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DNA microarray analysis of genes in highly metastatic 4T1E/M3 murine breast cancer cells following exposure to cannabidiolic acid

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ABSTRACT — We previously identified cannabidiolic acid (CBDA), a major component of the fiber-type cannabis plant, as an inhibitor of MDA-MB-231 human breast cancer cell migration *in vitro* (Takeda *et al*., 2012). Although MDA-MB-231 is a widely used human breast cancer cell line in *in vitro* and *in vivo* studies, these cells have to be injected into nude mice (immunodeficient animals) in *in vivo* trials. Thus, we established the murine breast cancer cell line, 4T1E/M3, which is highly metastatic to bone in BALB/c mice (Takahashi *et al*., 2008, 2009; Sakai *et al*., 2012); this murine syngeneic tumor model may be useful for identifying molecular targets for therapeutic interventions. Prior to *in vivo* experiments using the murine tumor model, we herein performed DNA microarray analyses of 4T1E/M3 cells, treated with CBDA for 48 hr at a sub-toxic concentration (25 μM), in order to comprehensively analyze the effects of CBDA on the genes involved in the bone metastasis of breast cancers. The results obtained revealed that the expression of matrix metalloproteinase-9 (MMP-9), transforming growth factor-β (TGF-β) inducible gene H3 (BIGH3), and parathyroid hormone-related protein (PTHrP) was markedly down-regulated by 0.11-fold, 0.22-fold, and 0.15-fold, respectively; these molecules were mutually involved in the bone metastasis of breast cancer cells.

Key words: Cannabidiolic acid, 4T1E/M3 cells, Bone metastasis, Fiber-type cannabis plant

INTRODUCTION

The biological activities of cannabidiolic acid (CBDA), which exists in the acid form of CBD, have not been examined as extensively as those of cannabidiol (CBD), a major non-psychotropic constituent of the fiber-type cannabis plant (Fig. 1) (Yamauchi *et al*., 1967; Turner *et al*., 1980; Taura *et al*., 2007) because CBDA is recognized as the pharmacologically inactive form (Yamauchi *et al*., 1967; Razdan, 1986; Burstein, 1999). However, recent studies including ours demonstrated that CBDA by itself exhibited the following biological actions; antibacterial effects (Appendino *et al*., 2008), the inhibition of cyclooxygenase-2 (COX-2) (Takeda *et al*., 2008), and anti-nausea/emetic effects (Bolognini *et al*., 2013; Rock and Parker, 2013). In addition, we found that CBDA abrogated human MDA-MB-231 breast cancer cell migration by inhibiting the activity and/or expression of COX-2 (Takeda *et al*., 2012, 2014; Takeda, 2013), thereby demonstrating the usefulness of CBDA as an anti-migration agent. Although MDA-MB-231 cells are widely used in bone metastasis studies *in vivo*, difficulties are associat-

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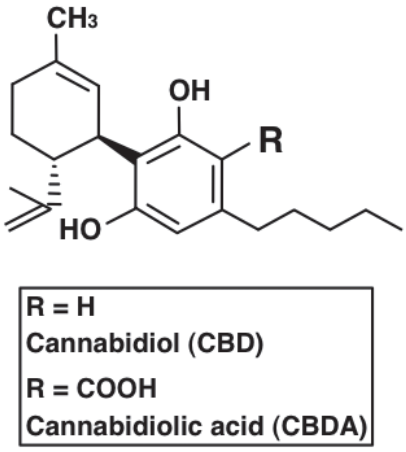

Fig. 1. Chemical structures of CBD and CBDA. The chemical structures of CBD and CBDA are shown. CBD (R = H) is formed from CBDA (R = COOH) by decarboxylation (–COOH).

ed with the preparation of intracardiac injections for mice and immunodeficient animals are also required in order to establish a human carcinoma in mice (Yoneda *et al*., 2001; Sheridan *et al*., 2006).

