Latrunculin A Has a Strong Anticancer Effect in a Peritoneal Dissemination Model of Human Gastric Cancer in Mice

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Abstract. *Peritoneal dissemination of gastric cancer is a refractory disease. This paper focuses on the efficacy of actin-binding marine macrolide latrunculin A, which quickly inhibits actin polymerization and disrupts the function of the actin cytoskeleton. The effects of latrunculin A on cell viability in vitro were evaluated by treatment of MKN45 or NUGC-4 cell cultures. An in vitro viability assay demonstrated an anti-cancer effect of latrunculin A in a dose-dependent manner. Latrunculin A induced acute cell injury and programmed cell death through activating the caspase-3/7 pathway. In vivo, MKN45 or NUGC-4 cells were intraperitoneally inoculated into nude mice, as a model of peritoneal dissemination. Intraperitoneal (i.p.) injection of latrunculin A significantly improved survival rate in mice without any major side-effects. Data indicated that latrunculin A has strong anticancer effects, and it may be a new candidate i.p. drug against peritoneal dissemination of gastric cancer in humans.*

More than 930,000 people annually suffer from gastric cancer worldwide, and the morbidity is fourth highest among all cancers. The incidence and mortality in most parts of the world have decreased markedly over several decades (1, 2). However, gastric cancer remains one of the most common types of cancer in Japan and Asia. There are still many refractory patients with gastric cancer with unresectable, locally advanced or metastatic lesions. The median survival of stage IV cancer is in the range of 6-10 months, and relative 5-year survival rate is only 6.2% (3, 4). Peritoneal dissemination is the most frequent cause of death (5, 6).

Various combinations of four key drugs, S-1, cisplatin, irinotecan and paclitaxel, are standard treatment for unresectable or recurrent gastric cancer, but there is no effective therapy for peritoneal dissemination (7-9). Signet-ring cell carcinoma and poorly differentiated adenocarcinoma are frequent in peritoneal dissemination, but these are sometimes resistant to most anticancer drugs. Therefore, other candidates with a new functional mechanism are necessary.

Actin is of fundamental importance for cell homeostasis, and it is responsible for maintaining signaling, adhesion, endo- and exocytosis, and motility in most cells. Signet-ring cell carcinoma is especially characteristic because it has less cell adhesive function and massive mucus storage. It is hypothesized that actin depolymerization may be effective as an anticancer therapy. As a result of the anticancer effect of several actin inhibitors in a preliminary study, it was found that latrunculin A, which is extracted from the sea sponge, had a strong anticancer effect.

This study focuses on the anticancer effect of latrunculin A, using human gastric cancer cell lines (MKN45 and NUGC-4), and evaluates its potency as an intraperitoneal (*i.p.*) anticancer drug as a new candidate for peritoneal dissemination of gastric cancer.

Materials and Methods

Reagents and cell lines. The human gastric carcinoma cell lines MKN45, poorly differentiated adenocarcinoma, and NUGC-4, signet-ring cell carcinoma, were obtained from Riken BRC Cell Bank (Tsukuba, Japan). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine

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serum and penicillin (100 U/mL)/streptomycin (100 μg/mL). Cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2. Latrunculin A and paclitaxel were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Latrunculin A was dissolved in dimethylsulfoxide (DMSO) and diluted to an appropriate concentration with PBS just before use. Cell Count Reagent SF (mixed solution of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt and 1-methoxy-5-methylphenazium) was purchased from Nacalai Tesque (Kyoto, Japan). Apo-ONE Homogeneous Caspase-3/7 reagent was purchased from Promega (Madison, WI, USA).

Animals. Ten- to twelve-week-old male BALB/c nude mice (Shimizu Laboratory, Kyoto, Japan) were maintained under specific pathogen-free conditions according to the Guidelines for Animal Experiments of Kyoto Prefectural University of Medicine, Japan.

Anti-cancer effect of latrunculin A on gastric cancer cells. MKN45 and NUGC-4 cells were seeded into 12-well flat-bottom tissue culture plates at a density of 1×10^4 cells and allowed to attach overnight. The wells were treated with latrunculin A at 5 μM (final concentration) for 24 or 72 h. Cellular morphological changes were observed using an inverted microscopy (model IX71; Olympus, Tokyo, Japan) and DP manager (Olympus).

