Inhibitory Effect of D-chiro-inositol on Both Growth and Recurrence of Breast Tumor from MDA-MB-231 Cancer Cells

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Abstract – D-chiro-inositol (DCI) is a secondary messenger in insulin signal transduction. It is produced in vivo from myo-inositol via action of an epimerase. In this study, we evaluated antitumor activity of DCI against human breast cancer both in vitro and in vivo. In order to determine the inhibitory effects of DCI on growth of human breast cancer cells (MDA-MB-231), two different assessment methods were implemented: MTT assay and mouse xenograft assay. MTT assay demonstrated downturn in cell proliferation by DCI treatment (1, 5, 10, 20 and 40 mM) groups by 18.3% (p < 0.05), 17.2% (p < 0.05), 17.5% (p < 0.05), 18.4% (p < 0.05), and 24.9% (p < 0.01), respectively. Also, inhibition of tumor growth was investigated in mouse xenograft model. DCI was administered orally at the dose of 500 mg/kg and 1000 mg/kg body weight to treat nude mouse for 45 consecutive days. On the 45th day, tumor growth of DCI (500 mg/kg and 1000 mg/kg) groups was suppressed by 22.1% and 67.6% as mean tumor volumes were 9313.8 ± 474.1 mm³ and 3879.1 ± 1044.1 mm³, respectively. Furthermore, breast cancer stem cell (CSC) phenotype (CD44+/CD24−) was measured using flow cytometry. On the 46th day, CSC ratios of DCI (500 mg/kg) and co-treatment with doxorubicin (4 mg/kg) and DCI (500 mg/kg) group decreased by 24.7% and 53.9% (p < 0.01), respectively. Finally, from tumor recurrence assay, delay of 5 days in the co-treatment group compared to doxorubicin (4 mg/kg) alone group was observed. Based on these findings, we propose that DCI holds potential as an anti-cancer drug for treatment of breast cancer.

Keywords – D-chiro-inositol, Breast cancer, MDA-MB-231, Cancer stem cell, Recurrence

Introduction

D-chiro-inositol (DCI) is a secondary messenger in insulin signal transduction. It is produced in vivo from myo-inositol via action of an epimerase. Clinical test results have shown that DCI treatment lowers testosterone level and increases insulin sensitivity and frequency of ovulation in women.

Breast cancer is a major health problem that is detrimental to the lives of millions of women. For the year 2015, it was estimated that 19,465 women in Republic of Korea will be diagnosed with breast cancer and that 2,367 women will succumb to it. With these numbers, breast cancer is the most commonly diagnosed cancer among Korean women and comes second in terms of total cancer incidence. From 1999 to 2012, annual percentage change of breast cancer incidence rate increased by 6.1% in Republic of Korea.

Recurrence is hardly rare in breast cancer, and is a major contributor to breast cancer-related deaths. Identified causes of recurrence are cancer stem cell (CSC), epithelial-mesenchymal transition (EMT), β1-integrin, notch signaling, wnt signaling, hedgehog signaling and miRNA. CSCs are group of cancer cells capable of self-renewing and producing heterogeneous lineages of cancer cells. A number of cell surface markers such as CD44+/CD24− are related to CSCs.

In this study, we evaluated the antitumor activity of DCI against human breast cancer was both in vitro and in vivo.

Experimental

Materials – The materials used and its vendors are as follows: d-chiro-inositol, doxorubicin hydrochloride, metformin, bovine serum albumin (BSA), 2,2,2-tribromoethanol, 2-methyl-2-buranol from Sigma (St. Louis, MO, USA); Rosewell Park Memorial Institute medium 1640 (RPMI 1640), fetal bovine serum (FBS), trypsin-
Tumor volumes were estimated by the following formula: $V = \frac{4}{3} \pi \rho \text{length} \times \text{width} \times \text{height} / 2$. To determine the toxicity of DCI, body weight changes of the mice were measured once every other day. On day 25 and 45, tumor was separated from mouse.

**Nude mouse xenograft assay for tumor recurrence** – In tumor recurrence assay, DCI at a dose of 500 mg/kg body weight was administered orally to treat nude mouse for 63 consecutive days. Doxorubicin hydrochloride was injected intratumorally once every five days (day 0, 5, 10, 15) at a dose of 4 mg/kg. Other conditions were the same as mouse xenograft assay for tumor growth.

**Cancer stem cell ratio analysis** – Single-cell suspensions were obtained from tumor tissue, and lysis red blood cell using ACK solution. Single cells were treated with CD44 antibody and CD24 antibody for 20 min. Breast cancer stem cell phenotype (CD44+/CD42−) was measured using BD canto II.

**Statistical analysis** – Statistical analysis of data were performed using IBM SPSS statistics 18 software (IBM corporation, Armonk, NY, USA). Statistical significance ($p < 0.05$) was assessed by one-way analysis of variance (ANOVA) coupled with scheffe test.

**Result and Discussion**

To determine the effects of DCI on the growth of human breast cancer cells, growth inhibitory effect was evaluated by MTT assay and mouse xenograft assay. MDA-MB-231 cells were treated with doxorubicin hydrochloride (final concentration: 1 µM), metformin (final concentration: 5 mM) and DCI (final concentration: 1, 5, 10, 20 and 40 mM) for 48 hr. Cell proliferation of doxorubicin hydrochloride group decreased by 21.7% ($p < 0.01$), compared to V.C group; metformin group decreased by 18.3% ($p < 0.01$), 17.2% ($p < 0.05$), 17.5% ($p < 0.05$), 18.4% ($p < 0.05$), and 24.9% ($p < 0.01$), respectively (Fig. 1).