Distant organ metastasis accounts for the majority of deaths caused by solid tumors; among organs, bone is a well-recognized site of metastasis (Mundy, 2002). Approximately 90% of breast cancer-related deaths have been attributed to metastasis to bone and the lungs (Hanahan and Weinberg, 2011). In order to investigate the molecular mechanisms of metastasis underlying the dissemination of breast cancer cells from a primary tumor to bone and overcome the unfavorable issues described above, murine syngeneic tumor models in which murine breast cancer cells exhibit high metastatic potency to bone need to be established. We successfully established the murine metastatic model cell line, 4T1E/M3 using parent BALB/c-derived 4T1 spontaneous mammary carcinoma cells (Aslakson and Miller, 1992; Takahashi *et al*., 2008). The frequency of spine metastasis was markedly higher in 4T1E/M3 cell-injected BALB/c mice than in parent 4T1 cell-injected BALB/c mice (*i.e.*, 14% vs. 77%) (Takahashi *et al*., 2008).

In the present study, we attempted to determine whether CBDA modulated the genes involved in bone metastasis in 4T1E/M3 cells treated with this molecule by performing DNA microarray analyses. We herein showed that CBDA abrogated the expression of matrix metalloproteinase-9 (MMP-9), transforming growth factor-β (TGF-β) inducible gene H3 (BIGH3), and parathyroid hormone-related protein (PTHrP), which are mutually responsible for breast tumor metastasis to bone (Yin *et al*., 1999; Buijs *et al*., 2012; Kim *et al*., 2012).

MATERIALS AND METHODS

Materials and cell culture

CBDA (purity: 96.5%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade, commercially available, and used without further purification. Cell culture (murine breast cancer 4T1E/M3 cells) conditions and methods were based on previously described procedures (Takahashi *et al*., 2008; Takeda *et al*., 2014). Briefly, the 4T1E/M3 cell line (Takahashi *et al*., 2008) was routinely grown in phenol red-containing minimum essential medium alpha (Invitrogen, Carlsbad, CA, USA), supplemented with 10 mM HEPES, 5% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a 5% CO_2–95% air-humidified incubator. Prior to the 24-hr CBDA treatments, the medium was changed to phenol red-free minimum essential medium alpha (Invitrogen) supplemented with 10 mM HEPES, 5% dextran-coated charcoal-treated serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. CBDA was prepared in ethanol. Control incubations contained equivalent additions of ethanol.

Preparation of total RNA and DNA microarray analyses

Total RNA was collected from 25 μM CBDA or vehicle-treated 4T1E/M3 cells or MDA-MB-231 cells (3×10^5 cells/well) 48 hr after the exposure using the RNeasy kit (Qiagen, Inc., Hilden, Germany), and was purified using RNeasy/QIAamp columns (Qiagen, Inc.). The specific gene expression patterns in 4T1E/M3 and MDA-MB-231 cells were examined by a DNA microarray analysis and compared with that in vehicle-controls. Total RNA was extracted from both cell types, and the synthesis of complementary DNA (cDNA) and cRNA labeling were conducted using a Low RNA Fluorescent Linear Amplification kit (Agilent, Palo Alto, CA, USA). Overall changes in gene expression were evaluated using a two-color microarray-based gene expression analysis (Takeda *et al*., 2014). Labeled cRNA (Cy3 to control, Cy5 to CBDA) was hybridized to mouse or human oligo DNA microarray slides (Agilent) that carried spots for mouse or human genes. Specific hybridization was analyzed using a Microarray scanner (Agilent) and evaluated as a scatter-plot graph for gene expression. Hokkaido System Science (Sapporo, Japan) provided assistance with the

experiments.

Analysis of reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from 4T1E/M3 cells using the RNeasy kit (Qiagen, Inc.) and was purified using RNeasy/QIAamp columns (Qiagen, Inc.). The subsequent synthesis of cDNA, RT, and PCR were performed using the SuperScript One-Step RT-PCR System with Platinum *Taq* polymerase (Invitrogen). The primers used for PCR were as follows: cannabinoid receptor type 1 (CB1) (sense), 5'-CTT GCA GAT ACC ACC TTC CGT-3'; CB1 (antisense), 5'-ACT GGA TGT TGT CCT CGT TCT-3'; (CB2) (sense), 5'-CAA CGC TAT CTT CCT GCT GA-3'; (CB2) (antisense), 5'-CAA TGA ATA GAA GCC AGC CCA-3'; β-actin (sense), 5'-CTG TGG CAT CCA TGA AAC TAC-3', β-actin (antisense), 5'-ACA CAG AGT ACT TGC GCT CA-3'. PCR products were separated by 1.5% agarose gel electrophoresis in Tris-acetate EDTA (ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid) buffer and stained with ethidium bromide. β-Actin was used as a housekeeping gene for RT-PCR.