Fluorescence microscopy of MKN45 cells. MKN45 gastric cancer cells were seeded on glass coverslips and attached for 24 h. The cells were incubated with 5 μM latrunculin A for 1, 12 or 24 h. Cells were fixed with 3% formaldehyde and permeabilized with 0.2% Triton® X-100 for 15 min. Actin filaments were stained with a solution of 9.0 μg/mL rhodamine–phalloidin (Cytoskeleton, Denver, CO, USA) for 1 h at room temperature. Cells were visualized and photographed with a fluorescence microscopy (model IX71; Olympus) and DP manager (Olympus).

Viability assay of MKN45 and NUGC-4 cells treated with latrunculin A. Cancer cells (1×10^4 cells) were seeded and incubated in a 96-well flat-bottom tissue culture plate for 24 h prior to latrunculin A treatment. After 24 or 72 h treatment with a final concentration of latrunculin A (0.01-10 μM), cell viability was evaluated using a Cell Count Reagent SF colorimetric assay. Cell viability was determined colorimetrically by measuring OD450, using a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA). The percentage cell viability was calculated as follows: percentage cell viability=$T/C\times100$, where C is the mean OD_{450} of the control group and T is that of the treated group. Dose–response curves were created and IC_{50} values were determined graphically from the plots.

Apoptosis and caspase-3/7 quantitative analysis. MKN45 or NUGC-4 cells (2,500 cells/well) were cultured in black flat-bottom 96-well plates (FluoroNunc; Nalgenunc International, Tokyo, Japan) for 48 h prior to *in vitro* apoptosis assay. Apoptosis was induced in each cell line by latrunculin A, paclitaxel (5 μM), or vehicle (negative control; 5% DMSO in PBS) for 12, 24 or 72 h. Caspase-3/7 activities were quantified by Apo-ONE Homogeneous Caspase-3/7 Assay reagent after 1 h. Fluorescence of each sample was measured in three replicate experiments by GENios Multifunctional Fluorescent Plate Reader (Tecan Japan, Kawasaki, Japan) at 485/535 nm wavelength.

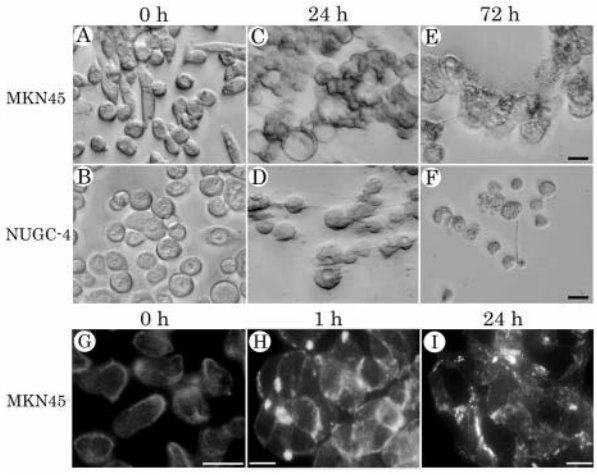

Figure 1. *Anticancer effect of latrunculin A. (A–F) Light microscopic observation of MKN45 and NUGC-4 cells after treatment with 5 μM latrunculin A for 0, 24 and 72 h. Control cells (A, B). Cells appeared round-shaped and swollen at 24 h (C, D), and almost all cells were ruptured and shrunken at 72 h (E, F). Cells showed morphological changes that led to cell death. Bar, 10 μm. Original magnification ×20. (G–I) Fluorescence microscopy. Effect of 5 μM latrunculin A on the distribution of actin in MKN45 cells after 1 and 24 h. (G) Control MKN45 cells with microvilli, philopodia, stress fibers and fine actin network in the periphery of the cell. (H) After 1 h treatment with latrunculin A, MKN45 cells lost their actin ultrastructure, which had a dot blot pattern. (I) After 24 h treatment with latrunculin A, most cells had an irregular shape. Latrunculin A disturbed actin polymerization and blocked its normal distribution. Latrunculin A induced a disruption of the actin fiber network and cell–cell and focal adhesion. Bar, 10 μm. Original magnification ×63 (G), and ×40 (H and I).*

Transmission electron microscopy. Confluent cells were doubly fixed with 2% glutaraldehyde in 0.1 M phosphate buffer and 1% osmium tetroxide in 0.1 M phosphate buffer, and dehydrated with a graded series of ethanol. Cells were then embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with an H-7500 electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 100 kV.

