Mechanism of Anti-tumor Effect of Combination of Bleomycin and Shock Waves

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We have previously reported marked enhancement of the cytoidal effect of bleomycin (BLM) on cancer cell suspensions in vitro by the combination with shock waves. In this study, we evaluated the synergistic effects on cancer cell proliferation and apoptosis in solid tumors. A spherical piezoceramic element was used as the shock wave source, with a pressure peak of 40 MPa. A human colon cancer cell line, SW480 was implanted onto the back of nude mice. Two thousand shock waves were administered to the tumor immediately following an intravenous injection of BLM at a dose of one-tenth of the LD50. The tumor was extirpated at 3, 6, 12, 24, 72 h and 1 week following shock exposure. Cell proliferation and apoptosis were detected by Ki-67 using antibody MIB-1 and by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) method. The lowest percentage (35.7%) of Ki-67-positive cells appeared 24 h following the treatment. The maximum apoptotic index was detected within 6 h following the treatment. Moreover, numerous large cells with enlarged nuclei were detected histologically. These results suggest that shock waves may enhance chemotherapeutic effects by increasing apoptosis and decreasing cell proliferation in the tumor tissue.

Key words: Shock wave — Bleomycin — SW480 — TUNEL — Mitotic death

A focused underwater shock wave can exert a high acoustic intensity in deep portions of the body without causing damage to adjacent tissues because of its high permeability through the tissues.1–3 The apparent feasibility of exposing a spatially limited region of the body to a potentially destructive form of mechanical energy has led to the idea of applying shock waves in tumor therapy.4–9 Previous results have not necessarily been promising, in cases of shock wave therapy alone,4–8 or in combination with various anti-cancer agents, for example, cis-platinum,9–13 mitomycin C (MMC),12, 13) actinomycin D,12) methotrexete13) and Adriamycin (ADR).12) However, we have reported that the combination of focused shock waves and bleomycin (BLM) reduced the IC50 of BLM to 1/10 000–1/100 000 in various human cancer cell lines, when compared to BLM alone.15, 16) This effect was detected even with a weak shock wave energy, which alone had almost no cytotoxic effect, and the degree of cytotoxicity enhancement was proportional to the amount of shock wave energy applied.15, 16) Immediately after shock wave exposure, cancer cells were examined under scanning and transmission electron microscopes. Numerous dimples (diameters distributed from 0.05 to 0.5 µm) became apparent on the cell surface.15, 16) These dimples were concluded to be pores penetrating through the cell membrane, because reagents such as propidium iodide and 5(6)-carboxyfluorescein could enter the cytoplasm of the cells treated with shock waves. The mechanism of this enhancement of chemotherapeutic effect was considered to be the entry of BLM molecules into cells through pores opened by the shock waves, but the details remain unknown.

When SW480, a human colon cancer cell line, was transplanted onto the back of nude mice, which were treated with a combination of intravenously (i.v.) injected BLM and regional exposure to shock waves, a significant enhancement in the chemotherapeutic effects was also detected in terms of tumor growth.15, 16) Therefore, in this study, we examined the synergistic effect on cancer cell proliferation and apoptosis in vivo using immunohistochemical staining with MIB-1 and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labelling (TUNEL) method, as well as transmission electron microscopy, in an effort to elucidate the mechanism involved.

MATERIALS AND METHODS

Mice Male BALB/c nude mice, 5 weeks of age, weighing 16–20 g, were purchased from CLEA Japan Inc. (Tokyo). Cells SW480 cells, histologically demonstrating adenocarcinoma features, derived from human colon cancer17) were obtained from the Cell Resource Center for Bimedi-
When tumors that were translocated at the focal area (Fig. 1). Degassed water and the mice were fixed so the tumor was reflected. A rubber bag filled with degassed water and is focused at the geometric center. An anesthetized mouse was fixed so its tumor was located at the focal area. The interface between the rubber bag and the skin of the mouse was dabbed with acoustic gel to adapt the acoustic impedance.

Shock wave exposure When tumors that were transplanted s.c. into nude mice had grown to approximately $1 \times 10^6$ SW480 cells were injected subcutaneously (s.c.) into the backs of nude mice to grow tumors.

Anticancer agent Water-soluble Bleomycin (BLM) was supplied by Nihon Kayaku Co., Ltd. (Tokyo).

