Naringin may alleviate doxorubicin cytotoxic effects in C2C12 myoblast cells

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Abstract. Doxorubicin is one of the first line chemotherapeutic agents used to treat different types of cancer. However, despite its efficacy, doxorubicin can be toxic to muscle tissue and worsen the prognosis of cancer patients. Previously, we investigated the effect of chemopreventive agents curcumin, naringin and epigallocatechin-3-gallate (EGCG), which show anticancer properties in cancer cells, in C2C12 myoblast cells. We found that naringin, a citrus flavonoid, showed no significant cytotoxic effect on C2C12 cells. In this study, we investigated doxorubicin cytotoxicity alone or in combination with naringin against C2C12 myoblast cells, which can be differentiated into multinucleated myotubes. Cell viability assays had been carried out by WST assay, whereas C2C12 differentiation was observed after incubation of C2C12 cells with 2% horse serum and stained with crystal violet. As a result, C2C12 cell viability decreased to 43.89% after treatment with 2 µM doxorubicin for 24 hours. Moreover, C2C12 differentiation is also inhibited by doxorubicin. In contrast, C2C12 cell viability was maintained at 90.45% after treatment with 500 µM naringin. Interestingly, naringin may alleviate doxorubicin cytotoxic effects on C2C12 cell myogenesis.

1. Introduction
Cancer is characterized by abnormal cell proliferation and identified as one of leading causes of mortality [1]. Cancer is generally treated with chemotherapy, surgery, or radiotherapy. Chemotherapeutic agents exert cytotoxic effect on cancer cells that actively proliferate, hence they are often referred to as cytotoxic or antineoplastic agents [2]. One of the first line chemotherapeutic agents widely used is doxorubicin. It is used in the treatment of several types of cancer including breast cancer, ovarian cancer and colon cancer [3,4].

Doxorubicin, an anthracycline drug, is one of the most prescribed chemotherapeutic agents. It works through DNA intercalation and inhibition of topoisomerase-II, an important enzyme for DNA repair during cell cycle progression. Inhibition of cell cycle will trigger cell cycle arrest, which eventually leads to cell death. Doxorubicin can also generate free radicals that interfere with the working of cellular proteins [5]. Doxorubicin has a high cytotoxic effect on cancer cells \textit{in vitro} as indicated by the loss of cell integrity [6].

In addition to its cytotoxic effects on cancer cells, doxorubicin also has side effects on normal cells. Doxorubicin can cause cachexia, which characterized by fatigue, loss of body weight and disturbance of body metabolism [7,8]. It shows side effects in muscle tissue, including cardiac muscle that may cause cardiomyopathy and heart failure [9]. Muscle is part of the musculoskeletal system for support and body movements. Muscle cells play an important role in glucose uptake and maintaining the...
homeostasis of glucose metabolism [10,11]. Failure of the metabolic system due to damage to muscle tissue can cause long term permanent effects that will worsen the condition of cancer patients [12]. Therefore, additional treatment is needed to increase doxorubicin’s efficacy at a lower dose and to reduce its toxic side effects.

To increase the effectiveness of chemotherapy, doxorubicin is generally prescribed as combination regimen with other antineoplastic agents such as 5-fluorouracil (5-FU) and cyclophosphamide [13]. However, administration of doxorubicin in combination with safer, less cytotoxic chemopreventive agents has not been clinically applied widely. Previously, we studied the effects of chemopreventive agents curcumin, naringin and epigallocatechin-3-gallate (EGCG) on C2C12 myoblast cells, where we showed that naringin displayed no significant cytotoxic effects on C2C12 cells at concentrations higher than those of curcumin and EGCG [14]. In this study, we investigated the effects of doxorubicin alone or in combination with naringin to see the potential of naringin in reducing the toxic effect of doxorubicin on C2C12 cells.

2. Material and Methods

2.1. Cell culture and reagents
C2C12 cells were maintained in growth medium containing DMEM medium (Sigma) supplemented with 15% FBS (Sigma) and 100 IU penicillin-100 μg streptomycin (Gibco) in 5% CO2 incubator at 37°C. The cells were obtained from Prof. Hiroshi Itoh, Nara Institute of Sciences and Technology (NAIST), Japan. To induce differentiation into myotubes, confluent C2C12 cells were incubated in differentiation medium containing DMEM medium supplemented with 2% horse serum (HS). Doxorubicin was obtained from Applichem, while 5-fluorouracil (5-FU) and naringin were obtained from Sigma. WST-1 reagent was purchased from Roche and crystal violet solution was purchased from Sigma.

