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Ultrastructure of Sarcoma 180 Cells After Ultrasound Irradiation in the Presence of Sparfloxacin

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Abstract. *Background: Sonodynamic synergistic antitumor effects have been well described to date, but detailed ultrastructural studies in this field are still lacking. Materials and Methods: A suspension of sarcoma 180 cells was exposed to ultrasound (US) in the presence of sparfloxacin (SPFX), one of the new quinolone antibiotics, at 2 W (0.64 W/cm^2) for 30 seconds. The antitumor effect was evaluated by the survival rate of cells and cell morphology by scanning and transmission electron microscopy. Results: After US irradiation, the survival rate of tumor cells in the SPFX-added group was 30.9%, significantly lower than 78.7% in the control group (p=0.0002). Most cells in the control group were spherical, but in the SPFX-added group the cells were aspherical. Cell membranes were often ruptured and numerous pores of various sizes were observed. Some cells were totally disintegrated. Conclusion: The most characteristic changes of the synergistic antitumor activities of US and SPFX were on the cell membrane.*

In the past few years, considerable progress has been made in the synergistic antitumor effects of ultrasound (US) in combination with sonodynamic chemicals (1-6). In contrast, there have been few reports on the ultrastructural study of tumor cells after US irradiation in the presence of sonodynamic compounds (7,8). In the present study, the survival rate of tumor cells was evaluated to determine if there is an enhancement of the cytotoxity of US in conjunction with sparfloxacin (SPFX), one of the new quinolone (NQ) antibiotics, against sarcoma 180 cells. Furthermore, scanning and transmission electron microscopic studies were performed to evaluate morphological changes induced by US irradiation in the presence of SPFX.

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Key Words: Sparfloxacin, ultrasound irradiation, sarcoma 180 cell ultrastructure.

Materials and Methods

Preparation of tumor cells. Ascitic sarcoma 180 was used as the experimental cell. A suspension of sarcoma 180 cells (about 1 ml) was injected intraperitoneally into 7-week-old ICR male mice (Shizuoka Laboratory Co., Shizuoka, Japan). After 7-10 days, 1.0 to 2.0 ml of ascitic fluid was collected and diluted in phosphate-buffered saline (PBS) so that the number of cells was set at $7.5x10^5$/ml ($5.3x10^5$/0.7ml) (hereinafter referred to as the stock solution). The control solution was prepared by diluting the stock solution 2-fold in 100 ml of PBS. The survival rate of tumor cells was evaluated by the trypan blue dye exclusion method using a hemocytometer (Kayagaki, Tokyo, Japan) under an optical microscope (Olympus BH-210, Tokyo, Japan, x400). To eliminate the influence of degeneration of tumor cells in the ascitic fluid, the stock solution with a mortality rate of less than 2.5% was used in the experiments to follow.

Chemicals. SPFX ($C_{19}H_{22}F_2N_4O_3$, molecular weight 392.41, supplied by Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) was used in the current study (Figure 1). To prepare a 0.2 mM solution of SPFX, 7.85 mg of SPFX was dissolved in 0.3 ml of DMF and diluted with 100 ml of PBS. The concentration of 0.2 mM was based on the amount of drug that could be dissolved in 0.3 ml of DMF, which was expected to have no influence on the experimental system (1,5).

Ultrasonic generator. The ultrasonic generator used in this study was made at the Department of Electronic Engineering, Akita University Mining College, Akita, Japan. Basically, the generator consists of a ceramic cylindrical transducer combined with a function generator. It can be used at a resonance frequency of 2 MHz on a piezo-electric element. The following components were used. A round ceramic plate of 20 mm in diameter and 1 mm in thickness (2Z 20D SYIC, Fuji Ceramics, Shizuoka, Japan) was used as the piezo-electric element. The resonance frequency after adhesion to aluminum is 2.256 MHz. A function generator (FG-350, Iwatsu Denshi, Tokyo, Japan) that can be operated over a frequency bandwidth of 0.1 Hz to 10 MHz was used. The sine wave function was employed in this experiment. The system also included a power amplifier (PA 40-2801, Someway, Shizuoka, Japan) with a frequency bandwidth of 100 kHz to 350 MHz, an output of 0 to 10 W, and a power meter (SX-200, Daiichi Denpa Kogyo, Tokyo, Japan) with a frequency range of 1.8 to 200 MHz and a power measurement range of 0 to 200 W.

