STUDY ON SUPPRESSION MECHANISM OF CANCER CELLS PROLIFERATION BY ULTRASOUND EXPOSURE FOR MINIMALLY INVASIVE CANCER TREATMENT

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Abstract

It had been found that proliferation of cancer cells was suppressed by appropriate ultrasound exposure in our study. We studied on the mechanism of the suppression of proliferation by ultrasound. It was confirmed by agarose gel electrophoresis and measurement method of enzyme activation that apoptosis induction in cancer cells exposed to ultrasound caused to the suppression of the cancer cell proliferation.

Introduction

Noninvasive cancer treatment has been considered to reduce patients' suffering in their treatments and aftereffects by medicines from various points of view. In particular, developments have been made in cancer treatment techniques with photosensitive anticancer agents, gene therapy accompanying changes in the gene characteristics of cancer cells using radiotherapy and hyperthermia, etc. We have studied the effects of ultrasound exposure on cancer cell proliferation and its mechanism [1]-[5]. The cancer cell proliferation rate in the culturing process was suppressed after the cells were exposed to ultrasound. The inertial cavitation generated by ultrasound exposure was verified by electrophoresis and an enzyme activation method to induce apoptosis of the cells.

Experimental procedures

Cancer cells and their proliferation curve

Cancer cells of mouse T lymphoma {EL-4} cultured in a culture medium of RPMI 1640 containing 10% fetal bovine serum were used in this experiment. Figure 1 is a typical photograph of the cells observed with a microscope. When the cancer cells EL-4 are cultured with a culture medium RPMI 1640 in CO_2 incubator, the number of cancer cells increases about 2.5 times in 24 hours. The proliferation curves of the cancer cells EL-4 are shown in Fig. 2.

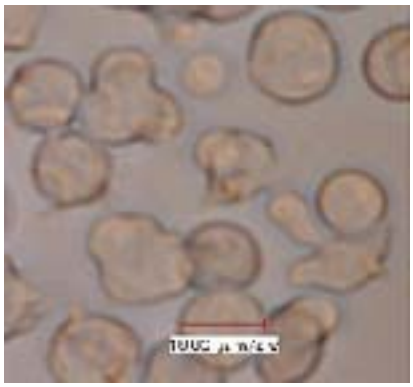

Fig.1 Photograph of cancer cells of mouse T type lymphoma (EL-4) used in this study

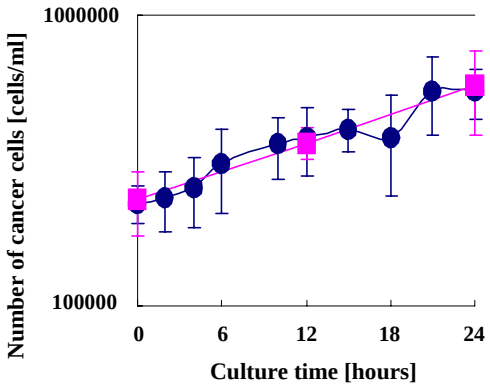

Fig. 2 Proliferation curves of the cancer cells EL-4

Experiment of ultrasound exposure

The ultrasound exposure system shown in Fig. 3 was used to expose the cells {EL-4}. A stainless steel vibrating disk with a Langevin type transducer was attached to the bottom of the water tank in the system using an O-ring. Output signal of function generator was amplified with a power amplifier of gain 50dB. The output signal was a continuous wave with an amplitude of 200mV and a frequency of 150 kHz. The amplified signal was applied to the transducer. The transducer had a nominal resonance frequency of 40kHz. However, a stacked piezoelectric ceramic vibrator with a resonance frequency of 150kHz was employed in the transducer. The distance between the bottom of the culturing flask with the cells and the

acoustic radiating surface of the transducer was 30 mm. Temperature in the water tank of the ultrasound exposure system was kept at 37 ℃. The ultrasound exposure times to the cells were 0, 10 and 15 minutes. The cells in the culturing flask after being exposed to ultrasound were cultured in a CO_2 incubator {temperature : 37 ℃, humidity : 100%, CO_2 concentration: 5%}.