2.2. Cell viability assays
C2C12 cells were seeded onto a 96-well plate at a cell density of 80,000 cells/ml. The following day, the cells were treated with serial concentrations of doxorubicin (25, 100, 250, 500, 1,000 and 2,000 nM), naringin (5, 10, 25, 50, 100, 250, 500 μM), 5-FU (50, 125, 250, 500, 600, 800, 1,000 μM) or DMSO (0.01, 0.02, 0.05, 0.10, 0.20, 0.50, 1.00%). After 24 h, the medium was changed with working solution of WST according to the manufacturer’s protocol and the plate was further incubated for 2 h. The absorbance of each well was directly measured at 450 nm and the cell viability was calculated using the following formula=(absorbance treatment-absorbance blank)/(absorbance nontreated-absorbance blank)x100%.

2.3. Combination assays
C2C12 cells were seeded onto a 96-well plate at a cell density of 80,000 cells/ml. To investigate the additional effect of naringin to doxorubicin-treated C2C12 cells, on the following day the cells were incubated with doxorubicin in combination with naringin and incubated for 24h, with or without 3h pre-incubation with naringin.

2.4. Differentiation assay
About 100,000 C2C12 cells/ml suspended in growth medium were seeded onto a 6-well plate. Two or three days later, the medium was removed and the cells were washed with PBS and differentiation medium containing doxorubicin alone or in combination with naringin. The medium containing tested compounds was changed every two days. At day four or six of differentiation, cells were washed with PBS and stained with 1% crystal violet solution. The differentiation state is defined by calculating the differentiation index and fusion index by using the following formulas. Differentiation index (DI): total nuclei of myotubes versus total nuclei. Fusion index (FI): total nuclei of myotubes with minimal 2 nuclei versus total nuclei.
2.5. Statistical analysis
Data were presented as averages and standard error of the means. t-tests were carried out to analyze the statistical significance of each treatment (* = P<0.05; ** = P<0.01; *** = P<0.001).

3. Results and Discussion
Doxorubicin possesses potent cytotoxic effects to cancer cells, but also causes irreversible and lethal cytotoxic effects to cardiac muscle, with its cytotoxic effect on skeletal muscle potentially leading to cachexia as marked by dramatic muscle loss [7-9]. One potential mechanism by which doxorubicin cytotoxic effects are mediated in skeletal muscle cells is through NF-kB activation [6]. Naringin, a citrus peel flavonoid, demonstrated a chemopreventive effect on HeLa cervical cancer cells through inhibition of NF-kB activity [15]. Our previous study showed that naringin did not show any significant cytotoxic effect on C2C12 myoblast cells [14]. Here, we studied the effect of doxorubicin in C2C12 cells alone or in combination with naringin.

![Figure 1](a) Graphs represent cell viability after treatment with indicated chemicals. Cells were incubated with doxorubicin, 5-FU, naringin or DMSO for 24 h then incubated with WST-1 for 2-3 h. Absorbance of each well was measured at 450 nm. (b) Representative images of cells after 24h treatment with doxorubicin, 5-FU, naringin or DMSO as control solvent.

**Figure 1.** C2C12 cell viability after treatment with doxorubicin, 5-FU and naringin. (a) Graphs represent cell viability after treatment with indicated chemicals. Cells were incubated with doxorubicin, 5-FU, naringin or DMSO for 24 h then incubated with WST-1 for 2-3 h. Absorbance of each well was measured at 450 nm. (b) Representative images of cells after 24h treatment with doxorubicin, 5-FU, naringin or DMSO as control solvent.
Figure 2. C2C12 cell viability after treatment with doxorubicin in combination with naringin. (a) Cells were incubated with doxorubicin (DOX) and naringin (NAR) for 24h. (b) Cells were pretreated with naringin for 3h before combination treatment with doxorubicin and naringin. ** = P<0.01
Figure 3. The effects of doxorubicin and naringin on C2C12 differentiation. C2C12 cells were incubated in the growth medium for two days, then the medium was changed to differentiation medium containing doxorubicin (DOX) or naringin (NAR). Representative images of C2C12 cells observed at day 6 of differentiation after treatment with doxorubicin or naringin alone. Red arrows indicate myotubes.

3.1. Cell viability assays
C2C12 myoblast cells are satellite cells that can generate multinucleated myotube in vitro. Satellite cells are quiescent adult muscle stem cells which can be activated to proliferate and differentiate to repair damaged muscle tissue [16]. To clarify the cytotoxic effect of doxorubicin in C2C12 cells, we examined the cell viability of C2C12 cells after treatment with doxorubicin in comparison with the combination agent 5-FU and the chemopreventive agent naringin. As expected, doxorubicin also showed cytotoxicity to C2C12 myoblast cells as it does to cancer cells. Out of the three compounds, doxorubicin showed the strongest cytotoxic effect to C2C12 cells. Two micromolar of doxorubicin reduced cell viability to 31%. 5-FU as doxorubicin adjuvant, works by inhibiting DNA synthesis and triggering cell cycle arrest at S-phase by inhibition of thymidylate synthase [17]. In combination with doxorubicin as topoisomerase-II inhibitor, they will synergistically inhibit DNA synthesis and repair which results in higher antineoplastic effects. 5-FU was found to show less cytotoxic effect than doxorubicin (Figure 1), yet at concentrations of 250 and 500 µM, 5-FU demonstrated higher cytotoxic effect than naringin. Therefore, as a chemopreventive agent, naringin exerted the least cytotoxic effects to C2C12 compared to the other compounds tested (Figure 1).