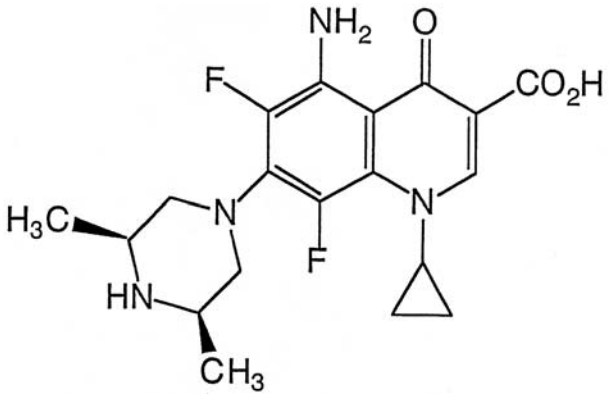


Figure 1. *Structural formula of sparfloxacin.*

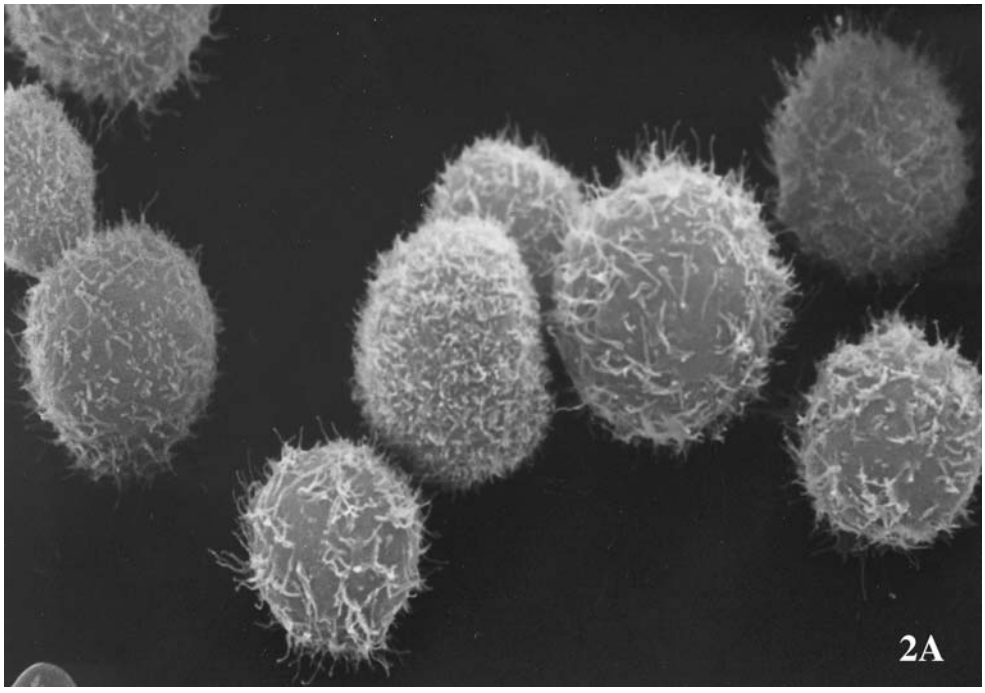
Ultrasonic irradiation experiment. 1.4 ml of the control solution and 0.7 ml of the stock solution mixed with 0.7 ml of 0.2 mM solution of SPFX were introduced separately into a glass cell 20 mm in diameter, 40 mm in height and with the base 1 mm in thickness (made at the Instrument Center, Akita University School of Medicine, Akita, Japan). The number of tumor cells contained in each glass cell was set at about $5.3x10^5$ as mentioned earlier. First, whether or not the survival rate of tumor cells in the stock solution changed by adding the drug itself was studied over time (n=10). Next, the control and the drug-added solutions were exposed to ultrasonic radiation of 2.0 W (0.64 W/cm^2) at a frequency of 2 MHz for 30 seconds respectively (n=10). To ensure close adhesion of the piezo-electric element with the glass cell, US transmission gel (Parker, Aquasonic 100, Fairfield, NJ, USA) was used. All procedures in the US irradiation experiment were performed within 1 hour after the aspiration of ascitic fluid of the mouse. The temperature of the solution in the glass cell was set at room temperature (22-26˚C). The cell survival rate in the experiment was calculated as (number of living cells after irradiation/number of living cells before irradiation) x100 (%), since the cells that had been destroyed by ultrasonic irradiation were counted as dead cells.