Shock wave generator The shock wave generator was designed to generate focused underwater shock waves at various frequencies and intensities by Toshiba Co., Ltd. (Tokyo). This instrument is comprised of a high-voltage electric current generator and a reflector. On the surface of the generator, piezo-ceramic elements were arranged to form part of a sphere. The pressure on the focal area (the center of the sphere) was proportional to the voltage applied to the elements. For in vivo experiments, the reflector was covered with a rubber bag filled with degassed water and the mice were fixed so the tumor was located at the focal area (Fig. 1).

Shock wave exposure When tumors that were transplanted s.c. into nude mice had grown to approximately $10 \times 10^3$, shock wave exposure and administration of BLM were performed following anesthesia with intraperitoneal pentobarbital sodium (40 mg/kg). The BLM dose was one-tenth of the LD$_{50}$ and the dose of the shock wave was 2000 shots at 40 MPa. For the combination treatment, BLM was administered i.v. just before exposure to the shock waves.

Tumors were extirpated 3, 6, 12, 24, 72 h and 7 days after the treatment. Each group consisted of at least 4 mice. Fixation was performed using 4% paraformaldehyde overnight.

Autoradiography Cobalt-57 was obtained as carrier-free $^{57}$CoCl$_2$ (37 MBq/ml) in 0.1 N HCl from the Japan Radioisotope Association (Tokyo). The labeling procedure has been described by Grove et al. The final concentration of $^{57}$Co-BLM was adjusted to 300 kBq/ml, 3 mg/ml. These labeled BLM complexes were injected into the tumor-bearing mouse i.v. (BLM 1 mg, 100 kBq/mouse). Immediately after the injection of $^{57}$Co-BLM, the tumor was exposed to 2000 shock wave shots at 20 MPa. A control mouse was treated with $^{57}$Co-BLM (i.v.) alone. Ten minutes after the injection, the mice were sacrificed and prepared for whole body autoradiography according to the method established by Ullberg. Mice were sliced into 35 µm sections and exposed to an imaging plate (Fuji Photo Film Co., Ltd., Tokyo) for 3 h, and then analyzed with a Bio-imaging analyzer, BAS 2000 (Fuji Photo Film Co., Ltd.).

Morphological analysis The paraffin-embedded blocks, fixed in 4% paraformaldehyde, were cut into 4 µm sections. One section from each sample was stained with hematoxylin and eosin (HE). For electron microscopy, treated tumors were fixed with 2% glutaraldehyde for 1 h, dehydrated, processed and observed using a H-600 (Hitachi Co., Ltd., Tokyo) electron microscope.

Immunohistochemistry Analysis of the synergistic effect of shock waves and BLM on cell proliferation was performed using a Ki-67 immunostain, employing MIB-1 antibody (Immunotech, Marseille, France) as the primary antibody. Tissue sections (4 µm), which were cut from 4% paraformaldehyde-fixed, paraffin-embedded blocks, were deparaffinized and autoclaved in 10 mM acetate buffer at 120°C for 5 min. After cooling, slides were incubated overnight at 4°C with a 1:50 dilution of the primary antibody MIB-1. The avidin-biotin-peroxidase complex method was used for immunohistochemical staining. Slides were incubated with the biotinylated anti-immunglobulins for 20 min at room temperature. The streptavidin-conjugated peroxidase was applied to the slides for 20 min, followed by exposure to 0.05% diaminobenzidine (DAB). Following immunostaining, sections were counterstained with hematoxylin and coverslipped with an aqueous mounting medium.

In situ detection of apoptosis To detect apoptotic cells in sections of paraformaldehyde-fixed, paraffin-embedded tissue, TUNEL method was used according to the procedures included in the Wako in situ apoptosis detection kit.

Fig. 1. Experimental apparatus for in vivo shock wave exposure. Piezo-ceramic elements were arranged in partial spherical form, and the generated shock wave propagates inside the rubber bag filled with degassed water and is focused at the geometric center. An anesthetized mouse was fixed so its tumor was located at the focal area. The interface between the rubber bag and the skin of the mouse was dabbed with acoustic gel to adapt the acoustic impedance.
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(Wako Co., Ltd., Osaka). Briefly, after deparaffinization, sections were incubated with a protein digestion enzyme for 5 min at 37°C. TdT with biotin-11-dUTP and dATP was applied to the slides for 10 min at 37°C in a moist chamber. Endogenous peroxidase was inactivated by covering the sections with 3% hydrogen peroxide for 5 min at room temperature. Then, anti-biotin-11-dUTP labeling was conducted for 10 min at 37°C, followed by exposure to DAB. Sections were counterstained with 0.5% methyl green solution. One thousand cells were counted in a visual field at random, and the average of four fields was used to determine the apoptotic index.

