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Consideration on suppression of cancer cell proliferation by ultrasound exposure using sonochemical and biological measurements

A Watanabe, H Nishimura, N Kawashima and S Takeuchi[1]
1614 Kurogane-cho, Aoba-ku, Yokohama-city, Kanagawa, 225-8502, JAPAN
shin1@cc.toin.ac.jp

Abstract. The suppression methods of cancer cells proliferation using ultrasound exposure are investigated to develop a new minimally invasive cancer treatment method. A stainless steel vibrating plate with a Langevin type transducer is attached to the bottom of a water tank of the ultrasound exposure system used in this study. Ultrasound was irradiated to cancer cells of mouse T lymphoma (EL-4) in a flask. A decreasing tendency of the number of viable cancer cells exposed to ultrasound of 150 kHz and acoustic intensity I_{SPTP} of 750 mW/cm^2 was confirmed in the culturing process. Then, the suppression mechanism of cancer cell proliferation by ultrasound exposure was considered through confirmation of apoptosis and necrosis with the exposed cancer cells by electrophoresis and enzyme activity measurements. It was found that the apoptosis was induced on the cancer cells after ultrasound exposure. We confirmed the generation of hydroxyl radical in water in the water tank by ESR device. When the hydroxyl radicals were scavenged by adding ethanol to the culture medium for cancer cells, the apoptosis was not induced and proliferation was not suppressed. Therefore, we found that generation of activated oxygen in the culturing medium by ultrasound exposure was caused to apoptosis induction and suppression of cancer cell proliferation. We will present the results of above consideration in this conference.

1. Introduction

The methods of suppressing cancer cell proliferation such as sonodynamic therapy (SDT) [1]-[5] and high-frequency focused ultrasound [6] have been studied actively for application to minimally invasive cancer treatments. We have studied a method of suppressing cancer cell proliferation by apoptosis induction in cancer cells using only ultrasound without anticancer agents. We have investigated the effects of ultrasound exposure on the proliferation properties of cancer cells [7], [8]. We also have proposed an ultrasound exposure system using a balloon and an ultrasound horn for a minimally invasive cancer treatment [9]. It was proved in this study that apoptosis was induced in cancer cells by hydroxyl radicals which are generated by ultrasound exposure, resulting

in the suppression of cancer cell proliferation.

2. Experiments

2.1 Cancer cells

Cancer cells of a mouse T lymphoma (EL-4) cultured in RPMI 1640 medium containing 10% fetal bovine serum were used in this experiment. This cancer cell is shown in Fig. 1. This is one of typical cancer cell for studies because easy to treat. The number of cancer cells increased approximately 2.5 times after 24 h incubation in a CO_2 incubator. The proliferation curves of the cancer cells are shown in Fig. 2.

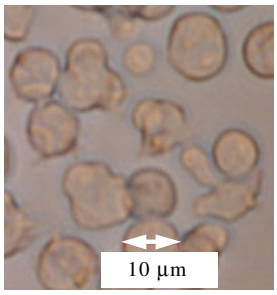

Fig. 1 Cancer cells used in this study (EL-4)

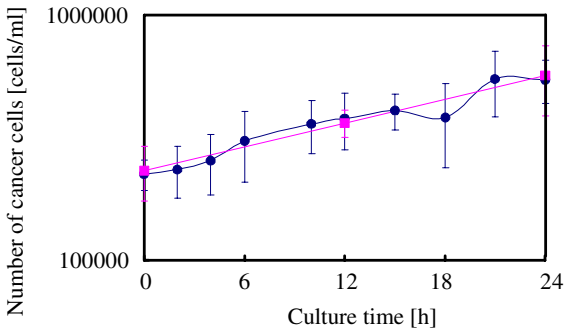

Fig. 2 Proliferation curves of cancer cells (EL-4) in a RPMI1640 medium in CO2 incubator.

2.2 Ultrasound exposure

Cancer cells (EL-4) were exposed to ultrasound using the ultrasound exposure system shown in photographs in Fig. 3. A stainless-steel diaphragm (160 mm in diameter, 2 mm in thickness) with a 40 kHz Langevin-type transducer (HEC-45402 Honda Electronics Co., LTD.) was attached to the bottom of a water tank. An output signal (continuous sinusoidal wave with an amplitude of 200 mV) from a function generator (HP 8116A) was amplified by a power amplifier (ENI 2100L) with a gain of 50 dB and applied to the Langevin-type transducer.