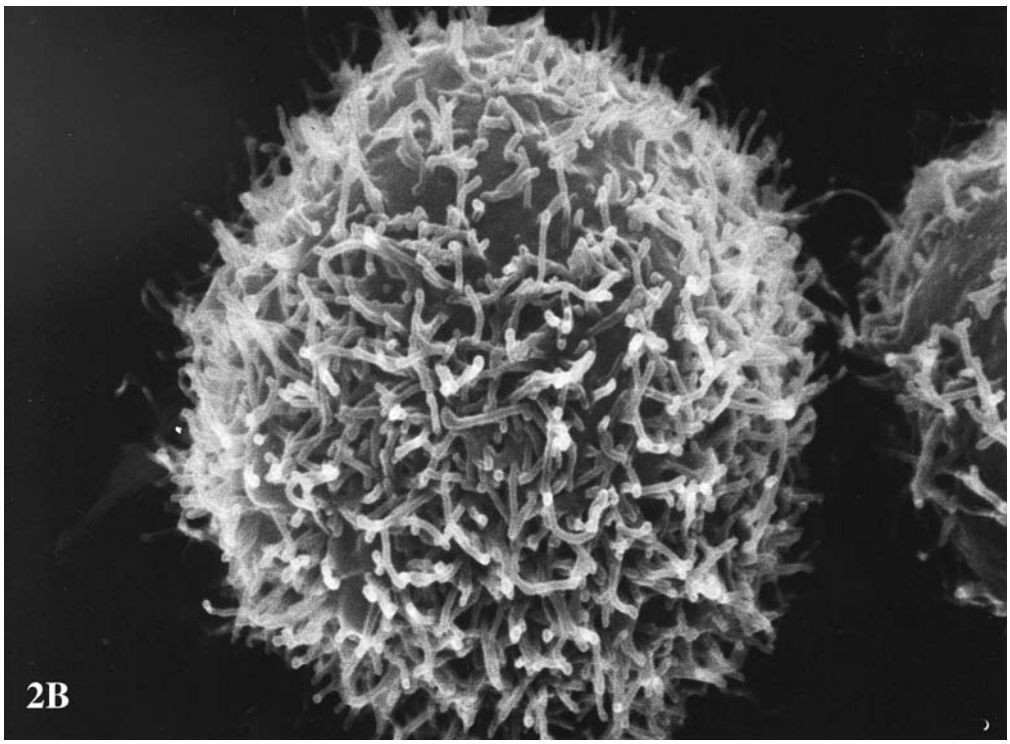
Light microscopic examination. A May-Giemsa smear was prepared from the cell suspension before and after the US irradiation to see the extent of degeneration and necrosis of cells (1,5).

Electron microscopy. For scanning electron microscopic study immediately after US irradiation, the cells were fixed for an hour in 3.0% glutaraldehyde solution buffered to pH7.2 and postfixation was performed with 1% osmium tetroxide for one hour. They then were gradually dehydrated with increasing concentrations of ethanol. Beginning with 50%, followed by 70%, 80%, 95% ethanol at 5 minutes each, and finally three 100% dehydrations for 5 minutes, before being transferred to a freeze-drier, coated with gold and examined with a 1200 EX scanning electron microscope (Electron-microscope Optical Ltd. JEOL, Japan) at 10 kV.

For transmission electron microscopy, the cell suspension was fixed with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and postfixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol and embedded in epoxy resin. Thin sections were cut and stained with uranyl acetate and lead citrate and viewed in a 1200 EX transmission electron microscope (Electron-microscope Optical Ltd.) at 80 kV.

Statistical analysis. The mean and standard deviation of survival rate of tumor cells were calculated for each group. Differences between the groups were considered significant when the *p* value of comparison by Wilcoxon test was 0.05 or smaller.