RESULTS

Introduction of ⁵⁷Co-BLM into the tumor analyzed by autoradiography

We performed autoradiography as a preliminary study to examine the difference of accumulation of labeled BLM between shock wave-treated mouse and the control. Significant accumulation of ⁵⁷Co-BLM was detected in the liver, bone marrow, skin, and lung 10 min following the i.v. injection. However, accumulation in the tumor was not marked (Fig. 2A). In the tumor exposed to shock waves, a two-fold higher level of radiation was detected compared to the control. The level of radioactivity in the tumor was divided by that of the liver in the same body to calculate relative radioactivity. Relative radioactivity of the tumor exposed to shock waves was 1.7-fold higher than that of the control (Fig. 2B).

Time course of suppression of cell proliferation caused by BLM with shock waves

The proliferation of SW480 was suppressed following shock wave exposure with BLM administration, based on counting of the positive ratio of Ki-67 expression (Fig. 3, A and B). The suppression began just following the treatment (closed circle, Fig. 3A) and was maintained until almost 24 h after treatment. The percentage of Ki-67 positive cells, which was 68.7% at beginning of treatment, reached a minimum of 35.7% after a time period corresponding roughly to the doubling time. Our findings show that the Ki-67-positive ratio decreased until 24 h and then gradually increased over the next 7 days.

Time course of apoptosis after exposure to shock waves with BLM

The percentage of TUNEL-positive cells, which was 1.22% at the beginning of treatment, reached a maximum of 6.21% at 6 h following the combined treatment with shock waves and BLM (Fig. 4A). We also analyzed the expression of an apoptotic cell marker 6 h following the treatment in four groups, control, BLM alone, shock waves alone and combination of shock waves and BLM (Fig. 4B). The apoptotic index in the combined therapy group was significantly higher than that of the other three groups (Fig. 4C).

Morphological analysis

SW480 tumor cells showed great morphological variation 6 h following combined treatment. There were many enlarged cells with a diameter more than twice the normal cell diameter (Fig. 5A). Some were binucleated and showed micronuclei (Fig. 5B), while others displayed arrest in mitosis (Fig. 5A). Electron
Fig. 3. (A) Positive rate of Ki-67 immunoreactivity in SW480 following the treatment. Time course of suppression of cell proliferation of control ( ■ ), BLM alone ( □ ), SW alone ( ○ ), and SW and BLM ( ● ) (mean±SD). The percentage of Ki-67-positive cells reached the minimum (35.7%) at 24 h after the combined treatment. (B) Time profiles of immunohistochemistry for Ki-67 in SW480 following combined treatment. (a) Control, (b) 12 h, (c) 24 h, (d) 7 days.

Fig. 4. (A) Expression of apoptotic cells on SW480 following the treatment. Time course of expression of TUNEL-positive cells in control ( ■ ), BLM alone ( □ ), SW alone ( ○ ), and SW and BLM ( ● ) (mean±SD). The apoptotic index reached the maximum (6.21%) at 6 h following the combined treatment. (B) TUNEL staining 6 h after treatment was compared in four groups: (a) control, (b) shock waves alone (SW alone), (c) BLM alone, and (d) shock wave with BLM (SW+BLM). Immunohistochemical staining for apoptotic features in SW480 after the treatment is shown. (C) Apoptotic index values in the four groups 6 h after treatment: control, SW alone, BLM alone, and SW+BLM. The apoptotic index of the SW+BLM group was significantly higher (P<0.05) than those of the other three groups.
microscopy studies with SW480 cells 6 h following combined treatment confirmed these observations (Fig. 5B).

