Synergistic effect of heat shock protein 90 inhibitor, 17-allylamino-17-demethoxygeldanamycin and X-rays, but not carbon-ion beams, on lethality in human oral squamous cell carcinoma cells

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The purpose of this study is to clarify the effect of a heat shock protein 90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG), in combination with X-rays or carbon-ion beams on cell killing in human oral squamous cell carcinoma LMF4 cells. Cell survival was measured by colony formation assay. Cell-cycle distribution was analyzed by flow cytometry. Expression of DNA repair-related proteins was investigated by western blotting. The results showed 17-AAG to have synergistic effects on cell lethality with X-rays, but not with carbon-ion beams. The 17-AAG decreased G2/M arrest induced by X-rays, but not by carbon-ion beams. Both X-ray and carbon-ion irradiation up-regulated expression of non-homologous end-joining-associated proteins, Ku70 and Ku80, but 17-AAG inhibited only X-ray-induced up-regulation of these proteins. These results show that 17-AAG with X-rays releases G2/M phase arrest; cells carrying misrepaired DNA damage then move on to the G1 phase. We demonstrate, for the first time, that the radiosensitization effect of 17-AAG is not seen with carbon-ion beams because 17-AAG does not affect these changes.

Keywords: heat shock protein 90; 17-allylamino-17-demethoxygeldanamycin (17-AAG); carbon-ion beam irradiation; radiosensitization

INTRODUCTION

High linear energy transfer (LET) carbon-ion radiotherapy has a superior dose distribution to, and higher biological effect than, X-rays. Radiosensitivity to X-rays is known to depend on the status of the tumor suppressor gene, p53, but some studies of the relationship between carbon-ion beams and p53 status have reported that irradiation effects do not depend on p53 status [1–3]. Carbon-ion beam irradiation inhibits phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways in tumor cells [4], and induces cell death by mitogen-activated protein kinase–extracellular signal-regulated kinase (MEK–ERK)-dependent multiple caspase activation [5]. These aspects of carbon-ion radiotherapy give excellent local control of radioresistant tumors [6]. However, control of metastases outside irradiated fields causes a problem when linking this local effect with an improvement in the overall survival rate [6]. Chemotherapy is one option for combined use with carbon-ion radiotherapy. Indeed, head and neck cancer is treated by a combination of carbon-ion radiotherapy and chemotherapy at the National Institute of Radiological Sciences (NIRS) [7].

In numerous tumor cells, heat shock protein 90 (Hsp90) is over-expressed and forms multi-chaperone complexes with client proteins that are involved in processes characteristic to malignant phenotypes, such as invasion, angiogenesis and
metastasis [8–10]. Moreover, Hsp90 stabilizes several proteins such as Raf-1 [11], Akt [12], ErbB2 [13] and hypoxia-inducible factor-1α (HIF-1α) [14], which are known to be associated with protection against radiation-induced cell death. Therefore, these results suggest that Hsp90 inhibitors could provide a promising strategy for implementing a multitarget approach to radiosensitization.

Actually, a number of studies have already explored Hsp90 as a potential molecular target for sensitization by X-rays of tumor cells [15–18]. However, there are no studies of the combined effects of Hsp90 inhibitor and high LET carbon-ion beams on cell lethality.

Here, we used Hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG), which is a derivative of geldanamycin, a benzoquinoid ansamycin compound. 17-AAG binds to the ATP binding site of Hsp90 protein and specifically inhibits its chaperone functions in tumor cells [19]. This study explores the combined effects of 17-AAG and carbon-ion beams in human squamous cell carcinoma cells in vitro. In particular, we have found, for the first time, that carbon-ion beam irradiation is less affected by 17-AAG than X-ray irradiation.

MATERIALS AND METHODS

Cell culture and treatments

Human oral squamous cell carcinoma (SCC) LMF4 cells were obtained from the Department of Oral and Maxillofacial Surgery, Tokyo Medical and Dental University (Tokyo, Japan) [20]. Cells were grown in RPMI-1640 (Life Technologies Japan Ltd, Tokyo, Japan), augmented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin and 100 μg/ml streptomycin (Life Technologies Corporation, Carlsbad, CA, USA). The 17-AAG (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was dissolved in 99.0% dimethyl sulfoxide (DMSO) to a stock concentration of 100 μM and stored at −20°C. This stock solution was diluted to reach a final concentration of 100 nM before use. X-ray irradiation was performed using a Faxitron X-650 X-ray machine (Faxitron Bioptics, LCC, Lincolnshire, IL, USA) operated at 100 kVp with a dose rate of 1.1–1.3 Gy/min. The broad beam of carbon particles was accelerated by the azimuthally varying field (AVF) cyclotron of Takasaki Ion Accelerators for Advanced Radiation Application (TIARA) at the Japan Atomic Energy Agency. Monolayered cells were irradiated with 18.3 MeV/u carbon particles, which provided a dose-averaged LET of 108 keV/μm.

