Therapeutic potential of ghrelin and des-acyl ghrelin against chemotherapy-induced cardiotoxicity

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Abstract. Cancer was considered an incurable disease for many years; however, with the development of anticancer drugs and state-of-the art technologies, it has become curable. Cardiovascular diseases in patients with cancer or induced by cancer chemotherapy have recently become a great concern. Certain anticancer drugs and molecular targeted therapies cause cardiotoxicity, which limit the widespread implementation of cancer treatment and decrease the quality of life in cancer patients significantly. The anthracycline doxorubicin (DOX) causes cardiotoxicity. The cellular mechanism underlying DOX-induced cardiotoxicity include free-radical damage to cardiac myocytes, leading to mitochondrial injury and subsequent death of myocytes. Recently, circulating orexigenic hormones, ghrelin and des-acyl ghrelin, have been reported to inhibit DOX-induced cardiotoxicity. However, little is known about the molecular mechanisms underlying their preventive effects. In the present study, we show the possible mechanisms underlying the effects of ghrelin and des-acyl ghrelin against DOX-induced cardiotoxicity through *in vitro* and *in vivo* researches.

Key words: Cardiotoxicity, Chemotherapy, Des-acyl ghrelin, Doxorubicin, Ghrelin

ONE-HALF of Japanese people receive a diagnosis of cancer during their life span, and one-third of Japanese people die of cancer [1]. Recent advances in the development of chemotherapy and early diagnosis of cancer have improved prognosis and life expectancy in cancer patients [2]. Recently, cardiovascular diseases in cancer patients with cachexia or at late stages in cancer survivors receiving chemotherapy have become a great concern [3]. Certain anticancer drugs and molecular targeted therapies induce cardiotoxicity, which

limit the widespread implementation of cancer treatment and decrease the quality of life in cancer patients significantly [3].

The anthracycline doxorubicin (DOX) induces cardiotoxicity, resulting in cardiomyopathy and subsequent congestive heart failure [4]. DOX-induced cardiotoxicity may be caused by several mechanisms, including apoptosis, myocardial fibrosis, mitochondrial dysfunction, inhibition of cardiomyocyte-specific gene expression, and molecular signaling-pathway alteration [5]. Recent reports indicate that the circulating orexigenic hormones ghrelin and des-acyl ghrelin, a non-octanoylated form of ghrelin [6], inhibit DOX-induced cardiotoxicity [7, 8]. However, little is

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known about their underlying molecular mechanisms. In this study, we show the possible mechanisms underlying the preventive effect of ghrelin and des-acyl ghrelin against DOX-induced cardiotoxicity through *in vitro* and *in vivo* experiments.

Materials and Methods

In vitro study

The effects of ghrelin and des-acyl ghrelin were evaluated in H9C2 cardiomyocytes (kindly gifted from Dr. Kishida, Kagoshima University, Japan.). The cells were seeded onto 96-well plates (0.5×10^4 cells/well) 24h and incubated with DOX (0 – 0.5 μ M) for 72 h in the presence or absence of ghrelin or des-acyl ghrelin. The changes in cell confluency were analyzed by the IncuCyte ZOOM[®] live-cell imaging system (Essen BioScience, Ltd., Japan) and cell viability was determined using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Japan). Apoptosis was determined using the CellPlayer 96-well kinetic Caspase-3/7 apoptosis assay kit (Essen BioScience) and analyzed with IncuCyte ZOOM[®].

In vivo study

To explore the therapeutic effect of des-acyl ghrelin against DOX-induced cardiotoxicity, male C57BL/6 WT mice were allocated to DOX + vehicle (saline) and DOX + des-acyl ghrelin groups. DOX (15 mg/kg) was administered once intraperitoneally (i.p.) to male C57BL/6 WT mice at 8 weeks of age. One day before DOX treatment, des-acyl ghrelin (100 mg/kg) or saline was administered subcutaneously, twice daily, for 8 consecutive days. Transthoracic echocardiography (M-mode) (Vevo[®]2100, Primetech Corporation, Japan) was performed under inhalation anesthesia with isoflurane before and after DOX administration. Seven days after DOX administration, the mice were deeply anesthetized with pentobarbital sodium (100 mg/kg i.p.), and then the heart and lung tissues were collected from each group.