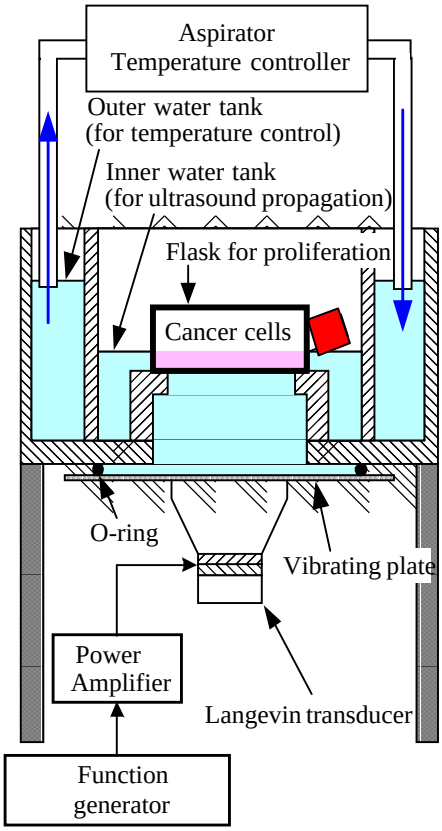

Fig.3 Basic structure of ultrasound exposure system for suppression of cancer cells proliferation

Agarose gel electrophoresis

Biological changes occurred in the DNA of cancer cells exposed to ultrasound were observed by agarose gel electrophoresis. This method is one of the most common methods to confirm the apoptosis induced in cells. Figure 4 is a schematic diagram of the apparatus for agarose gel electrophoresis.

An agarose gel block was immersed in the tank of the electrophoresis apparatus filled with buffer solution. DNA samples of cancer cells were injected in the pits called well at a side of the agarose gel block, and the voltage was applied at each electrode of the gel. DNA in the apoptosis induced cells were segmented. The segmented DNA fragments were moved from the cathode to the anode in the gel due to the electric charge.

Smaller sized fragments of segmented DNA move a longer distance. As the DNA in the apoptosis induced cells were segmented to the DNA fragments with the molecular size of integral multiple of 180 bp to 200 bp, the moved distances of the DNA fragments were integral multiple of the particular constant distance determined by the fundamental molecular size of segmented DNA fragments. Cyclic ladder patterns could therefore be seen at the positions with DNA fragments in the gel, as the segmented DNA fragments in the gel fluoresced with UV exposure. This ladder patterns are generally used as the evidence of apoptosis induction in cells in the field of biology.

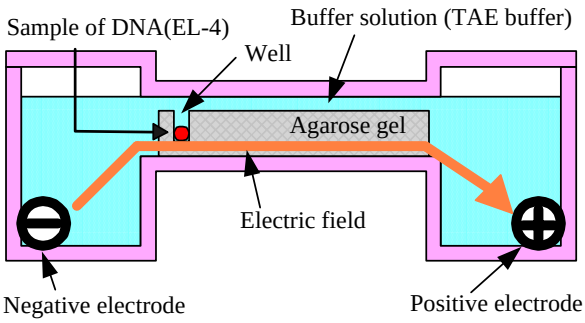

Fig. 4 Apparatus for agarose gel electrophoresis

Measurement method of enzyme activation

DNA in cancer cells are segmented to DNA fragments when DNA is activated by some external factors and apoptosis enzymes. Caspase 3 is one of the apoptosis activating enzyme. Apoptosis in cancer cells induced by ultrasound exposure is verified by detecting the enzyme of caspase 3. Activation of caspase3 is detected with a fluorescent substrate called Ac-Asp-Glu-Val-Asp-MCA. The peak wave-length in the spectrum of a chromophore MCA in the substrate (methylcholanthrene) is about 400 nm. The peak wavelength in the spectrum shifts from about 400 nm to about 440 nm when MCA is hydrolyzed by the activation of caspase3 to give free AMC (aminomethylcoumarin). Then, the activation of caspase3 can be detected by measuring peak wavelength shift under UV exposure (wavelength : 325 nm). Apoptosis in cancer cells induced by

ultrasound exposure can be confirmed by detecting the activation of caspase3.

