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Modification of thermosensitivity by amrubicin or amrubicinol in human lung adenocarcinoma A549 cells and the kinetics of apoptosis and necrosis induction

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Abstract. The effects of amrubicin (AMR) and its active metabolite, amrubicinol (AMROH), on the sensitivity of human lung adenocarcinoma A549 cell line to hyperthermia at 44°C were investigated. The cell phase response as well as the kinetics of apoptosis and necrosis induction were also analyzed. The cytotoxic effects of 44°C hyperthermia on A549 cells exhibited low thermosensitivity with a T0 value of 12 min. The slope of the survival curve in the exponential phase, described semilogarithmically, in 44°C hyperthermia combined treatment with AMROH (0.02 μg/ml) was approximately parallel to 44°C hyperthermia alone. The initial shoulder shape portion of the survival curve from 44°C hyperthermia alone, indicating the repair of sublethal thermal damage (SLTDR), was reduced with the sequential combined treatment of AMR or AMROH. Sequential treatments with AMR or AMROH prior to 44°C hyperthermia resulted in additive thermo-enhancement effect by reducing not only survival but was shoulder wide. Furthermore, like AMR and AMROH, adriamycin (ADM) and etoposide (VP-16) are DNA topoisomerase II inhibitors, and the effects of these 4 agents on 44°C hyperthermia were compared. All 4 agents exhibited comparable thermo-enhancement effects. Using synchronized A549 cells, AMR or AMROH did not elicit cell phase responses, irrespective of the concentration. The induction of apoptosis was investigated at 48 and 72 h after AMROH treatment, 44°C hyperthermia or the combined treatment, in which apoptosis was not significantly induced after any treatment. Furthermore, the incidence of necrosis was examined as well as apoptosis. The incidence of necrosis at 48 and 72 h after AMROH was 2.4 and 4.3%, respectively; after 44°C hyperthermia was 3.3 and 4.0%, respectively; and after the combined treatment it was 10.7 and 8.7%, respectively. The necrosis induced after the combined treatment was circa 3 times higher than that in either of the single treatments.

Introduction

Amrubicin hydrochloride (AMR) is the first totally-chemically synthesized anthracycline derivatives (1,2), and it exhibits antitumor activity against human non-small cell lung, small cell lung and superficial bladder cancer (3-5). AMR exerts its antitumor effects by inhibiting topoisomerase II activity and suppressing DNA synthesis (6,7). AMR is metabolized to AMROH in vivo (2), and the results of our in vitro study using human lung cancer A549 cells evidenced that the cytotoxic effects of AMROH were about 10 times more potent than those of AMR. The survival curves of A549 cells treated with isotoxic doses of AMR and AMROH showed the same profile. When cancer cells are exposed to hyperthermia, the cells usually acquire thermo-tolerance during heating or afterwards, which has been one of the problems associated with thermotherapy (8-10). Thermo- and chemo-therapy, a combined treatment with hyperthermia and chemical agents, has been adopted as an interdisciplinary treatment strategy to overcome the problems of thermod tolerance.

We previously reported that the lethal sensitivity in the simultaneous combined treatment with 42°C hyperthermia and adriamycin (ADM) for the initial short time was markedly higher but followed by reduced lethal sensitivity, the sequential treatment showed the tolerant lethal sensitivity (11). In this study, the modification of thermo-sensitivity by AMR or AMROH was investigated using A549 cells in vitro. A549 cell line originating from human lung adenocarcinoma was established by Giard et al (12,13). Several studies have been conducted on the sensitivity of A549 cells to radiation and hyperthermia (14-17).

It has been clarified at the molecular level that A549 cells have a mutated K-ras gene (18) and the wild-type p53 gene (19). When DNA is damaged, wild-type p53 protein is...
activated by phosphorylation on signaling pathway, and the activated p53 exhibits antitumor effects by inducing either apoptosis or G1 arrest (20-25). The ras gene was one of the first oncogenes discovered, and it is involved in cellular proliferation and differentiation (26-28), but there have not been any studies investigating the relationship between the ras gene and thermosensitivity.