Scanning electron microscopy. MKN45 cells were cultured on plastic cover slips (Celldesk; Sumitomo Bakelite, Tokyo, Japan) and exposed to 5 μM latrunculin A for 1, 12 or 24 h. Confluent cells were doubly fixed with 1.2% glutaraldehyde in 0.1 M PBS and 1% osmium tetroxide in 0.1 M PBS, and dehydrated with a graded series of ethanol. The dehydrated samples were critical-point dried, followed by sputter coating with gold. The preparations were examined using a JSM-5800LV scanning electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 10 kV.

In vivo treatment with i.p. latrunculin A against peritoneal metastasis of MKN45 and NUGC-4 cells. Mice bearing peritoneally disseminated MKN45 or NUGC-4 tumors were prepared by an *i.p.* inoculation of 1×10^7 cells in 5% DMSO/0.7 mL PBS. On day 3, 10 and 17 after tumor inoculation, each mouse was given an *i.p.* injection

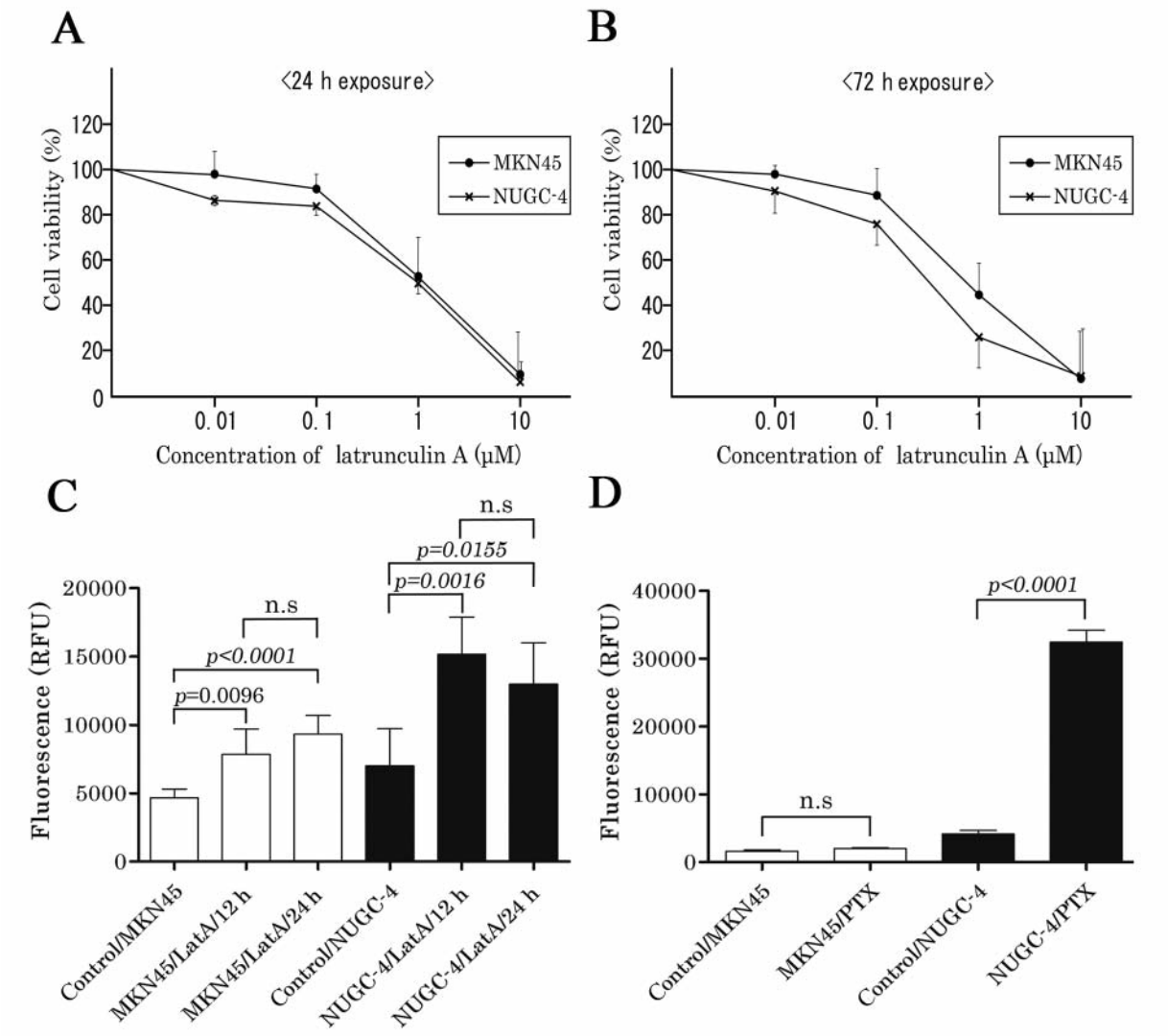

Figure 2. *In vitro viability assay of MKN45 and NUGC-4 cells. Anticancer effect of latrunculin A (LatA) induced apoptosis. MKN45 and NUGC-4 cells were treated with different drug concentrations for 24 (A) and 72 (B) h. Cell viability was determined using a WST-8 colorimetric assay. The results are expressed as percentages of cell growth relative to that of untreated control cells. IC_{50} (24/72 h) in MKN45 and NUGC-4 cells was (1.14/0.76 μM) and (1.04/0.33 μM), respectively. (C-D) Caspase-3/7 quantitation assay. Apoptosis was induced in MKN45 or NUGC-4 cells