3.2. Combinatorial effect of doxorubicin and naringin on cell viability
To understand the effect of naringin in reducing doxorubicin cytotoxicity in C2C12 cells, we performed a cell viability assay of C2C12 after combination treatment with doxorubicin and naringin. Doxorubicin at 10, 25, 50, 100, 500, 1,000 and 2,000 nM were each combined with 5 and 50 µM naringin. After 24 h incubation, there were no significant difference between doxorubicin alone and its combination with naringin at various doxorubicin concentrations (Figure 2A). An exception was observed of the following combinations: doxorubicin 25 nM-naringin 5 µM and doxorubicin 100 nM – naringin 50 µM, where cell viability seemed to be higher than those exposed to a single doxorubicin treatment. Thus, we performed the combination assay after 3h preincubation of C2C12 cells with 5
µM naringin. As a result, naringin significantly increased C2C12 cell viability upon treatment with 25 nM doxorubicin (Figure 2B). However, doxorubicin cytotoxicity seems to be too strong in C2C12 cells, which meant that a longer pretreatment with naringin may be necessary before treatment with a combination of doxorubicin and naringin.

Figure 4. Naringin may alleviate doxorubicin inhibitory effect on C2C12 differentiation. C2C12 cells were incubated for three days in the growth medium, followed by incubation in differentiation medium containing doxorubicin alone or in combination with naringin. Cells were pretreated with naringin for 3 h before combination treatment. (a) Representative images of C2C12 cells observed at day 4 of differentiation. (b) Graph represents the quantification of differentiation index (DI) and fusion index (FI). Red arrows indicate myotubes. *P<0.05.

3.3. Effect of doxorubicin on C2C12 differentiation
Confluent C2C12 cells will differentiate to myocyte then gradually fuse with other cells to form multinucleated myotube [16]. To examine the effects of doxorubicin on C2C12 differentiation, we treated confluent cells with doxorubicin dissolved in differentiation medium two days after cell seeding. The results showed that 100 nM doxorubicin inhibited cell differentiation compared to 10 nM doxorubicin, which may be related to its effect on cell death. On the other hand, exposure to 5 and 50 µM naringin resulted in a better C2C12 differentiation than doxorubicin-treated cells (Figure 3). There were more myotubes observed in naringin-treated cells compared to doxorubicin-treated cells. Because of the cytotoxicity of doxorubicin, we incubated the cells three days prior to incubation with differentiation medium. Then, we observed the combination effect of 100 nM doxorubicin and 5 µM
naringin and calculated the differentiation index (DI) and fusion index (FI). DI of doxorubicin alone and combination with naringin were 0.17 and 0.23, whereas FI of doxorubicin alone and combination were 0.08 and 0.12, respectively. Significantly more myotubes were observed in combination treatment group than in doxorubicin single treatment (P<0.05) (Figure 4), which indicated that naringin may alleviate doxorubicin cytotoxicity in myogenesis.

In this study, the effects of doxorubicin on C2C12 myoblast cells have been investigated through cell viability and cell differentiation assays to determine the effects on myoblast and myogenesis. As expected, doxorubicin showed cytotoxicity by reducing cell viability and inhibiting myogenesis. Interestingly, this study not only demonstrated that naringin had no significant effect on C2C12 cell viability, but it may also alleviate doxorubicin cytotoxicity on myogenesis. Reduction of doxorubicin’s toxic effects by naringin has been shown previously in nude mice bearing HeLa cells, highlighting the potential uses of naringin as a combination agent with doxorubicin [18]. To further clarify the effect of doxorubicin and naringin in C2C12 cell myogenesis, investigation of differentiation marker as well as elucidation of molecular mechanism underlying the doxorubicin cytotoxicity and naringin effect related to NF-kB activation need to be performed.

4. Conclusion
Doxorubicin showed cytotoxic effects on C2C12 myoblast as demonstrated by inhibition of cell viability and C2C12 differentiation. Naringin, which is non-toxic to C2C12 cells, may be beneficial to alleviate doxorubicin cytotoxicity in C2C12 myoblast cells.

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