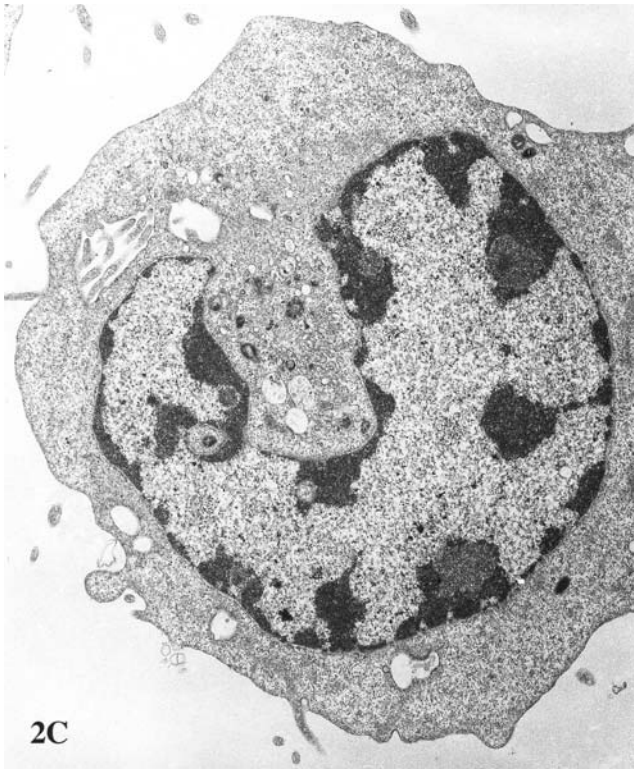


Figure 2. *Non-irradiated sarcoma 180 cells. A. The cells are round or oval with microvilli on the plasma membrane (original x2000). B. The surface of cell is covered with numerous microvilli (original x7500). C. The cytoplasm contains numerous regularly distributed cell organelles and nucleus (original x5000).*

Results

Influence of drugs on tumor cell viability over time. The survival rate of tumor cells in the control group (98.3±13.1%) was not significantly different from that of the 0.2 mM SPFX-added group (97.7±12.6%) without US irradiation within 3 hours (p=0.7806).

Influence of ultrasonic irradiation on survival rate of tumor cells. The survival rate of tumor cells was significantly lower in the SPFX-added group (30.9±15.4%) compared with that in the control group (78.7±2.3%) after US irradiation (p=0.0002).

Light microscopic observation. In the control group, sarcoma 180 cells were seen diffusely in the May-Giemsa smear. Both the nucleus and cytoplasm were well maintained. In the SPFX-added group, the cells were mostly lost or broken into small pieces. The remaining cells showed concentrated nucleus and bright cytoplasm.

Electron microscopic observation

Sarcoma 180 cells before irradiation: Nonirradiated cells, both in the control and SPFX-added groups, were round or oval with microvilli on the plasma membrane, contained large nuclei and prominent nucleoli. The chromatin was homogeneous and cell organelles showed a regular distribution. The endoplasmic reticulum arranged in single cisternae occupied only small areas of the cytoplasm (Figure 2).

Sarcoma 180 cells after US irradiation in the control group: In the control group, most sarcoma 180 cells were spherical in shape and the number of projected microvilli decreased, although round or spherical cells without detectable microvilli were sometimes encountered. The surface membrane was continuous and neither disruption, pore, nor pit was observed. The cytoplasm became vacuolated, but mitochondria and the plasma membrane remained intact during the course of these morphological changes. The chromatin of these cells was a little condensed at the nuclear periphery (Figure 3).

Sarcoma 180 cells after US irradiation in the SPFX-added group: The sizes of the cells varied. Most cells lost microvilli, developed numerous blebs on the plasma membrane and were characterized by their irregular shape. The surface membrane had been disrupted and numerous pores of various sizes were observed in the cell membrane. The ruptured cells tightly adhered to each other and formed clusters. The cytoplasm became vacuolated and the mitochondria showed an excessive swelling or total breakdown. The contour of the nucleus was remarkably irregular and the nuclear membrane was often ruptured. The chromatin condensed to clumps and some cells showed a fragmented nucleus. Some cells were totally disintegrated and individual cellular components could no longer be distinguished due to a complete membrane breakdown. Numerous separated components of disintegrated cells were found among the cells (Figure 4).

