity of SAS cells to SDT using 5-aminolevulinic acid. Data are presented as the mean \pm standard deviation. (A) Survival rates of SAS cells in different cell cycle phases following SDT with ultrasound for 1, 2 and 3 min (n=6). (B) Effect of different cell cycle phases on the SDT-induced apoptotic rates of the SAS cells, determined using fluorescence-activated cell sorting *in vitro* (n=6). Following synchronization to the G₁, S and G₂/M phases, the cells in each group were treated with SDT for 1 min. *P<0.05, vs. N group; *P<0.05, vs. G₁-phase group. SDT, sonodynamic therapy; N, normal cycling.





Figure 5. Effect of cell cycle on the expression levels of caspase-3 and Bcl-2 and cyclin A following SDT treatment. Data are presented as the mean \pm standard deviation. (A) Cells in each cell cycle group were lysed and subjected to western blot analyses to determine the expression of caspase-3 and Bcl-2 (n=6). ; *P<0.05, vs. N + SDT group; *P<0.05, vs. G1-phase + SDT group. (B) Expression levels of cyclin A were examined using western blot analysis prior to and following SDT treatment in four groups (n=6).. *P<0.05, vs. N group; *P<0.05, vs. G₁-phase group; *P<0.05, vs. S + SDT-, &P<0.05, vs. G₂/M + SDT-, SDT, sonodynamic therapy; Bcl-2, B-cell lymphoma 2; SDT-, no SDT; SDT+, 1 min SDT; N, normal cycling.

Discussion

Cell cycle synchronization is a well-established technique to augment the efficacy of conventional cytotoxic anticancer therapy (24). The metabolic activity of a cell and the activity of cellular enzymes can vary with the phase of the cell cycle (12,13). The effect of cancer treatment, including chemotherapy, radiotherapy, photodynamic therapy (PTD) and oncolytic virus therapy is associated with the cell cycle (13,15,16,25,26).

In the present study, the levels of 5-ALA-induced PpIX were higher in the cells in the S and G_2/M phases, compared with those in the G1 phase and in the unsynchronized cells (Fig. 2), similar to findings reported by Wyld et al (13). Physiological processes, including macromolecule synthesis, metabolism and DNA synthesis enzyme activation are more active in the S and G₂/M phases than in other phases, which may be a major determinant of increased PpIX production in these phases. The varying cell surface area in different phases of the cell cycle has been considered to contribute to the cell cycle-dependent uptake of a sonosensitizer (14), however, the increase in low-density lipoprotein receptors and tumor-specific glycoprotein may also contribute (27). Different levels of PpIX, produced from 5-ALA, may elicit differing sensitivities to SDT in certain phases of the cell cycle.

In the present study, SAS cells were partially synchronized in vitro using serum starvation, double thymidine block and nocodazole to arrest the cells at the G₁, S and G₂/M phases, respectively. The subsequent analysis of the synchronized DNA content analyses indicated a high level of synchronization and no damage to the cells (Fig. 1). The rate of cell survival following SDT treatment, following release from synchronization, was measured. The CCK8 assays revealed that, compared with the G1 phase and normal cycling SAS cells, the cells in the S and G₂/M phases were significantly more sensitive to SDT following treatment with 5-ALA (Fig. 3). The susceptibility to SDT was also positively correlated with the level of PpIX in the cells in different phases. This variability in sensitivity to SDT with cell cycle is in agreement with previous observations on the susceptibility of cells to radiotherapy (28) and PDT with several photosensitizers, including 5-ALA (13), photofrin II (26) and ATX-S10 (Na) (14).

In the present study, cancer cells with a high percentage of cells in the S-phase or high proliferative activity were more sensitive to SDT-induced apoptosis (Fig. 3B). The results revealed that the levels of Bcl-2 and caspase-3 fluctuated in a cell cycle-dependent manner. SDT treatment induced cells in the S and G_2/M phases to produce less Bcl-2 and more caspase-3 than the levels in relative resting cells (Fig. 4A). Caspase-3 is a critical effector in mediating several forms of apoptosis, and our previous study demonstrated that SDT activates caspase-3 to induce SAS cell apoptosis through the mitochondrial signaling pathway (19). The level of SDT-induced caspase-3 increased in the S and G_2/M phases, which may be due to these phases having a lower threshold for caspase-3 activation (16).

Cell cycle protein regulation and the induction of cell death may be closely associated, and these two events may account for how the phase of the cell cycle affects tumor cell sensitivity to SDT (29). Cyclin A begins to accumulate during the S phase and maintains high levels until metaphase (30). With the exception of its functions in mitosis, cyclin A is involved in the initiation and progression of DNA synthesis during the S phase (31) and in the regulation of apoptosis (32). The induction of apoptosis is uniformly associated with the activation of cyclin A, but not with cyclins E or B (33,34). In addition, knockdown of the expression of cyclin A in K562 cells suppresses doxorubicin-induced growth arrest and apoptosis (35). Therefore, the present study investigated the role of cyclin A in the alteration in the sensitivity of cells to SDT in different cell cycle phases. The results confirmed that the protein expression levels of cyclin A were higher in cells in the S and G₂/M phases compared with those in the G1-phase cells following synchronization, and SDT caused a significant upregulation of cyclin A in all groups, particularly the S and G₂/M cells (Fig. 4B). The increased expression of cyclin A has been previously observed to correlate well with the activation of caspase-3 and increase in apoptotic rate (36,37), in which the overexpression of cyclin A circumvents the anti-apoptotic capacity of the Bcl-2 oncogene. Therefore, the increase in cyclin A in cells in the S and G₂/M phases may also explain why cells in these phases exhibiter higher apoptotic rates and sensitivity to SDT compared with those in the G₁ phase.

In conclusion, the present study demonstrated that synchronizing SAS cells to the S or G_2/M phase can significantly enhance SDT-induced cell growth arrest and apoptosis. This may be due to an increase in the production of PpIX in the S and G_2/M phases. In addition, increasing cyclin A in cells in the S and G_2/M phases may enhance the sensitivity of the cells to SDT by inhibiting Bcl-2 and promoting caspase-3. The results of the present study suggest the possibility of combination therapy with SDT and chemotherapy. To enhance the effect of SDT on cancer therapy and reduce tumor recurrence, a tumor-cell synchronizing agent may be administered to induce the cells into a more sensitive cell cycle phase prior to SDT treatment.

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