Cytotoxic effects of chemopreventive agents curcumin, naringin and epigallocatechin-3-gallate in C2C12 myoblast cells

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Abstract. Muscle tissues make up about 40-50% of the human bodies. Satellite cells, which present in between the basal lamina and myofiber, are the adult muscle stem cells or myoblasts which are important for the regeneration of muscle tissues. Anticancer agents generally possess high cytotoxicity to either cancer cells or normal cells. Their effects on muscle cells generate cachexia or the deterioration of muscle tissues. Chemopreventive agents which possess lower cytotoxic effects are expected to show higher safety in normal cells. Therefore, we investigated the effects of chemopreventive agents curcumin, naringin, and epigallocatechin-3-gallate (EGCG), which show anticancer properties in cancer cells, in C2C12 myoblast cells. We observed the C2C12 cell viability by MTT and WST assays, cell migration by wound healing scratch assay, as well as differentiation assay after treatment with the chemopreventive agents. The results indicated that curcumin showed highest cytotoxicity compared to naringin and EGCG. In addition, naringin and EGCG inhibited C2C12 cell migration at cell density 150,000 cells/ml. Whereas, at cell density 100,000 cells/ml, there was no significant effects of naringin as well as EGCG. Altogether, the results suggest that naringin and EGCG possess lower cytotoxic effect on C2C12 myoblast cells whereas curcumin showed stronger cytotoxicity at concentration higher than 20 μM.

Key words: C2C12 myoblast, curcumin, naringin, epigallocatechin-3-gallate, EGCG.

1. Introduction
Metastatic cancer is one of life threatening diseases [1]. Chemotherapeutic agents such as doxorubicin is commonly used for the chemotherapy, including cancer metastasis [2][3]. However, its high cytotoxic effects on cancer cells have raised its side cytotoxic effects to the normal cells. Doxorubicin can generate cachexia or deterioration of skeletal muscle tissues [4][5] and cardiomyopathy to the cardiac muscles as the more serious effect [6].

Muscle tissues make up about 40% of human body weight [7]. Satellite cells, which present in between the basal lamina and myofiber, are the adult muscle stem cells or myoblasts which are important for the regeneration of muscle tissues [8]. Cancer cachexia contributes to 20-30% of chemotherapeutic associated patient morbidities [9]. Thus, cancer adjuvants are important to minimize the doses and side effects of chemotherapeutic agents.

Chemopreventive agents are the compounds that are able to reverse, delay, or prohibit the carcinogenesis [10]. Combination of chemotherapeutic agents and chemopreventive agents have been reported to enhance anticancer effects of the chemotherapeutic agents in vitro or in vivo [11][12]. Furthermore, cytotoxicity tests of those chemopreventive agents to normal cells, such as myoblast cells, are important to determine their safety as cancer adjuvant. In this study, we investigated the effects of chemopreventive agents curcumin, naringin and epigallocatechin-3-gallate (EGCG), which show anticancer properties in several cancer cells, in C2C12 myoblast cells.
2. Material and Methods

Cell culture and reagents. C2C12 myoblast cells were obtained from Prof. Hiroshi Itoh, Nara Institute of Sciences and Technology (NAIST), Japan. The cells were grown in DMEM complete medium (Sigma) (supplemented with 15% FBS (Sigma) and 100 IU penicillin-100 µg streptomycin (Gibco)) in a humid incubator at 37°C in the presence of 5% CO₂. To induce differentiation, after confluence, the cells were incubated in DMEM medium supplemented with 2% horse serum (HS). Curcumin, naringin and EGCG were obtained from Sigma. MTT was purchased from Gibco whereas WST was from Roche.

Cell viability assays. C2C12 cells were seeded onto 96-well plate at a cell density 800,000 cells/ml. One day after seeding, the cells were treated with serial concentration of curcumin (5, 10, 20, 50, 75, 100 µM), naringin (10, 25, 50, 100, 250, 500 µM), EGCG (5, 20, 50, 100, 150, 200 µM) or DMSO as control solvent. After 24 hours, the medium was changed with MTT (0.5 µg/ml) or WST (diluted 10x from stock) solution in DMEM complete medium. For MTT assay, after 2-3 hour incubation, the stopper reagent (10% SDS in 0.01M HCl) was added to each well then the plate was incubated overnight in the dark. The absorbance was measured at 570 nm which is the peak absorbance for formazan [13]. For WST assay, after 2 hours, the absorbance was directly measured at 450 nm. Cell viability was measured as followed: (absorbance treatment-absorbance blank)/(absorbance nontreated-absorbance blank) x 100%.