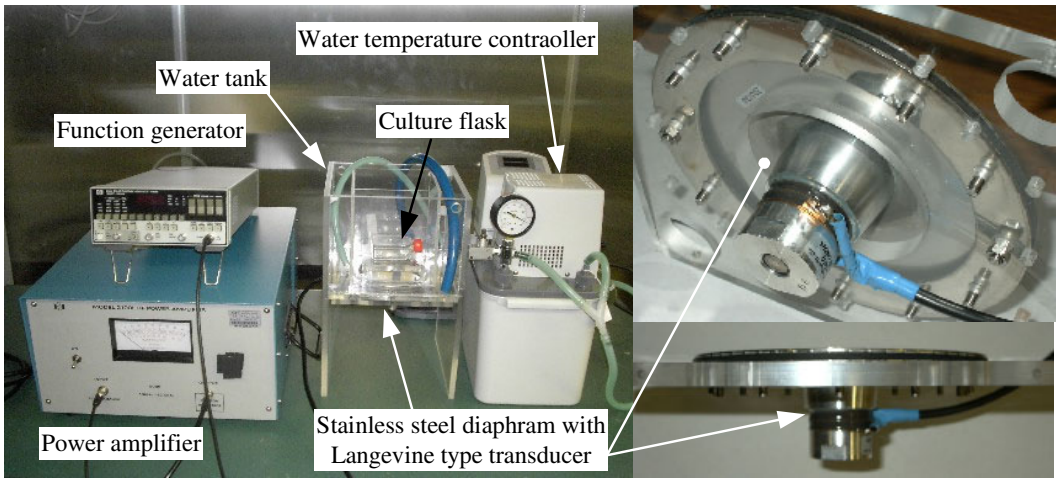

Fig. 3 Ultrasound exposure system for suppression of cancer cell proliferation used in this study.

We intended to suppress cancer cell proliferation by acoustic cavitation and sonochemical reaction such as the generation of active oxygen. Therefore, the frequency characteristics of the intensity of sonochemical luminescence were measured to determine the ultrasound exposure conditions for efficient generation of active oxygen using our ultrasound exposure system. Sonochemical luminescence is one of the typical sonochemical phenomenon. The intensity of sonochemical luminescence was measured in luminol solution in the water tank using a photomultiplier. As a result, highest intensity of light emission was observed at 150 kHz despite using the 40 kHz Langevin-type transducer. Therefore, ultrasound was irradiates to cancer cells using ultrasound exposure system driven at 150 kHz in this study. The maximum sound pressure in the water tank was about 150 kPa. It was difficult to measure the sound pressure accurately, because acoustic cavitation was generated and a standing wave acoustic field was formed. Therefore, this value is shown for reference. The maximum sound pressure of 150 kPa corresponded to the peak acoustic intensity (I_{SPTP}) of 750 mW/cm^2. Temperature in the water tank was maintained at 37 degrees in centigrade. The distance between the bottom of the culture flask with cancer cells and the acoustic radiation surface of the stainless-steel diaphragm was set at 30mm. The ultrasound exposure times of cancer cells were 0 min, 10 min and 15 min. Cancer cells in the culture flask were cultured in the CO_2 incubator after ultrasound exposure and the number of viable cells was counted.

2.3 Biochemical consideration

We investigated biochemical changes that occurred in cancer cells by agarose gel electrophoresis and enzyme activity measurement. These are the most general methods of confirming apoptosis induction in cells.

An agarose gel block was immersed in an electrophoresis tank filled with buffer solution. The extracted DNA molecules from cancer cells were loaded into the wells on one side of the agarose gel block. Then, a voltage was applied to electrodes on each side of the agarose gel. When apoptosis was induced in the cancer cells, the DNA molecules of these cancer cells become fragmented. The fragmented DNA molecules migrated from the cathode to the anode in the agarose gel due to an electric field. Small-size fragmented DNA molecules migrate a longer distance. When apoptosis was induced in cancer cells, the DNA molecules of these cancer cells were segmented to DNA fragments with molecular sizes of integral multiples of 180 bp (base pairs) to 200 bp. Thus, the distances the DNA fragments migrated were integral multiples of a particular constant distance determined on the basis of the fundamental molecular sizes of the DNA fragments. The cyclic ladder patterns were observed on the agarose gel, as the DNA fragments in the agarose gel were fluoresced by UV exposure. These ladder patterns are generally used as the evidence of apoptosis induction in biochemistry.

DNA molecules from cancer cells are fragmented when apoptosis enzymes are activated by some external stimuli. Caspase-3 is one of the apoptosis enzymes. In this study, apoptosis induced in cancer cells was investigated by detecting activation of caspase-3. The activation of caspase-3 was detected using a fluorescent substrate called Ac-Asp-Glu-Val-Asp-MCA. A peak was observed at a wavelength of about 400 nm in the fluorescence spectrum of chromophore 4-methyl-coumaryl-7-amide (MCA), exposed to UV with an excitation wavelength of 325 nm. The peak in the fluorescence spectrum shifted from a wavelength of about 400 nm to about 440 nm, when MCA was hydrolyzed to 7-amino-4-methyl-coumarin (AMC) by the activation of caspase-3. Therefore, the activation of caspase-3 can be detected by measuring the peak shift, and apoptosis induced in cancer cells by ultrasound exposure can be confirmed by detecting the activation of caspase-3.