by latrunculin A for 12 and 24 h (C), and in NUGC-4 cells by paclitaxel for 72 h (D). Latrunculin A strongly induced caspase-3/7 activation in MKN45 and NUGC-4 cells. The data represent the means±SD from three separate experiments. n.s (not significant). PTX, Paclitaxel.*

of latrunculin A. The *i.p.* injection group were treated with latrunculin A (0.05 mg/kg, 5% DMSO in 0.7 mL PBS, n=14, MKN45; and n=10, NUGC-4), and the control group was treated with 5% DMSO in 0.7 mL PBS (n=19, MKN45; and n=9, NUGC-4). Negative (n=10) group was given latrunculin A without tumor inoculation. After inoculation of cancer cells, all mice were carefully observed every day until they all died.

Statistical analysis. The Chi square test was performed to determine correlations among the various parameters, and Fisher's exact test was also used, as necessary. Survival curves were analyzed using the Kaplan–Meier log-rank test. Significance was established using Student's *t*-test and analysis of variance. Results are expressed as Mean±SD. Difference was considered significant when p was less than 0.05. The data were analyzed by Graph Pad Prism5 (GraphPad Software, San Diego, CA, USA).

Results

Effect of latrunculin A on gastric cancer cells. It was found that latrunculin A had a strong anticancer effect. MKN45 and NUGC-4 lost cell adhesion complex within 24 h after latrunculin A treatment. Most cells appeared round-shaped and swollen at 24 h (Figure 1C, D). After 72 h treatment,