Results

Both ghrelin and des-acyl ghrelin significantly reduced DOX-induced cell damage and cell death, with des-acyl ghrelin showing greater beneficial effect (Fig. 1A, B, C). In addition, apoptosis assay showed that DOX-induced cell damage and cell death were due to

apoptosis (data not shown). Both the hormones significantly inhibited DOX-induced apoptosis (data not shown). The *in vivo* experiment showed that systolic dysfunction was induced in mice treated with DOX, while mice treated with DOX and des-acyl ghrelin showed significantly improved systolic dysfunction (data not shown).

Discussion

In this study, we determined the therapeutic potential of ghrelin and des-acyl ghrelin against DOX-induced cardiotoxicity. H9C2 cell lines do not express ghrelin receptor (GHSR-1a) [7], and des-acyl ghrelin does not bind to GHSR-1a [6]. To date, specific receptors for des-acyl ghrelin have not been identified yet [6]. We showed that DOX-induced damage and apoptotic death of H9C2 cells were inhibited by ghrelin and des-acyl ghrelin, with des-acyl ghrelin exhibiting greater beneficial effect. Based on these results, it is likely that receptors for both ghrelin and des-acyl ghrelin and/or receptors specific for des-acyl ghrelin may exist in H9C2 cells. Ghrelin might bind to the receptors for both ligands or receptors specific to des-acyl ghrelin after deacylation from its ghrelin residue. This hypothesis is based on the result that des-acyl ghrelin showed greater beneficial effects than ghrelin on DOX-induced events. CellKey assay system that evaluates the activities of almost all receptors including G protein-coupled receptors, has been developed in our laboratory and applied for the screening of new receptors [9]. The identification of des-acyl ghrelin specific receptors in H9C2 cells by this system is under way in our laboratory.

As anticancer drug therapy is a scheduled treatment, des-acyl ghrelin could be administered from the day before DOX administration. From the results of *in vivo* experiments, it can be suggested that des-acyl ghrelin may have a therapeutic potential against DOX-induced cardiac dysfunction.

Previous animal studies showed that cardiac dysfunction and cardiac muscle atrophy were paralleled by skeletal muscle atrophy in cancer cachexia model [10, 11]. We previously reported that the traditional Japanese medicine rikkunshito, prescribed for the treatment of gastrointestinal disorders [12, 13], improved symptoms of cancer cachexia by reducing ghrelin resistance through the enhancement of ghrelin receptor-mediated signaling in our cancer cachexia model

Fig. 1 Effects of ghrelin and des-acyl ghrelin treatment in H9C2 cardiomyocytes against DOX-induced cell damage and cell death (A) Images from the IncuCyte ZOOM[®] live-cell imaging with H9C2 cells are shown (scale bar, 300 μm). (B) Ghrelin and des-acyl ghrelin protected against DOX-induced cell damage and cell death in H9C2 cardiomyocytes. ** $p < 0.01$ for each group vs. DOX treatment (one-way ANOVA followed by the *post hoc* Dunnett's comparison test); N=5 per group. (C) Cell viability was measured using the Cell Counting Kit-8. ** $p < 0.01$ for each group vs. DOX treatment (one-way ANOVA followed by the *post hoc* Dunnett's comparison test); N=4 per group.

(Fig. 2A) [12, 13]. Cardiac dysfunction might have occurred in this model along with skeletal muscle loss or atrophy or both, although this has not been investigated so far. Other studies demonstrated that ghrelin improved cardiac cachexia [14]; thus, cardiac dysfunction occurring in cancer cachexia might be improved by the activation of ghrelin signaling. We speculate

that ghrelin and des-acyl ghrelin may improve cardiac dysfunctions in the cancer cachexia model and experiments are currently being performed in this model. Clarifying the mechanisms of cancer cachexia symptoms, including cardiac dysfunction, will be important in the development of a new therapy to improve QOL in cancer patients (Fig. 2 B).

Fig. 2 A novel cancer cachexia model induced by human gastric cancer-derived 85As2 cell inoculation

(A) Ghrelin resistance. Rikkunshito improved ghrelin resistance and the symptoms of cancer cachexia by enhancement of ghrelin signaling. FFM, fat-free mass; GHSR, growth hormone secretagogue receptor; NPY/AgRP, The neuropeptide Y / agouti-related protein neurons; NST, nucleus solitary tract. (B) Cardiac dysfunction is also expected in this model; however, the cardiac functions in this model remain unclear.

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Conflicts of Interest

Yasuhito Uezono, who received a research grant and lecture fees from Tsumura & Co. Kiyoshi Terawaki is an employee of Tsumura & Co.

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