Results and Discussion

Morphological observation

Figure 5 shows a photograph of cancer cells taken under a digital microscope after being exposed to ultrasound. Although no remarkable change in the shape of the cells was observed just after the cells were exposed to ultrasound, the cells shrunk gradually with increasing culturing time.

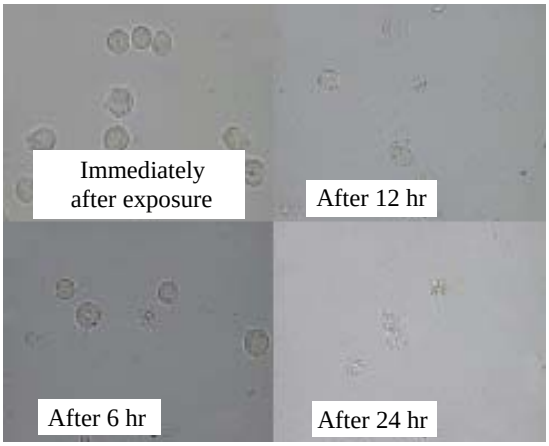

Fig.5 Photograph with digital microscope of changing shapes of cancer cells (EL-4) after ultrasound exposure

Figure 6 shows the proliferation curves of cancer cells. The number of cancer cells exposed to ultrasound was confirmed to decrease with increasing culturing time.

Since quality degradation of culturing medium by sonochemical reactions is considered to cause this phenomenon, the cancer cells were cultured in the culture medium exposed to ultrasound before culturing in the same conditions as those used in Fig. 6. No such phenomenon was observed in Fig. 7.

When necrosis occurred in the cancer cells exposed to ultrasound exposure, the cells were broken and the number of the cells decreased immediately after ultrasound exposure. However, the shape and number of the cells showed no change just after ultrasound exposure. The cells shrunk and the increase in the number was suppressed as a function of time after they were exposed to ultrasound as shown in Figs. 5 and 6. Apoptosis may contribute to this effect

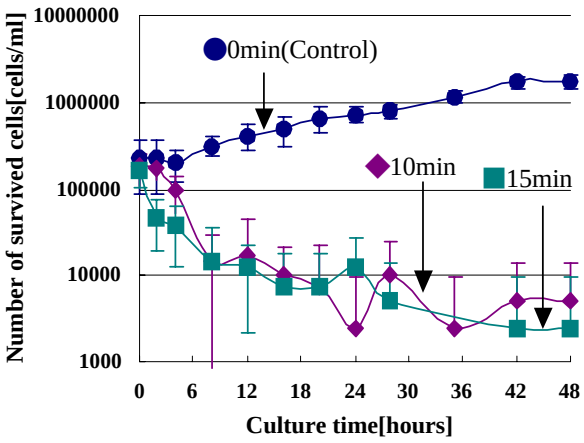

Fig.6 Relationship between culturing time and number of survived cells exposed to continuous ultrasound

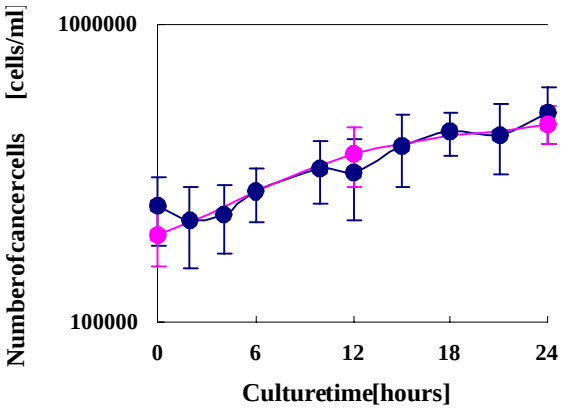

Fig.7 Proliferation curve of cancer cells cultured in culturing medium exposed to ultrasound

Confirmation of apoptosis with electrophoresis

A 2.0% agarose gel was used in the electrophoresis measurement. Figure 8 shows the results on the cancer cells cultured 8 hours after ultrasound exposure.