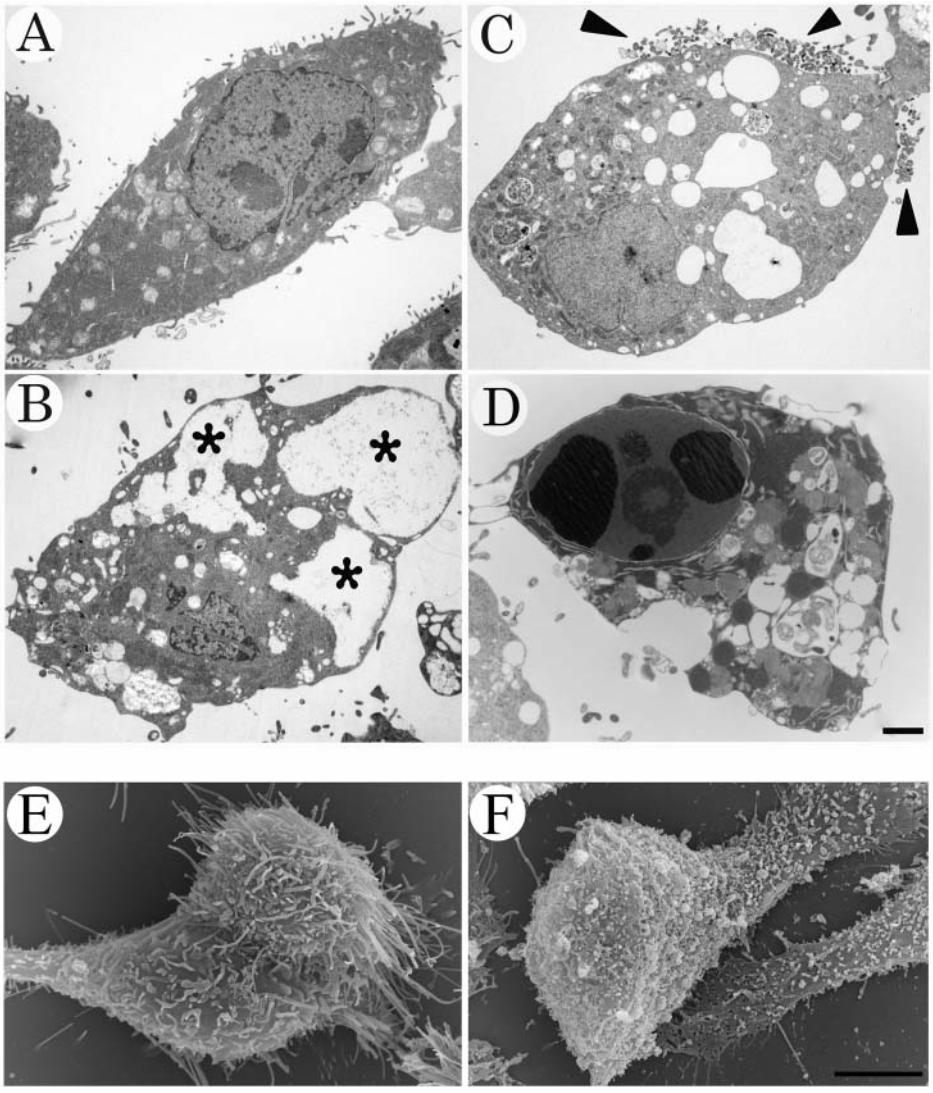

Figure 3. *Electron microscopy of MKN45 cells. A-D, transmission, and E-F, scanning electron microscopy. A, Control MKN45 cells had dense actin fibers in their filopodia and peripheral actin network. B, Latrunculin A induced acute cell injury caused by breakdown of the plasma membrane and rapid cell ballooning (asterisk) after1 h. C, Cytoplasmic organelles were compressed and erupted from the cells (arrowhead, 12 h). D, Latrunculin A induced apoptosis (12 h). Bar, 2 μm. Original magnification ×5000. E, Control MKN45 cells had numerous lamellipodia, microvilli and filopodia. F, Latrunculin A (5 μM) immediately disrupted actin polymerization. MKN45 cells lost their protruding structures after 1 h. Bar, 10 μm. Original magnification ×1500.*

MKN45 and NUGC-4 cells lost focal contact and many cells had burst or shrunk (Figure 1E, F). These result suggested that latrunculin A induced dynamic morphological changes to MKN45 and NUGC-4 cells.

Fluorescence staining of actin cytoskeleton of MKN45 cells. To further investigate the morphological change after latrunculin A treatment, the actin filament network was visualized by rhodamine–phalloidin (Figure 1G). As shown in Figure 1H, latrunculin A treatment of MKN45 cells reduced the peripheral actin network, and the distribution of actin had a dot blot pattern. Most cells became irregular in shape, and microspikes or philopodia at the cell surface disappeared after 1 h treatment (Figure 1H). Twenty-four hours later, the fine actin network and stress fibers had completely disappeared (Figure 1I). Latrunculin A induced a disruption of the stress fibers and focal adhesions. Fixed cell samples of NUGC-4 were not obtained because all treated cells were detached.

Viability of MKN45 and NUGC-4 cells after latrunculin A treatment. A low concentration of latrunculin A (0.01 μM) was not cytotoxic to MKN45 and NUGC-4 cells after 24 h