Clonogenic assay and cell cycle analysis

Cells were treated with or without 17-AAG for 24 h at 37°C, then irradiated with X-rays or carbon-ion beams. Shortly after irradiation, for colony formation assay, cells were trypsinized, diluted, counted and seeded in 60-mm dishes at various cell densities. After 2 weeks of incubation, cells were fixed by 100% ethanol and stained with 2% crystal violet. Colonies that consisted of more than 50 cells were counted. Plating efficiencies of untreated cells ranged from 43.6 to 52.8%. All radiation dose–response curves were analyzed by linear regression analysis for survival slopes versus radiation dose curves. For cell-cycle analysis, cells treated with the drug and radiation were subsequently fixed by 70% ethanol to which 500 μl/ml RNase A (Sigma-Aldrich Corporation, St. Louis, MO, USA) was added, and stained with propidium iodide (Sigma-Aldrich Corporation) at each indicated time point (0, 6, 12, 24, 36 and 48 h) after irradiation. Cell-cycle distribution was analyzed by FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) using the CellQuest program.

Western blots

At 24 h after irradiation, cells were lysed with Cell Lysis buffer (Millipore, Billerica, MA, USA) containing phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich Corporation) and protease inhibitor cocktail 3 (Calbiochem, Darmstadt, Germany). The protein levels in supernatants obtained after centrifugation (15 000 g) were quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins (30 μg per lane) were resolved by electrophoresis on 4–15% Mini-Protein TGX gels (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Thereafter, the proteins were transferred onto nitrocellulose membranes. Target protein levels were assessed using antibodies to Ku70, Ku80 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or actin (Sigma-Aldrich Corporation). Primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies and the electrochemiluminescent (ECL) detection system (Amersham, Piscataway, NJ, USA). Quantification was done using image acquisition and analysis software, Labo 1D (Kurabo, Osaka, Japan). Results are expressed as means and SD relative to internal controls of actin.

RESULTS

Effect of 17-AAG on radiosensitivity

Survival curves for LMF4 cells treated with 17-AAG and irradiation are shown in Fig. 1. Data points and bars represent the averages and SDs, respectively, from three separate experiments. The 17-AAG (100 nM) cytotoxicity (compared with DMSO) was 30–40% (data not shown). The radiation doses to reduce the surviving fraction to 10% (D10) and D0 (A dose of D0 reduces survival from 1 to 0.37) values of X-rays or carbon-ion beams with and without 17-AAG treatment are shown in Table 1. Notably, carbon-ion irradiation was more lethal than X-ray irradiation plus 17-AAG.

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Effect of 17-AAG on radiation-induced G2/M phase arrest

The combination of 17-AAG with X-rays additively induces G2/M phase arrest [18], while the same is not reported with carbon-ion beams. Therefore, we analyzed the percentage of cells in the G2/M phase after treatment with 17-AAG and/or exposure to X-rays or carbon-ion beams. The cell percentage in the G2/M phase was calculated from the resulting histograms. When treated with 17-AAG for 12 h, the percentage of G2/M phase cells increased from 26.5 ± 1.3% to 49.6 ± 1.2% (Fig. 2A). The G2/M phase cells significantly increased after X-ray and carbon-ion beam irradiation (Fig. 2B, C). The G2/M arrest induced by irradiation thus showed dose-dependency for both X-ray and carbon-ion beam irradiation, and reached a maximum after 12 h of irradiation, followed by a decrease thereafter (Fig. 2B, C).

Twelve hours after cells were irradiated with 10-Gy X-rays or 2-Gy carbon-ion beams, their cell cycle phases were analyzed (Fig. 2D). These doses were selected as their relative biological effectiveness value was around 5. On the other hand, cells in the G2/M phase had decreased with treatment of 17-AAG alone (Fig. 2Db). The fraction of untreated cells in the G2/M phase was 32.5 ± 2.8%, while in cells 12 h after exposure to X-rays or carbon-ion beams it was 71.2 ± 1.8% or 72.7 ± 6.5%, respectively, meaning that X-rays and carbon-ion beams produced an identical degree of G2/M arrest (Fig. 2Dc, e). However, when 17-AAG was added, X-rays or carbon-ion beams decreased the number of cells in the G2/M phase to 49.6 ± 8.8% or 62.2 ± 1.2%, respectively, 12 h after irradiation (Fig. 2Dd, f). A significant difference was seen between 10-Gy X-rays (Fig. 2Dc) and 10-Gy X-rays combined with 17-AAG (Fig. 2Dd).

Effect of 17-AAG on relative amount of radiation-induced NHEJ-related proteins

It is well known that radiosensitivity is determined by the repair of radiation-induced DNA double-strand breaks (DSBs). To determine whether DNA DSB repair proteins are affected by 17-AAG, we investigated the expression levels of non-homologous end-joining (NHEJ)-associated proteins (Ku70 and Ku80) after treatment with irradiation and 100 nM 17-AAG (Fig. 3). Expression of Ku70 and Ku80 proteins increased 24 h after X-ray irradiation; however, 17-AAG reduced these X-ray-induced increases in Ku70 and Ku80 proteins. Expression of the Ku70 protein increased in carbon-ion beams, with or without 17-AAG treatment. However, Ku80 protein expression did not change. No significant change in Ku70 or Ku80 protein levels was detected between those treated with carbon-ion beam irradiation alone and those treated with carbon-ion beam irradiation in combination with 17-AAG.