DNA molecules moved in the network structure of agarose gel in the electric field between the anode and cathode of the apparatus. DNA molecules with smaller sizes have a higher movility in agarose gel at the same applied voltage.This phenomenon is callled the molecular sieve effect. Typical segmented DNA fragments by apoptosis were detected using the molecular sieve effect. DNA molecules were segmented to smaller fragments with sizes of integral multiple of 180 bp to 200 bp. Typical ladder petterns of segmented DNA fragments were then shown on the agarose gel with apotosis induced cells. The electrophoretogram of cancer cells unexposed to

ultrasound is shown as the control on the lane 1 in Fig. 6. The electrophoretogram of the cells exposed to ultrasound for 15 minutes is shown on the lane 1 in Fig.8. Ladder patterns were obseved on the lane 2. Thus, apoptosis was verified to be induced in cancer cells exposed to ultrasound.

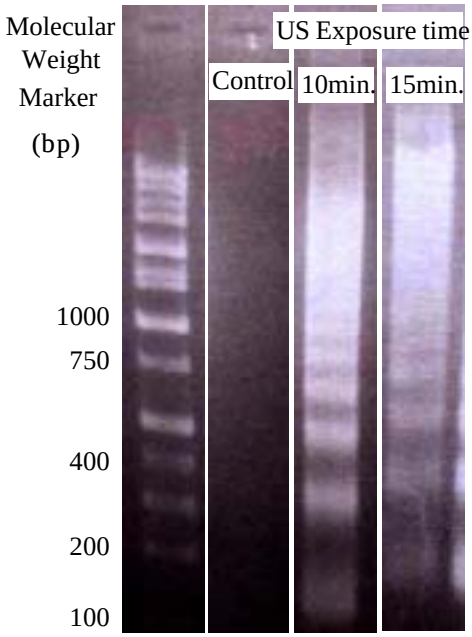

Fig.8 Electrophoretogram after 6hours incubation of this experiment showing apoptosis on cancer cells by ultrasound exposure

Confirmation of apoptosis with enzyme activation

We tried to detect activation of the enzyme (caspase3) to confirm apoptosis induction in the cancer cells exposed to ultrasound. The cells exposed for 15 min. were cultured for 2 hours. The fluorescence spectrum of the cells exposed to ultrasound was compared with that of the unexposed control cells.

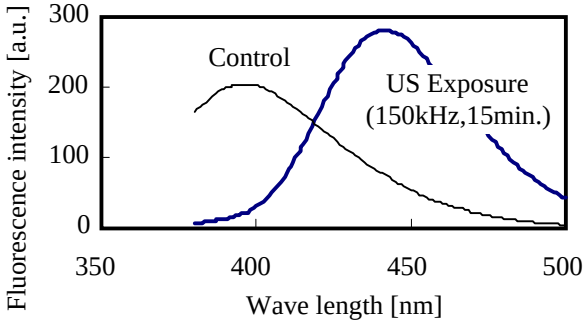

Fig. 9 Change of fluorescence spectrum by activation of enzyme caspase3 with apoptosis induction in cancer cells exposed to ultrasound

The peak wavelength of the fluorescence spectrum shifted from 400nm to 440nm as shown in Fig. 9. This result shows activation of the apoptosis enzyme caspae3. Consequently, the cancer cells were verified to dye from apoptosis induced by ultrasound exposure.

Conclusion

Cancer cells cultured in a flask with a culturing medium were exposed to a standing wave acoustic field of ultrasound formed in a water tank using a stainless steel vibrating disk. The vibrating disk was driven by a bolt clamped Langevin type transducer with a resonant frequency of 40 kHz. Continuous sinusoidal signal with a frequency of 150 kHz and an amplitude of about 60 V was applied to the transducer. Exposure to ultrasound of cancer cells for 10 and 15 minutes suppressed cell proliferation in the culturing process after the exposure. The causes of the observed remarkable suppression of cancer cell proliferation by ultrasound exposure were investigated by morphological observations, agarose gel electrophoresis and activation of the apoptosis enzyme of caspase 3. The suppression of cancer cell proliferation with ultrasound exposure was considered to be caused by apoptosis induction with segmented DNA in cancer cells.

Reference

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