Also, suppression of tumor growth was investigated in mouse xenograft model. DCI at a dose of 500 mg/kg and 1000 mg/kg body weight was administered orally to treat nude mouse for 45 consecutive days. Metformin at a dose of 500 mg/kg was administered by the same method as DCI group. Doxorubicin hydrochloride was injected intratumorally once every five days (day 0, 5, 10, 15) at a dose of 4 mg/kg. On day 0, MDA-MB-231 cells (9 × 10⁶ cell/mouse) in 100 µl of PBS were injected subcutaneously into nude mice. When the tumor size reached 50 mm³,
mice were randomly distributed into equal groups (7 mice per group). The body weights of tumor-bearing nude mice were measured to evaluate overall toxicity of DCI. After drug administration, body weights had been reduced insignificantly, disproving the toxicity of DCI (Fig. 2.B). On day 25, tumor growth of doxorubicin (4 mg/kg) group was inhibited by 94.1%; and mean tumor volume was $190.8 \pm 105.2 \text{mm}^3$ ($p < 0.05$), compared to V.C group. Tumor growth of metformin (500 mg/kg) group was inhibited by 77.3%; and mean tumor volume was $736.3 \pm 409.2 \text{mm}^3$. Tumor growth of DCI (500 mg/kg and 1000 mg/kg) groups were inhibited by 22.8% and 66%; and mean tumor volumes were $2503.1 \pm 714.5 \text{mm}^3$ and $1101.4 \pm 564.1 \text{mm}^3$, respectively. Tumor growth of co-treatment with doxorubicin (4 mg/kg) and metformin (500 mg/kg) was inhibited by 96.4%; and mean tumor volume

Fig. 1. Growth inhibition of DCI on breast cancer cell lines. MDA-MB-231 cell lines were treated with 1 - 40 mM DCI for 48 hours. Doxorubicin (1 µM) and metformin (5 mM) were used as comparisons. Cell viability of MDA-MB-231 cell lines was estimated by MTT assay ($p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Fig. 2. Inhibition of breast tumor growth by DCI in nude mouse xenograft assay. On day 0, nine million MDA-MB-231 cells were implanted subcutaneously into nude mice. DCI at a dose of 500 mg/kg or 1000 mg/kg body weight was administered orally for 45 consecutive days. (A) The body weights of the tumor-bearing nude mice were measured to evaluate overall toxicity of DCI. (B) Tumor volumes were estimated by the formula: length (mm) × width (mm) × height (mm)/2. Photographs of representative MDA-MB-231 tumor mass on (C) day 25 and (D) day 45 were shown. On (E) day 25 and (F) day 45, relative tumor size was measured ($p < 0.05$, $**p < 0.01$, $***p < 0.001$).
was 117 ± 17 mm$^3$ ($p < 0.05$). Tumor growth of co-treatment doxorubicin (4 mg/kg) and DCI (500 mg/kg or 1000 mg/kg) were inhibited by 93.1% and 98.5%; and mean tumor volumes were 821.1 ± 27.9 mm$^3$ and 184.9 ± 110.2 mm$^3$ ($p < 0.05$), respectively (Fig. 2 A, D, F).

Furthermore, the population of the CSCs was investigated using flow cytometry. Single-cell suspensions were obtained from tumor tissue, and lysis red blood cell using ACK solution. Single cells ($1 \times 10^6$ cell) were stained with CD44 antibody and CD24 antibody for 20 min on ice. Breast cancer stem cell phenotype (CD44$^+$/CD24$^-$) was measured using BD canto II. On day 25, CSCs ratio of doxorubicin (4 mg/kg) group decreased by 62.7% ($p < 0.05$), compared to V.C group; and DCI (500 mg/kg) and co-treatment with doxorubicin (4 mg/kg) and DCI (500 mg/kg) decreased by 23.8% and 70.2% ($p < 0.05$), respectively (Fig. 3A) On day 46, CSCs ratio of doxorubicin (4 mg/kg) group increased by 1.3%, compared to V.C group; and DCI (500 mg/kg) and co-treatment with doxorubicin (4 mg/kg) and DCI (500 mg/kg) decreased by 24.7% and 53.9% ($p < 0.01$), respectively (Fig. 3B).

Moreover, mouse xenograft assay was implemented to determine the effect of DCI on recurrence of breast cancer. Tumor recurrence assay, DCI at a dose of 500 mg/kg body weight was administered orally for 63 consecutive days. Doxorubicin at a dose of 4 mg/kg was injected within breast tumor four times a day for 15 days.

In conclusion, inhibitory effect of DCI was confirmed...
by MTT assay in MDA-MB-231 cells. Also, compared to the untreated control group of mice, tumor growth was hindered and CSCs ratio diminished in DCI administered nude mice. Additionally, tumor recurrence lagged in co-treatment group with DCI and doxorubicin compared to doxorubicin administration group. Thus, we propose DCI as a potential anti-cancer drug for the treatment of breast cancer.

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References


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