Table 1. D10, D0 and relative biological effectiveness (RBE) values of X-rays or carbon-ion beams with or without 17-AAG treatment

| 17-AAG (nM) | X-rays D10 (Gy) | Carbon-ion beams D10 (Gy) | RBE | X-rays D0 (Gy) | Carbon-ion beams D0 (Gy) | RBE |
|-------------|----------------|--------------------------|-----|----------------|--------------------------|-----|
| 0           | 7.73 ± 0.62    | 1.51 ± 0.23              | 5.12| 3.23 ± 0.01    | 0.61 ± 0.08              | 5.30|
| 100         | 4.69 ± 0.34    | 1.32 ± 0.20              | 3.55| 2.14 ± 0.02    | 0.54 ± 0.08              | 3.96|
| Sensitization rate | 1.65 | 1.14 | - | 1.51 | 1.13 | - |
| P           | P < 0.05       | P = 0.22                 | -   | P < 0.01       | P = 0.14                 | -   |

RBE: relative biological effectiveness. Sensitization rate = (D10 or D0 values of 0 nM 17-AAg)/(D10 or D0 values of 100 nM 17-AAG).
DISCUSSION

The cytotoxic mechanism of Hsp90 inhibitor 17-AAG affects multiple pathways that relate to programmed cell death and cell-cycle regulation. We showed here that 17-AAG sensitized cells to the lethal effect of X-rays but not those of carbon-ion beams in SCC LMF4 cells (Fig. 1). The X-ray sensitizing effects of 17-AAG in the present study could partly be due to activities exerted by 17-AAG, including DNA DSB restoration inhibition [17], modification of cell-cycle progression [18], Raf-1 and Akt inhibition [21] and PI3K/Akt inhibition [22]. However, the sensitizing effects of 17-AAG on carbon-ion beam irradiation have not been examined till now. High-LET irradiation is more effective in both killing cells [23–26] and delaying the G2 phase than photon irradiation, whereas DNA damage repair before cells enter mitosis is critical after photon irradiation [24]. 17-AAG decreased the X-ray-induced G2/M accumulation more prominently than the carbon-ion-induced G2/M accumulation (Fig. 2). As radiosensitivity increases when cells are released from the G2/M phase [27], we hypothesize that modification of G2/M delay by 17-AAG would have a different effect on cell killing caused by photon irradiation from carbon-ion beam irradiation. Another Hsp90 inhibitor is also known to abrogate the G2- and S phase arrest induced by X-ray irradiation [28]. On the other hand,
high LET irradiation is more effective in increasing phosphorylation of c-jun NH₂-terminal kinases (JNK) than low LET [29]. JNK induces the degradation of Cdc25B and Cdc25C, which play a key role for cells entering into mitosis [30, 31]. Therefore, JNK phosphorylation increased by carbon-ion beams may prevent G₂ phase cells from progressing into metaphase. This prevention activity could have overwhelmed another function of 17-AAG, i.e. inhibition of proteins Chk1 and Wee1, which are clients of Hsp90 and important for G₂ arrest.

The response of tumor cells to radiation often depends on DNA DSB repair [17, 18, 32]. DNA DSB can be repaired by two basic processes: homologous recombination repair (HR), requiring an undamaged DNA strand as a participant in the repair as a template; and NHEJ, which mediates end-to-end joining [32]. High-LET irradiation affects only the Ku-dependent NHEJ but not HR, a feature totally different from low-LET irradiation [33]. The key proteins associated with NHEJ are Ku70, Ku80 and DNA-PKcs [32]. In this report, both Ku70 and Ku80 were up-regulated by X-rays, while 17-AAG inhibited these up-regulations (Fig. 3). Falsone et al. [34] report that DNA-PKcs is a client of Hsp90. DNA-PKcs levels in the cytosol of HeLa cells are degraded by treatment with Hsp90 inhibitor [34]. The Ku/DNA-PK complex is necessary for NHEJ repair and binds DNA ends first [32]. In the present study, if 17-AAG also suppressed DNA-PKcs activity, the Ku/DNA-PK complex would be inactivated. This results in inhibition of NHEJ. Therefore, it seems likely that the mechanism for radiosensitization caused by 17-AAG could be its inhibitory effect on Ku70 and Ku80, which otherwise would be up-regulated by X-ray irradiation. This inference agrees with the lack of influence of 17-AAG on Ku-protein up-regulation that is induced by carbon-ion beams treatment (Fig. 3).

We conclude that the radiosensitizing effect of 17-AAG is not seen when combined with carbon-ion beams probably because 17-AAG does not affect NHEJ of DNA DSB induced by carbon-ion beams.

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