2.4 Sonochemical consideration

In this study, we considered the effects of active oxygen generated together with acoustic cavitation by ultrasound on cancer cells. The generation of active oxygen was investigated by the spin trap method using an electron spin resonance (ESR) device (JEOL JES-FA200) and a spin trap agent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). Active oxygen (hydroxyl radical) has a very short lifetime. Therefore, we should trap active oxygen by a spin trap agent such as DMPO. When a hydroxyl radical is generated, it can be trapped by DMPO and DMPO-OH is generated. DMPO-OH has a longer lifetime than the hydroxyl radical. The concentration of hydroxyl radicals generated in water in the culture flask can be estimated by comparing the ESR spectrum of DMPO-OH and that of TEMPOL used as a standard. Ethanol is known as a scavenger for hydroxyl radicals. The ESR spectrum was measured in water with 50 µl of ethanol (99.5%) with ultrasound exposure. The cancer cells in the culture medium with ethanol were exposed to ultrasound and measurements of the proliferation curve and electrophoresis were accomplished. We considered the effects of hydroxyl radicals on apoptosis induction in cancer cells and on the suppression of cancer cell proliferation.

3. Results and Discussion

3.1 Proliferation curve

Figure 4 shows the proliferation curves of cancer cells with ultrasound exposure for 10 and 15 min and without exposure as control. The number of cancer cells exposed to ultrasound decreased with culture time.

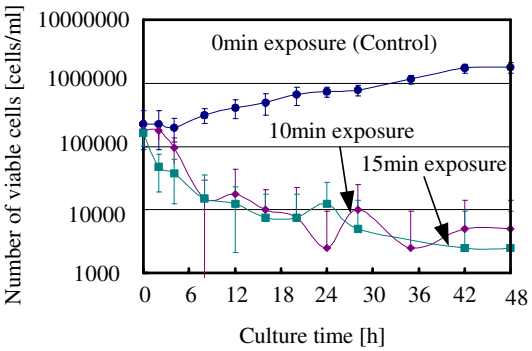

Fig. 4 Proliferation curves of cancer cells in culture medium exposed to ultrasound before culturing.

3.2 Biochemical consideration of apoptosis induction

Figure 5 shows the relationship between the lapse of time after 15 min ultrasound exposure and ladder patterns on the agarose gel electrophoresis. The results show that apoptosis started 2 h after ultrasound exposure and finished at about 12 h after exposure. We attempted to detect the activation of caspase-3 to confirm apoptosis induction in cancer cells exposed to ultrasound. Cancer cells exposed for 15 min were cultured for 2 h. The fluorescence spectrum of cancer cells exposed to ultrasound was compared with that of unexposed control cells. The peak in the fluorescence spectrum shifted from a wavelength of 400nm to 440nm, as shown in Fig. 6. This result shows the activation of caspase-3 in cancer cells exposed to ultrasound. Consequently, apoptosis induced by ultrasound exposure was verified by this method.

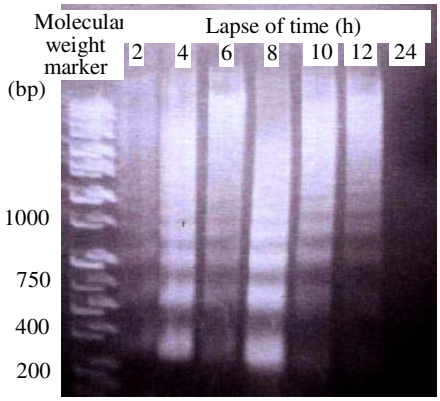

Fig. 5 Confirmation of apoptosis induction in cancer cells with 15 min ultrasound exposure by electrophoresis.

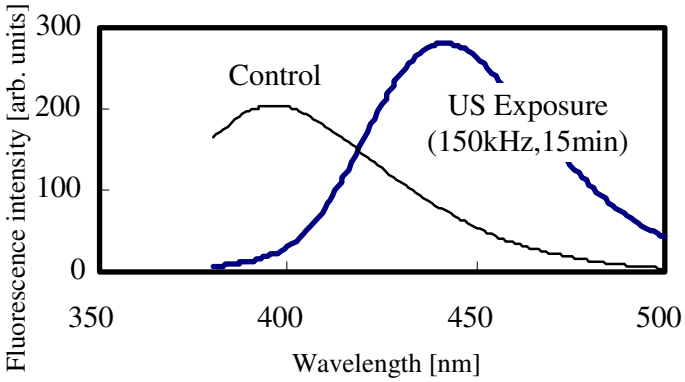

Fig. 6 Change in fluorescence spectrum by activation of enzyme caspase-3 with apoptosis induction in cancer cells exposed to ultrasound

3.3 Sonochemical confirmation of apoptosis

We considered the effects of active oxygen generated together with acoustic cavitation by ultrasound on cancer cells. A typical ESR spectrum of DMPO-OH can be observed in Fig. 7. It was confirmed by comparison with Fig. 7 and the ESR spectrum of TEMPOL as the standard that the concentration of hydroxyl radicals generated in water in the culture flask is about 10 mM.