Wound healing assay. The cells were seeded 100,000 or 150,000 cells/ml per well of a 96 well-plate and incubated overnight. The next day, the cells were scratched by using white tips to introduce cell free gap. Then, the cells were incubated for 6 hours. The pictures were taken after 0 and 6-hour incubation by using Nikon eclipse Ti-S inverted microscope and Nikon DS-Ri2 camera. The wound areas were analyzed by using Image J to calculate wound closure by the formula: (Area₀h - Area₆h)/Area₀h x 100%.

Differentiation assay. C2C12 cells were seeded at a density 100,000 cells/ml in a 6-well plate in DMEM complete medium with 15% FBS. After 2 days, the medium was changed with 2% horse serum (HS)/DMEM containing 50µM naringin, 50µM EGCG or control solvent and incubated for 6 days. The medium containing tested compounds were changed every 2 days.

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

Cell viability assays. To observe the cytotoxic effects of chemopreventive agents curcumin, naringin and EGCG, cell viability assays on C2C12 cells were performed by MTT and WST assay. MTT assay works based on the conversion of yellow MTT into purple formazan crystal. Moreover, WST assay was performed to confirm the results of cell viability assay using MTT assay. The data showed that the trend of cell viabilities of C2C12 cells after treatment with 3 different compounds either with MTT or WST were similar. Curcumin significantly decreased about 19% of cell viability at a concentration of 20 µM based on both MTT and WST results (P<0.01). On the other hands, naringin only declined about 9% of cell viability at a concentration 250µM (P<0.05) as shown by WST assay. In addition, 200 µM of EGCG reduced 25% cell viability compared to control solvent (P<0.01) (Figure 1 and 2). Overall, these results indicate that curcumin showed highest toxicity among the chemopreventive compounds tested. Whereas naringin showed lowest cytotoxicity among the tested compounds.

Wound healing assay. The ability of the myoblast cells to heal the wound also depends on their ability to migrate [14]. Thus, we carried out wound healing migration assay to analyze the migratory capacity of C2C12 myoblast cells after treated with the less toxic compounds which were naringin and EGCG. The data indicated that at a seeding density of 100,000 cells/ml, naringin did not significantly enhance cell migration. Whereas at a seeding density 150,000 cells/ml, both naringin and EGCG
significantly inhibited C2C12 cell migration (P<0.05 or P<0.01) (Figure 3). Naringin may have different effect on cell migration which depends on the density of C2C12 cells. 

**Differentiation assay.** Myoblast cells or the adults muscle stem cells are able to differentiate to form multinucleated myotubes [15]. Thus, cytotoxicity of the chemopreventive agents may affect the differentiation ability of myoblasts into myotubes. Among the tested compounds, naringin might promote cell differentiation compared to control solvent as represented by black arrows pointing the myotubes. In addition, EGCG might inhibit cell differentiation and induce cell death (Figure 4).

**Figure 1.** Investigation of C2C12 cell viability by MTT assay. Cells were incubated with chemopreventive agents or control solvent for 24 hours then incubated with MTT for 2-3 hours. SDS 10% in 0.01 M HCl was used to dissolve formazan crystal. Absorbance was measured at 570 nm.

**Figure 2.** Investigation of C2C12 cell viability by WST assay. Cells were incubated with chemopreventive agents or control solvent for 24 hours then incubated with WST for 2 hours. Absorbance was measured at wavelength 450 nm.
Chemopreventive agents curcumin, naringin and EGCG exhibit inhibitory effects in cancer cells. Curcumin, the main compound of turmeric, inhibits cancer cell proliferation, cell cycle, as well as metastasis [16]. In addition, curcumin promotes apoptosis through inhibition of NF-kB [17]. Naringin, a polyphenolic compound found in citrus peel, exhibited inhibitory effect on HeLa cervical cancer cells growth as well as induction of apoptosis which also depends on the inhibition of NF-kB activity [18]. Furthermore, EGCG, a catechin type polyphenol isolated from green tea, also shows inhibitory effect on NF-kB activity. EGCG has been reported to delay cancer cell proliferation and induce cell death in caspase dependent or independent manners [19]. It has been known that chemotherapeutic agents such as doxorubicin exerts side effects to cancer patients. Despite its potent cytotoxic effects to cancer cells, it also causes cytotoxicity to the normal cells including the skeletal as well as the cardiac muscle cells [4][5][6]. Doxorubicin effects on the skeletal muscle cells cause cachexia which marked by the shrinkage of muscle tissues. Activation of NF-kB is one of the mechanisms by which doxorubicin induces skeletal muscle damage [20]. Therefore, those three compounds are selected in this study to be investigated to elucidate their cytotoxic effects on C2C12 myoblast cells.