Discussion

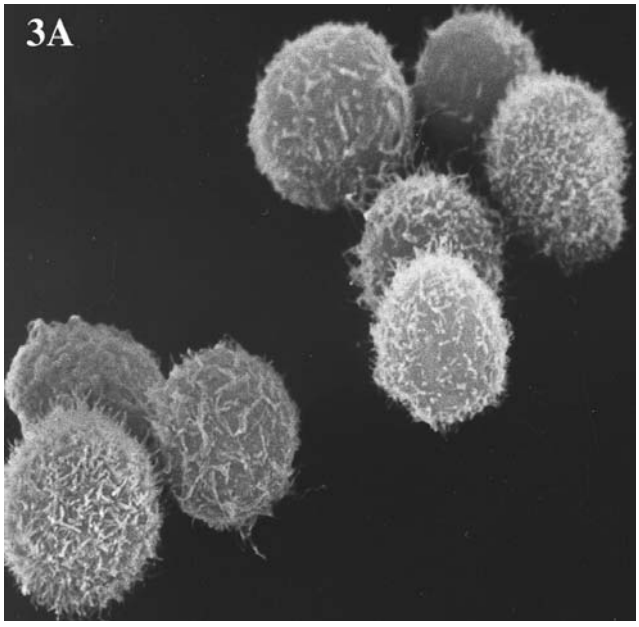
Recently, some drugs such as hematoporphyrin and its derivatives and some of the nonsteroidal anti-inflammatory drugs were found to have the ability to enhance the cell-damaging effect of US irradiation at concentrations at which the chemical alone causes no cell damage (1-6). However, most of them are still far from ideal for clinical use in terms of severe side-effects or high dosage (5,9).

NQ antibiotics are a highly effective class of antibiotics and used clinically as a first choice for combating general bacterial infectious diseases. Some of them show weak side-effects dependent on their structures. Our hypothesis was that NQ antibiotics with functional groups, such as fluorine and methoxyl groups at the C_8 position, could also be promising sonodynamic agents because of their radical formation at the C_8 position under ultrasonic irradiation, followed by a reaction with oxygen to provide the unstable perhydroxyl compounds, leading to generation of singlet oxygen (10-12).

SPFX is one of the NQ antibiotics with the functional group of fluorine at the C_8 position, widely used clinically and causing weak photodermatitis. This compound has the following pharmacological advantages: 1. Great penetration of the dense, largely lipid-containing outer capsule and cell wall; 2. Superior tissue penetration, resulting in levels 2 to 11 times higher than those obtained in plasma; 3. Superior accumulation within macrophages; and 4.Long half-life in plasma in mice, which is also obtained in humans (10). With these excellent pharmocokinetics, SPFX was expected to be not only a useful antimicrobial agent but also a sonodynamic agent.

In the present experiment, after US irradiation at 2 W (0.64 W/cm^2) for 30 seconds, the survival rate of tumor cells was significantly lower than that in the control group. From this result, it seems that the intensity of 2 W was not enough to induce a significant antitumor effect with US alone, however a considerable antitumor effect of US could be achieved at the same power in a shorter period of time in the presence of SPFX.

Recent fine ultrastructural studies described cellular reactions and changes after US irradiation in the presence of a sonodynamic agent. Tachibana *et al.* studied the structure of the surface of HL-60 cells (human promyelocytic leukemia cell) with scanning electron microscopy. Cells exposed to US in the presence of merocyanine 540 (MC 540) showed multiple surface pores and dimple-like craters. In contrast, cells exposed to identical ultrasound conditions in the absence of MC 540 showed none of the above features. Ogawa *et al*. used a high resolution scanning electron microscope and a transmission electron microscope to evaluate the morphological changes of HL-60 after US irradiation in the presence of the photo sensitizing drug MC 540. Exposure of HL-60 cells to US without MC540 resulted in a decrease of finger-like processes in the cells. Cells exposed to US in the presence of MC 540 were spherical in shape and the surface was relatively smooth. Dimple-like craters of various sizes were observed in the surface membrane in many cells. Various sized pores were noted in the cell membranes of more damaged cells (7,8). In these studies, the duration of irradiation was 30 seconds and the intensity of US was 0.4 W/cm^2.