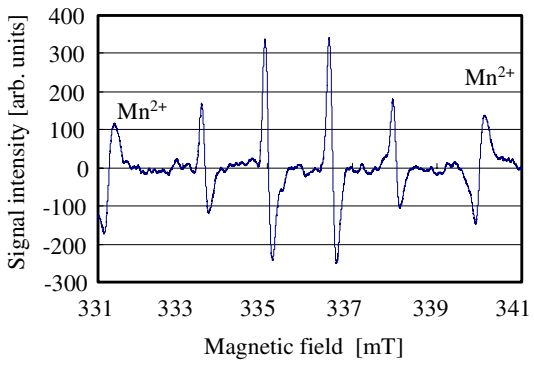

Fig. 7 ESR spectrum of DMPO-OH measured in water in culturing flask with 15 min ultrasound exposure.

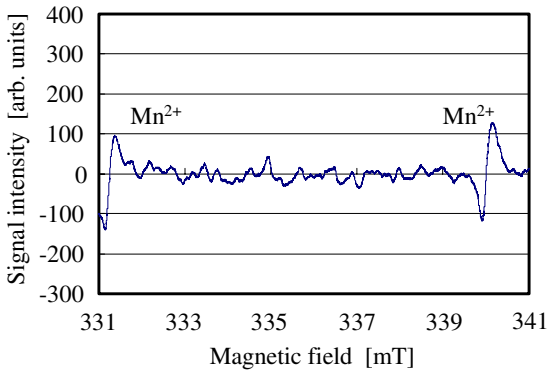

Fig. 8 ESR spectrum of DMPO-OH measured in water with ethanol in culturing flask with 15 min ultrasound exposure.

Ethanol is known as a scavenger for hydroxyl radicals. The ESR spectrum was measured in water with 50 µl of ethanol (99.5%) with ultrasound exposure. Figure 8 shows that a typical ESR spectrum pattern of DMPO-OH disappeared from the measured data. Therefore, 50 µl of ethanol was added into the culture medium in the culture flask to scavenge the active oxygen of hydroxyl radicals which was generated by ultrasound exposure. The proliferation curve of cancer cells in the above-mentioned culture medium was measured after ultrasound exposure. We obtained a proliferation curve with a tendency similar to that of a normal proliferation curve. The number of the above-mentioned cancer cells increased about 2.5 times after 24 h incubation. Thus, it was confirmed that the proliferation of the above-mentioned cancer cells is not suppressed by ultrasound exposure

in the culture medium with 50 µl of ethanol. Furthermore, apoptosis induction in the above-mentioned cancer cells was investigated by agarose gel electrophoresis. When the generation of hydroxyl radicals was inhibited by ethanol, we could not observe the typical ladder pattern on the agarose gel block caused by apoptosis induction.

The results are summarized as follows. When cancer cells in the above-mentioned culture medium were exposed to ultrasound, hydroxyl radicals were generated and DNA molecules from cancer cells became segmented because of the hydroxyl radical. Then, apoptosis was induced in cancer cells and the proliferation of cancer cells was suppressed.

4. Conclusions

An ultrasound exposure system with a stainless-steel diaphragm driven by a Langevin-type transducer was developed. Cancer cells (EL-4) in culture medium in a culture flask were exposed to ultrasound in the water tank of the ultrasound exposure system filled with water. A standing wave acoustic field with a maximum acoustic pressure of about 150 kPa and a temporal peak spatial peak sound intensity (I_{SPTP}) of about 750 mW/cm^2 was formed by illuminating a 150 kHz continuous sinusoidal ultrasound wave. Electrical input power to the Langevin-type transducer was about 10W. Active oxygen of a hydroxyl radical was generated together with acoustic cavitation in the acoustic field. Cancer cells (EL-4) were exposed to such an acoustic field for 10 and 15 min. It was confirmed by agarose gel electrophoresis and enzyme activity measurement that apoptosis could be induced in cancer cells. It was found that the proliferation of cancer cells exposed to ultrasound is markedly suppressed by apoptosis induction in the culturing process after ultrasound exposure.

Furthermore, when the same experiments were performed in the culture medium with ethanol as a scavenger for hydroxyl radicals, apoptosis was not induced and the proliferation of cancer cells was not suppressed. Therefore, it can be considered that active oxygen of a hydroxyl radical plays an important role in apoptosis induction and in the suppression of proliferation by ultrasound exposure.

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