In the present study, we analyzed the effects of AMR and AMROH on the thermosensitivity of A549 cells with the above-mentioned genetic backgrounds. We examined the thermo-enhancement effects of isotoxic doses of AMR and AMROH on the sensitivity of A549 cells to 44°C hyperthermia and compared the effects of AMR and AMROH with those of two other DNA topoisomerase II inhibitors (ADM and VP-16). With regard to cell phase responses to hyperthermia, the survival curve for mild hyperthermia at 40°C was flat and responses were not appreciable for any phase (16), but the responses for experimental hyperthermia at 44°C showed marked cytotoxicity in the S phase (30,31).

We conducted a study using bleomycin, a DNA synthesis inhibitor, and confirmed that the survival curve for synchronized cells was flat regardless of drug concentration, and that cell phase responses were not appreciable during cell phase progression (30). The cell phase responses to AMR and AMROH have not been clarified. Therefore, we investigated the cell phase responses of A549 cells synchronized with hydroxyurea to AMR and AMROH. In murine L cells having wild-type p53 that show high thermosensitivity, hyperthermia effectively induced apoptosis (8).

Here, in A549 cells that have wild-type p53 gene and are not thermosensitive, the induction of apoptosis (32,33) and necrosis (34,35) was investigated following AMROH treatment combined sequentially with 44°C hyperthermia. The effects of AMR and its active metabolite, AMROH, on the sensitivity of A549 cells to 44°C hyperthermia were analyzed at the kinetic and molecular levels.

Materials and methods

Cells and culture medium. A549, a human lung adenocarcinoma cell line, cultured in Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) containing NCTC-135 (Gibco Laboratories, Grand Island, NY, USA), lactalbumin hydrolysate solution (Difco Laboratories, Detroit, USA) and 15% newborn calf serum (Gibco) (hereinafter referred to as MLN-15), was used (12,13).

Chemicals, hyperthermia and treatments. AMR or AMROH (Sumitomo Pharmaceuticals Co., Ltd. Osaka, Japan) was dissolved in the culture medium to an appropriate final concentration for the treatment. The adhered cells in the flasks were treated with AMR or AMROH by replacement with 6 ml of the AMR or AMROH solution in MLN-15 for graded
For cell phase responses, A549 cells were treated with 4 mM hydroxyurea (HU) (Sigma Chemical, St. Louis, MO, USA) (36,37) for 5 h to synchronize them at the G1-S border, and after an interval of 1-9 h, cells were treated with AMR or AMROH for 4 h. Cell phase responses were also analyzed by the colony formation methods.

**Analysis of apoptosis and necrosis.** Induction of apoptosis was analyzed at 48 and 72 h after treatment with 0.02 µg/ml AMROH for 4 h. 44°C hyperthermia for 35 min. or the combined treatment. Trypsinedized cells were placed in MLN-15 to stop trypsinization, rinsed with PBS(-), fixed overnight in 1% glutaraldehyde (Nacalai Tesque Inc., Kyoto, Japan), and placed in PBS(-). For observation, cells were stained with 0.2 mM Hoedt 33342; Bisbenzimide H33342 Fluorochrome, Trihydrochloride (Calbiochem, Merck KGaA, Darmstadt, Germany) and were analyzed under a fluorescence microscope (Nikon Eclipse E600, Nikon Co. Ltd, Tokyo, Japan). A total of 1,000 cells were counted, and the percentage of typical apoptotic cells was calculated. The induction of necrosis was also determined after the above-described treatments. After double-staining the cells using acridine orange (Sigma-Aldrich Co. St. Louis, USA) and ethidium bromide (Nacalai Tesque Inc., Kyoto, Japan) (AO/EB) (38), cells were observed in real-time under a fluorescence microscope, and the percentage of typical necrotic cells was calculated.