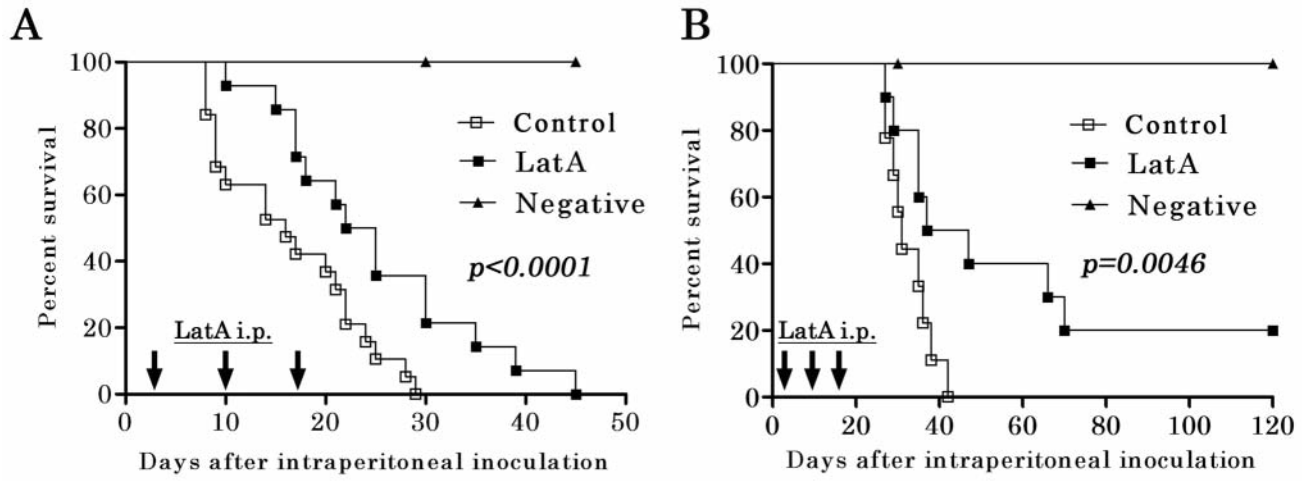

Figure 4. *Anticancer effect of latrunculin A (LatA) improved survival in a mouse model of peritoneal dissemination. Survival of mice was analyzed Kaplan–Meier log-rank test. Mice received i.p. injection of 0.05 mg/kg latrunculin A or vehicle (control) after inoculation with 1×10^7 MKN45 (A) or NUGC-4 (B) cells. Negative group was given latrunculin A without inoculation of cancer cells. A, MKN45 peritoneal dissemination model. Median survival was 23.5 days in the latrunculin A group and 16 days in the control group. n=14-19 mice. B, NUGC-4 peritoneal dissemination model. Median survival was 42 days in the treated and 31 days in the control groups; in both cell lines, median survival was significantly prolonged in the treated group (MKN45, p<0.0001, and NUGC-4, p=0.0046).*

(Figure 2A). Cell viability after 72 h treatment was similar to that at 24 h (Figure 2B). At higher concentrations of 1-10 μM, cell viability was reduced. These data suggested that latrunculin A affected cell viability in a concentration-dependent manner. Drug sensitivity varied in each cell line: IC_{50} (24/72 h) in MKN45 and NUGC-4 cells was (1.14/0.76 μM) and (1.04/0.33 μM), respectively.

Apoptosis and caspase-3/7 quantitative analysis. To clarify whether the cause of non-viability was acute cell injury or apoptotic cell death, the activity of caspase-3/7 was detected in cells treated with latrunculin A for 12 and 24 h, and with paclitaxel for 72 h. Treatment of both MKN45 and NUGC-4 cells with latrunculin A for 12 and 24 h significantly increased caspase-3/7 activity, while there was no significant difference between 12 and 24 h (Figure 2C). Anticancer effect of latrunculin A consisted of apoptotic cell death, and latrunculin A acted more quickly than paclitaxel. Paclitaxel increased caspase-3/7 activity about 7.8-fold in NUGC-4 cells, but not in MKN45 cells after 72 h (Figure 2D).

Electron microscopy. Transmission electron microscopy showed acute cell injury. Some cells treated with 5 μM latrunculin A for 1 h were swollen with abnormal accumulation of vesicles within the cytoplasm (Figure 3B, asterisk). After 12 h treatment, intracellular organelles were squeezed and erupted from the cells, caused by injury to the plasma membrane (Figure 3C, black arrowhead). Finally apoptotic changes in cells were found after 24 h treatment with latrunculin A (Figure 3D). Scanning electron microscopy showed that MKN45 cells had an irregular surface. On their surface, lamellipodia, microvilli and filopodia protruded, and most cancer cells displayed cell–cell and cell–substratum adhesion and motility (Figure 3E). Short-term exposure to latrunculin A (5 μM, 1 h) disrupted most of these actin-filament-based structures. There still remained cell adhesion at the basal surface (focal contact), but there was loss of the cell-surface protrusion and the plasma membrane was perforated (Figure 3F).