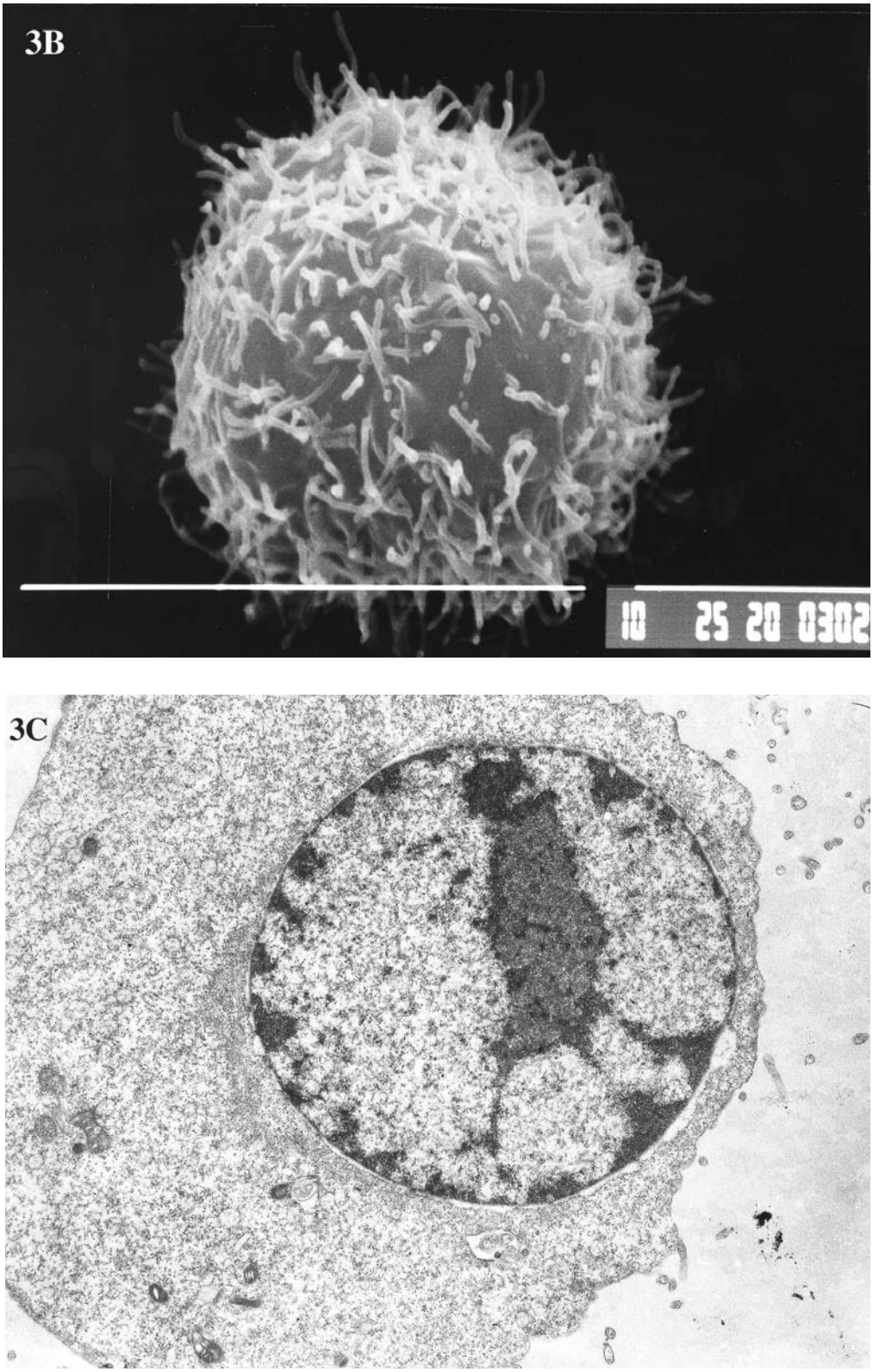
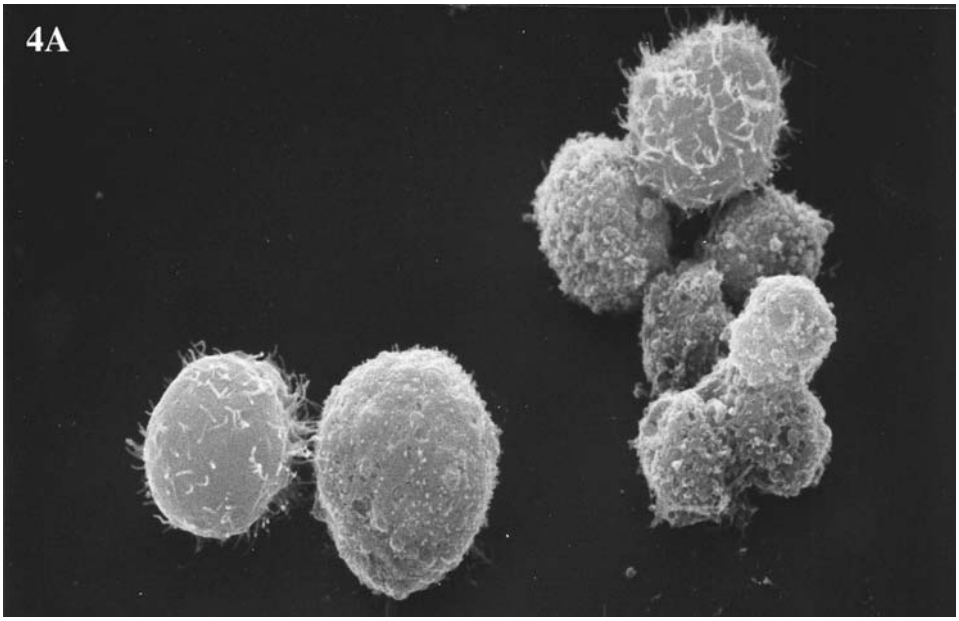