**Results**

**Cytotoxic effects of AMROH.** A549 cells were treated with graded concentrations of AMROH (0.01, 0.02, 0.1 or 0.2 µg/ml) for 1-10 h in order to determine its cytotoxic effects as shown in Fig. 1. For each concentration, the surviving fraction of cells were plotted against treatment time on a semilogarithmic chart. All curves showed steeply a slope during the initial stages of treatment. At concentrations of 0.01 and 0.02 µg/ml, the curves gradually reached a plateau after 4 to 6 h, and the plateau was maintained ≤10 h at all concentrations. AMROH was markedly cytotoxic during the early stages of treatment, but no marked changes in sensitivity were seen beyond 4 h.

**Heating period (44°C) - survival relationships of A549 cells.** With regard to the thermosensitivity of A549 cells, the T0 value (dose required to reduce the survival by 1/e), which is the reciprocal of the slope of the survival curve in the exponential phase, was 12 min for 44°C hyperthermia. As is the case with the Elkind recovery associated with low-dose radiotherapy, the repair of sublethal thermal damage (SLTDR), which is seen as a curve shoulder, was identified during the early stages of hyperthermia. The Tq value, a sort of cellular capacity for SLTDR, was 6.5 min as shown in Fig. 2.

**The effects of AMROH on hyperthermia.** Using A549 cells, the thermo-enhancement effects of AMR and AMROH were investigated based on the survival curves drawn after 44°C hyperthermia and AMROH combination therapy in Figs. 2 and 3. The survival curve of the cells performed with 44°C hyperthermia alone elicited a shoulder, indicating the repair of SLTD, whereas the curve of sequentially combined
treatment with AMR or AMROH was reduced. The SLTDR associated with short-term hyperthermia was blocked by AMR or AMROH. The slope of the survival curve in the exponential phase for 44°C hyperthermia and the combination therapy with AMR or AMROH were parallel, i.e. administration of AMR or AMROH prior to hyperthermia showed additive thermo-enhancement effects. The thermo-enhancement effects of 4 topoisomerase II inhibitors, ADM, VP-16, AMR and AMROH, on 44°C hyperthermia for 30 min were compared as shown in Table I. The effects of 4 agents in isotoxic doses were comparable, markedly enhancing the effects of hyperthermia.

Cell phase responses to AMR and AMROH. A549 cells synchronized at the G₁-S border by treatment with 4 mM HU for 5 h were incubated for graded periods of time, and were further exposed to AMR and AMROH in order to analyze the cell phase responses as shown in Fig. 4. A control, synchronized cells were incubated for graded periods of time and were treated with 4 mM HU for 2 h, not shown in the figure. Cell survival was plotted in order to compare the results with the standard cell-cycle phase. After a specified interval, synchronized A549 cells were treated with 3 graded concentrations of AMR or AMROH for 4 h respectively, and the survival curve was largely flat for all cell-cycle phases at all concentrations. AMR and AMROH did not elicit cell phase responses, irrespective of the concentration.

Effects on apoptosis induction. The thermo-enhancement effects of AMROH on A549 cells were investigated based on the induction of apoptosis as shown in Fig. 5. After AMROH for 4 h, 44°C hyperthermia for 35 min or combination therapy, cells were harvested at 48 and 72 h after incubation and were fixed with glutaraldehyde. Fixed cells were stained with Höchst 33342 solution, and cellular morphology was observed under a fluorescence microscope in order to determine the percentage of apoptotic cells. At 48 and 72 h after treatment with AMROH alone, the incidence of apoptosis was 0.9% and 1%, respectively; circa 13% (at both time points) for 44°C hyperthermia alone; and 2.4 and 1.7% for combination therapy, respectively.

Effects on necrosis induction. As was the case with apoptosis, the thermo-enhancement effects of AMROH on A549 cells were investigated in the induction of necrosis. The incidence of necrotic cells following AMROH treatment, 44°C hyperthermia or the combination therapy was investigated by AO/EB double staining at intervals of 48 and 72 h after treatment. The incidence of necrosis at 48 and 72 h after treatment with AMROH was 2.4 and 4.3%, respectively, after 44°C hyperthermia was 3.3 and 4.0%, respectively, and after combined treatment it was 10.7 and 8.7%, respectively as shown in Fig. 6.