In vivo treatment with i.p. latrunculin A against peritoneal dissemination of MKN45 and NUGC-4 cells. In a peritoneal dissemination model of MKN45, mice receiving *i.p.* latrunculin A exhibited a median survival of 23.5 days (n=14), compared to control survival of 16 days (n=19), which was significantly prolonged (p<0.0001). All mice died with hemorrhagic ascites by 45 days after cancer cell inoculation (Figure 4A). In the NUGC-4 peritoneal dissemination model, the mean survival time was 31 (control, n=9) and 42 (treated group, n=10) days (Figure 4B). Four out of 10 mice survived for >50 days, and two were cancer free in the abdominal cavity until 6 months, although all untreated mice died with massive hemorrhagic ascites at up to 42 days after inoculation. The survival of the treated group was significantly prolonged (p=0.0046). These data suggested that latrunculin A had strong antitumor effect in peritoneal dissemination models of poorly differentiated adenocarcinoma (MKN45) and signet-ring cell carcinoma (NUGC-4).

Discussion

Latrunculin A, a natural toxin purified from the red sea sponge *Latrunculia magnifica,* binds to and stabilizes actin monomers; it thereby causes a net depolymerization of actin filaments both *in vitro* and *in vivo* rapidly and reversibly in normal cells (10, 11). Latrunculin A forms 1:1 complexes with G-actin and inhibits its polymerization. In this study, latrunculin A immediately destroyed the cell–cell adhesion complex, focal contact and motility. It also induced various morphological changes. It was found that the intracellular fine network of actin disappeared, there was perforation of the plasma membrane, and cancer cells were swollen (Figures 1 and 3). As a result of disruption of actin polymerization, it led to cell death as a result of cell swelling and shrinkage. Latrunculin A also induced apoptosis through the activation of caspase-3/7 cascade. It is well known that paclitaxel induces apoptosis in many cancer cells by activating the CD95 ligand pathway. This effect is dose- and time-dependent, because paclitaxel has been shown to induce apoptosis in many types of cancer, on account of cell cycle arrest in the G_2/M phase. Paclitaxel has been widely used to treat some specific tumors, including gastric cancer (12). The presented data suggested that latrunculin A strongly and quickly induced apoptosis. Although the mechanism of caspase activation by latrunculin A is still unclear, the cytoplasmic translocation of actin and ERM proteins as crosslinkers is responsible for the microvillar breakdown at an early phase of apoptosis (13). These data is believed to be the first in which latrunculin A up-regulated caspase-3/7 in cancer cells (Figure 2). The data indicate that latrunculin A has two different means of exerting its anticancer effects, including acute cell injury and apoptosis. Therefore, it is thought that signet-ring cell carcinoma may be a good target for actin inhibitors for two reasons. First, they have very weak cell adhesion caused by abnormalities in *E-cadherin* and *catenin* gene expression (14, 15). Generally complete disruption of cell adhesion should cause growth inhibition or cell death of epithelial tumor. Second, they have massive accumulation of vesicles and mucus in the cytoplasm. Latrunculin A accelerates mucus collection and cell swelling. Data showed that latrunculin A had a strong anticancer effect on NUGC-4 cells, and it improved survival in an *in vivo* model (Figure 4D). No major side-effects of latrunculin A were observed after *i.p.* injection to C57BL/6J mice, in terms of survival and blood analysis (data not shown). Therefore a strong/irreversible anticancer effect on MKN45 and NUGC-4 cells may depend on a peculiarity of each cell line. Intraperitoneal injection of latrunculin A against peritoneal dissemination of NUGC-4 and MKN45 cells effectively improved survival of nude mice. Latrunculin A (Mw, 421.6) is a hydrophobic molecule, which is another feature in its favor for treating peritoneal dissemination, since hydrophobic molecules remain longer in the abdominal cavity than hydrophilic small molecules such as cisplatin (Mw, 300.05) or 5-fluorouracil (Mw, 130.08).

Actin and microtubules play opposing roles in fine-tuning the mobility and localizing organelles at target sites on the plasma membrane. In the current study, disruptions of the actin cytoskeleton induced a loss of cytoplasmic organella, smooth endoplasmic reticulum, Golgi stacks, endosomes, lysosomes and mitochondria from the cells. Further study will be required to confirm clinical utility, but *i.p.* injection of latrunculin A in the perioperative period for gastric cancer patients or in the early phase of peritoneal recurrence might be a good therapeutic method. In conclusion, latrunculin A is anticipated to be a novel anticancer drug for peritoneal dissemination of gastric cancer.

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