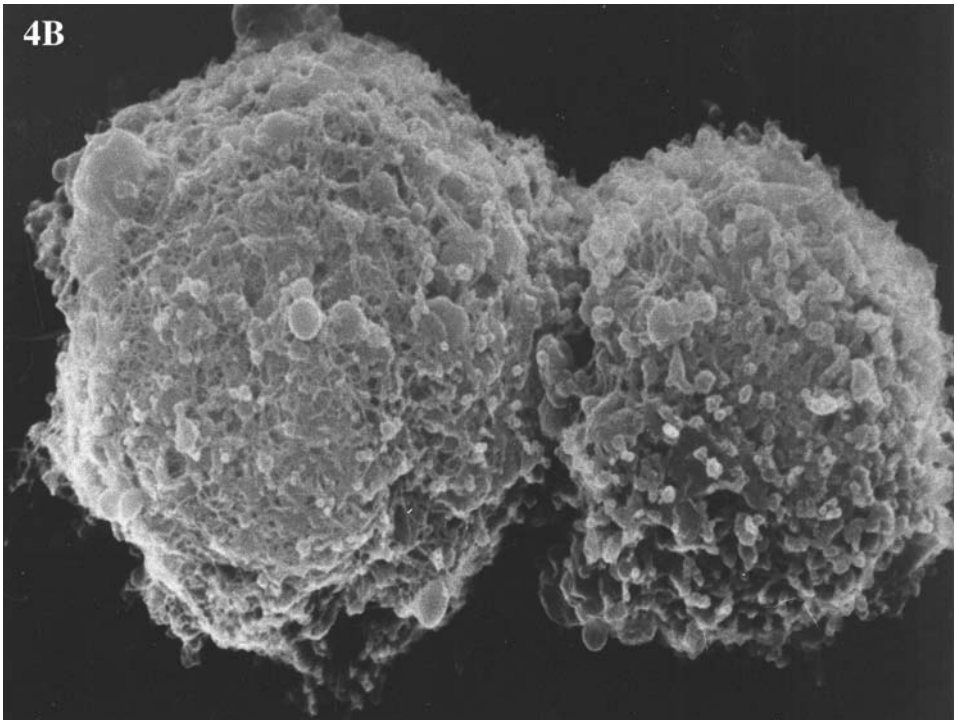