The cytotoxic effects of curcumin, naringin and EGCG on C2C12 myoblast cells had been performed by both MTT and WST assays. Among the tested compounds, curcumin showed the highest cytotoxic effect on C2C12 cells which indicated by inhibition of cell viability. In addition, naringin exerted lowest inhibitory effect on C2C12 cell viability. EGCG showed inhibitory effect in both migration and differentiation assay. It also promoted cell death during cell differentiation. Naringin also inhibited cell migration at a higher cell density, whereas at lower cell density, it might induce cell migration. Furthermore, naringin may promote differentiation of C2C12 cells. Naringin’s inhibitory effect on cell migration at a higher cell density may occur because it induced myocyte differentiation. Cytotoxic effect of doxorubicin in C2C12 cells is higher than curcumin, naringin and EGCG. Low concentration of doxorubicin, as low as 2 µM, causes cell death which indicated by the loss of cell integrity [20]. However, the cytotoxic effects of doxorubicin in C2C12 cells by MTT or WST, as single agent or in combination with curcumin, naringin or EGCG still need to be investigated.

Cancer adjuvant has been implicated in cancer therapy to enhance the outcome of the treatment including to increase the effectivity of anticancer effect as well as to reduce the side toxic effects of chemotherapeutic agents. Curcumin as a chemopreventive compound has been widely explored as cancer adjuvant. The combination treatments of curcumin and chemotherapeutic agents such as doxorubicin have been investigated in cancer cells [21]. However, curcumin also showed cytotoxicity in C2C12 cells. Yu et al. [22] reported that curcumin at concentration 10-50 µM exhibited cytotoxicity and ROS generation in C2C12 cells. Whereas, at lower concentration which does not affect the cell viability, curcumin may play a role as antioxidant. Further studies are needed to clarify the effect of low concentration of curcumin in myoblast cells to study its potency to treat cachexia. Naringin and EGCG which exerted lower cytotoxic effects on C2C12 cells have also been investigated as cancer adjuvant. Combination of doxorubicin with naringin in HeLa cervical cancer cells enhances the inhibitory effects on cell viability compared with single doxorubicin treatment. In addition, naringin attenuate doxorubicin toxicity effect in nude-mice bearing HeLa cells [12]. On the other hand, EGCG has also been reported to show synergistic effects with doxorubicin [11]. Elucidation of the appropriate concentrations of curcumin, naringin or EGCG in combination with doxorubicin to decline the doxorubicin cytotoxic effect on C2C12 cells or skeletal muscle in vivo are required to minimize the occurrence of cachexia.
Figure 3. Representative images of wound healing assay on C2C12 cells. Cells were seeded at cell density 100,000 (A) or 150,000 cells/ml (B). After introduction of wound, cells were treated with naringin or EGCG and incubated for 6 hours. (C) Graphs represented wound closures (%) after analyses by using image J.
Figure 4. C2C12 differentiation assay of naringin and EGCG. Cells were grown in DMEM complete medium supplemented with 15% FBS in a 6-well plate until confluence, then the medium was changed into 2% HS/DMEM containing 50µM naringin, 50µM EGCG or control solvent. The multinucleated myotubes were observed at day 4 and 6. ◆: myotube, ♦: group of death cells.

4. Conclusion

To conclude, our study revealed the cytotoxic effect of curcumin, naringin and EGCG in C2C12 myoblast cells. All compounds showed different level of cytotoxicity on cell viability. Thus, further determination of the optimal concentration of each compound to eliminate its cytotoxic effect while showing the beneficiary effects on C2C12 cells are need to be carried out. Curcumin possesses higher cytotoxic effect than naringin and EGCG. Meanwhile, naringin showed the lowest cytotoxic effect and may be potential for the treatment of muscle regeneration.

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6. References

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