**DISCUSSION**

This study shows that anti-proliferative and pro-apoptotic effects of BLM combined with shock wave treatment are seen not only *in vitro*, but also *in vivo* in a solid tumor. Immunohistochemical staining, using MIB-1 antibody, revealed that the suppression of cancer cell proliferation began just after the treatment and the proliferation ratio reached the minimum at 24 h after the treatment, almost equivalent to the doubling time. The maximal apoptotic index was detected 6 h after the combined treatment and at the same time, clusters of enlarged cells, polynucleated cells and mitosis were also detected morphologically. It was reported that when several million molecules of BLM are internalized, morphological changes, identical to those usually associated with apoptosis, are observed, as well as very rapid DNA fragmentation into oligonucleosomal-sized fragments. On the other hand, when only a few thousand BLM molecules are internalized, cells display an arrest in the G2-M phase of the cell cycle and become enlarged and polynucleated before dying. These observations correspond to mitotic death, generally a slow process that is dependent upon mitotic activity, during which cells will usually complete at least one mitosis prior to their disintegration. Thus, the clusters of enlarged and polynucleated cells we observed 6 h following the combined treatment may be the result of mitotic death. This is the first report, to our knowledge, of mitotic death observed *in vivo.*
BLM is a water-soluble antibiotic that was first isolated by Umezawa et al., and exhibits cytotoxic activity against mammalian cells. The cytotoxicity of BLM depends greatly on its ability to induce double-strand breaks in DNA. BLM cytotoxicity is considerably potentiated in vitro when cultured cells are exposed to appropriate electric pulses. Antitumor effects of the compound are also highly increased in vivo by electric pulses delivered locally to the tumor site. The plasma membrane is known to limit BLM uptake and electropermeabilization is an efficient means of circumventing this barrier, thus allowing the direct internalization of BLM molecules into the cytoplasm.

Although electrochemotherapy on human skin tumors has shown good results, direct application of electric current to cancerous tissue deep inside the human body can cause unacceptable side effects. Moreover, many difficulties remain before routine clinical use of electrochemotherapy can be considered in human patients. On the other hand, focused high-energy shock waves were first applied to ureteral stones in humans in 1980. This is now a standard procedure for ureteral stone treatment. Focused shock waves have successfully introduced high energy into deep portions of the human body without causing damage to adjacent tissues. We have demonstrated that under-water shock waves can open the micropores on the surface of suspended cells, thereby increasing the permeability of the cell membrane, like electroporation. Therefore, clinical application of the combination therapy, BLM and shock waves, may be feasible. Our study suggests that this combination therapy may be effective in adenocarcinomas, which are normally refractory to BLM, and that this method should be effectively applicable to multidrug-resistant tumors expressing multidrug-resistance genes (MDRs) and/or multidrug-resistance-associated proteins (MRPs). In vivo gene transfection into solid tumors using shock waves has recently been reported. These studies demonstrate that shock waves can insert reagents into the cytoplasm, not only in vitro, but also in vivo. In this study, we also performed whole-body autoradiography to visualize the promotion of BLM accumulation caused by shock waves.

Some of the biological properties of Co-BLM, such as its cell membrane permeability and cytotoxicity, differ from those of BLM. However, we employed Co-BLM for autoradiography to investigate BLM movement because of the close resemblance in molecular weight and chemical structure to those of BLM and the high specific radioactivity of the chelate, as well as the very high stability of the Co-BLM coordination complex. In this study, the accumulation of Co-BLM in the shock wave-treated tumor was 1.7-fold higher than that of control. This suggests that insertion of BLM caused by shock waves occurred not only in vitro, but also in vivo. Our previous study showed the anticancer effect on a cancer cell suspension in vitro and the suppression of tumor growth in vivo caused by shock wave chemotherapy, but histological evaluation of the effects on solid tumors in vivo was not done. There appear to be differences in the effect of shock waves from the viewpoint of acoustics between cells suspended in a solution and cells tightly grouped in a solid tumor. In this study, we showed that shock wave chemotherapy suppresses cell proliferation and induces apoptosis not only in vitro, but also in solid tumors in vivo. Furthermore, some of the cell death appeared to be quite similar to mitotic death.

Several reports have shown that shock wave exposure of a solid tumor promotes necrosis. However, in this study, we could not evaluate whether the extent of necrosis was affected by shock wave chemotherapy or not, because the implanted SW-480 tumor usually has central necrosis of varying degree.

The response of cancer cells in vivo is affected by many factors, for example, the vascular system, immune system and so on. This study deals with early events in the expression of anticancer effects by shock wave chemotherapy. Further studies are planned to investigate the longer-term efficacy of this approach.

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