Discussion

In the present study, the modification of AMR and AMROH on the thermosensitivity of A549 cells was investigated.

Cytotoxicity of AMROH. In our study, AMROH exhibited marked cytotoxic activity during the early stages. AMR and its active metabolite, AMROH, are DNA topoisomerase II inhibitors (3,4). DNA topoisomerase II is reported to be vital for essential biological reactions such as replication, transcription and genetic recombination (39-41). In 1984, Liu et al discovered that mAMSA [4-(9-acridinylamino) methanesulfonylaminidazole], an antitumor agent, inhibited topoisomerase II
effects on hyperthermia. When A549 cells were treated with AMR or AMROH prior to 44°C heating in vitro, the SLDTR seen during the early stages of hyperthermia was inhibited. AMR and AMROH additively enhanced the effects of 44°C hyperthermia. In thermotherapy for cancer, the time required to achieve 10% survival (0.1 on a survival curve) for hyperthermia alone was 30 min, but that for the combination therapy was 10 min. Comparable results were obtained in about one-third of the time by combining hyperthermia with AMR or AMROH. The fact that combining AMR or AMROH cuts the duration of hyperthermia to one-third is beneficial in multidisciplinary anticancer therapy. We have investigated the ability of various chemicals to modify the effects of hyperthermia using different cells at the kinetic and molecular levels (11,30,45-47). The effects of AMR, AMROH, ADM (48) and VP-16 (49), which target DNA metabolism, on the thermosensitivity of A549 cells were compared. When A549 cells were treated with isotoxic doses of AMR, AMROH, ADM or VP-16 for 3 h and then sequentially subjected to 44°C hyperthermia for 30 min, comparable degrees of enhancement were noted. These findings clarify that all topoisomerase II inhibitors increase the thermosensitivity of A549 cells.

Cell phase responses. In order to clarify the mechanisms of thermal enhancement by AMR or AMROH, we investigated the survival curves through cell phase progression, the cell phase response of A549 cells to 0.1, 0.5 and 2.5 µg/ml of AMR or 0.004, 0.02 and 0.1 µg/ml of AMROH were analyzed. All survival curves were flat, i.e., cell phase responses were not seen regardless of the concentrations of AMR or AMROH. We reported that bleomycin (29), which inhibits DNA synthesis, was cell cycle non-specific regardless of concentration (30). When exposed to more than moderate hyperthermia or radiation, cells are most sensitive to hyperthermia during the G1-S phase and to radiation during the G2-M phase (50,51). There was no cell phase response to mild hyperthermia that could be assessed uniformly in all phases for thermosensitivity (30), but with 44°C experimental
Induction of apoptosis and necrosis. The induction of apoptosis following 44°C hyperthermia or 0.02 µg/ml AMROH increased slightly with time, but there was no significant increase following sequential combination therapy. However, the induction of necrosis following 44°C hyperthermia increased with time, and there was a significant increase in the induction of necrosis after 4 h of AMROH exposure followed by 44°C hyperthermia. p53-mediated apoptosis is thought to be cell cycle dependent (55, 56). We reported that apoptosis was induced effectively by 44°C hyperthermia in thermosensitive murine L cells with wild-type p53 (8). The results of the present study were unexpected in that the cytotoxicity of AMROH against A549 cells having wild-type p53 in hyperthermia was due to increased necrosis, not increased apoptosis. In addition, with A549 cells, cell phase responses were not seen. This suggests that AMR and AMROH directly act on DNA topoisomerase II without affecting the p53 cascade. AMR and AMROH were shown to dynamically enhance the thermosensitivity of A549 cells. Thus, in multidisciplinary anticancer therapy, the antitumor effects of hyperthermia can be improved by administering AMR or its metabolite, AMROH (57).

Acknowledgements

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