Figure 3. *US-irradiated cells in the control group. A. Most of the cells remained intact and one cell is round without detectable microvilli (original x2000). B. The cell demonstrates a decrease in the number of projected microvilli (original x7500). C. The cell membrane is continuous. However, the cytoplasm became vacuolated and the chromatin was condensed in the nucleus (original x5000).*





To further identify the site and degree of damage to tumor cells by sonication in the presence of a sonodynamic agent, we studied the cell structure with scanning and transmission electron microscopy. Within 30 seconds of US irradiation in the presence of SPFX, the morphological alterations of sarcoma 180 cells were evident. The sizes of the cells varied. The surface membrane in most cells were disrupted and numerous pores or blebs of various sizes were observed in the cell membrane. The ruptured cells tightly adhered to each other and formed clusters. Some cells were totally disintegrated. The separated components of disintegrated cells were observed among cells. However, in the control group, most of the cells remained intact under the same conditions. These findings confirmed the cellular morphological changes seen under light microscope in this and our former studies (1,5) and further support the idea that the cell killing mechanism involves both US and a sonodynamic agent. In the current study, the morphological changes seemed more severe than in the former studies by

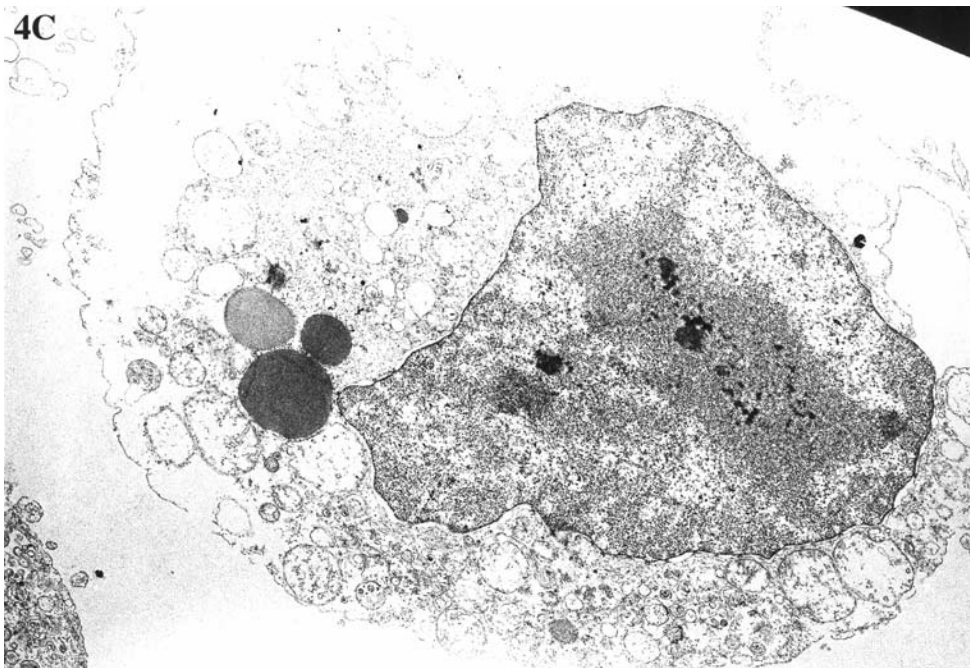


Figure 4. *US-irradiated cells in the SPFX-added group. A. The sizes of the cells varied and disrupted cells adhered to each other (original x2000). B. Numerous pores and blebs of various sizes formed on the cell membrane (original x7500). C. The cell membrane was severely ruptured. The contour of the nucleus was irregular and the chromatin condensed to clumps (original x4000).*

Tachibana *et al*. and Ogawa *et al*. These differences may be due to the different intensity of US, the cell line, the sonodynamic agent and others. In this study, the intensity of US was 2W (0.64 W/cm^2) and the irradiation period was 30 seconds, based on our former studies (1,5).

In conclusion, a synergistic antitumor effect of US in combination with SPFX was observed. Obvious morphological changes or disintegration of tumor cells, especially on the cell membrane, were demonstrated by scanning and transmission electron microscopy.

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