RESULTS AND DISCUSSION

As shown in Fig. 2 (Control), cultured 4T1E/M3 cells exhibited a typical cobblestone-like morphology. Although no marked morphological change was observed at 5 μM CBD and CBDA, 4T1E/M3 cells became elongated without significant cell death (~70% viability) when exposed to a sub-toxic concentration of CBDA (25 μM) for 48 hr. However, the same concentration of CBD resulted in the complete death of cells. This was expected because we and other studies previously reported that the anti-proliferative potential of CBDA on various cancer cells, including MDA-MB-231 cells, was markedly less than that of CBD (IC_{50} values of CBD and CBDA = 1~10 μM and > 25 μM, respectively) (Ligresti *et al*., 2006; McAllister *et al*., 2007; Shrivastava *et al*., 2011: Takeda *et al*., 2012, 2014). The elongated morphology was also observed in MDA-MB-231 cells treated with CBDA (Takeda *et al*., unpublished observation). The implications of CBDA-evoked morphological changes in 4T1E/M3 cells (including MDA-MB-231 cells) are currently being investigated.

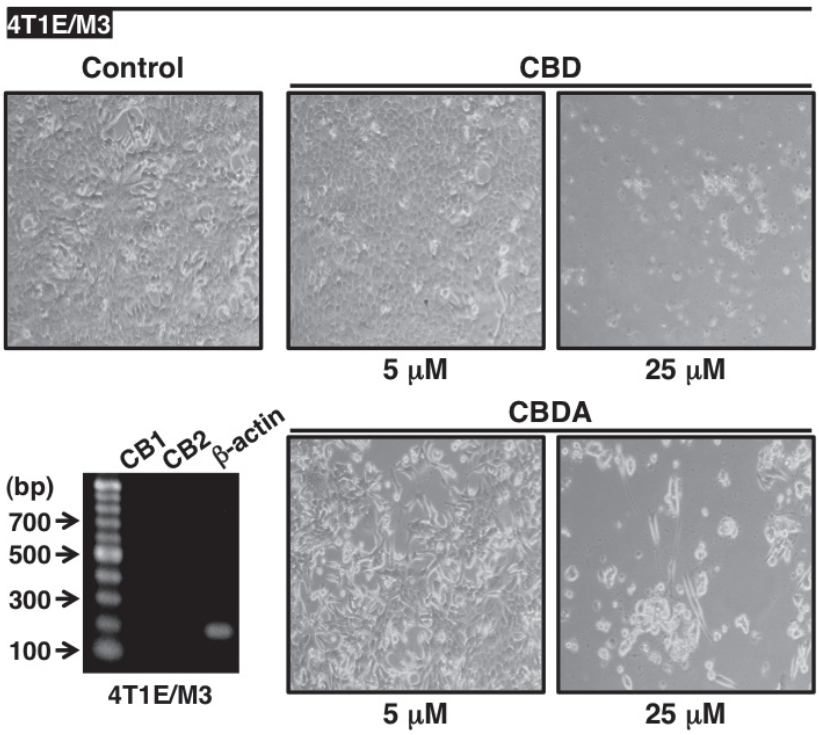

Fig. 2. Effects of CBD and CBDA on the morphology and viability of highly aggressive murine 4T1E/M3 breast cancer cells. The morphology of 4T1E/M3 cells 48 hr after the CBD (5, 25 μM) or CBDA (5, 25 μM) treatment. Control cells were treated with vehicle alone. (*Inset*) RT-PCR analyses of basal CB1 and CB2 levels in 4T1E/M3 cells. β-Actin was used as a housekeeping gene for RT-PCR.

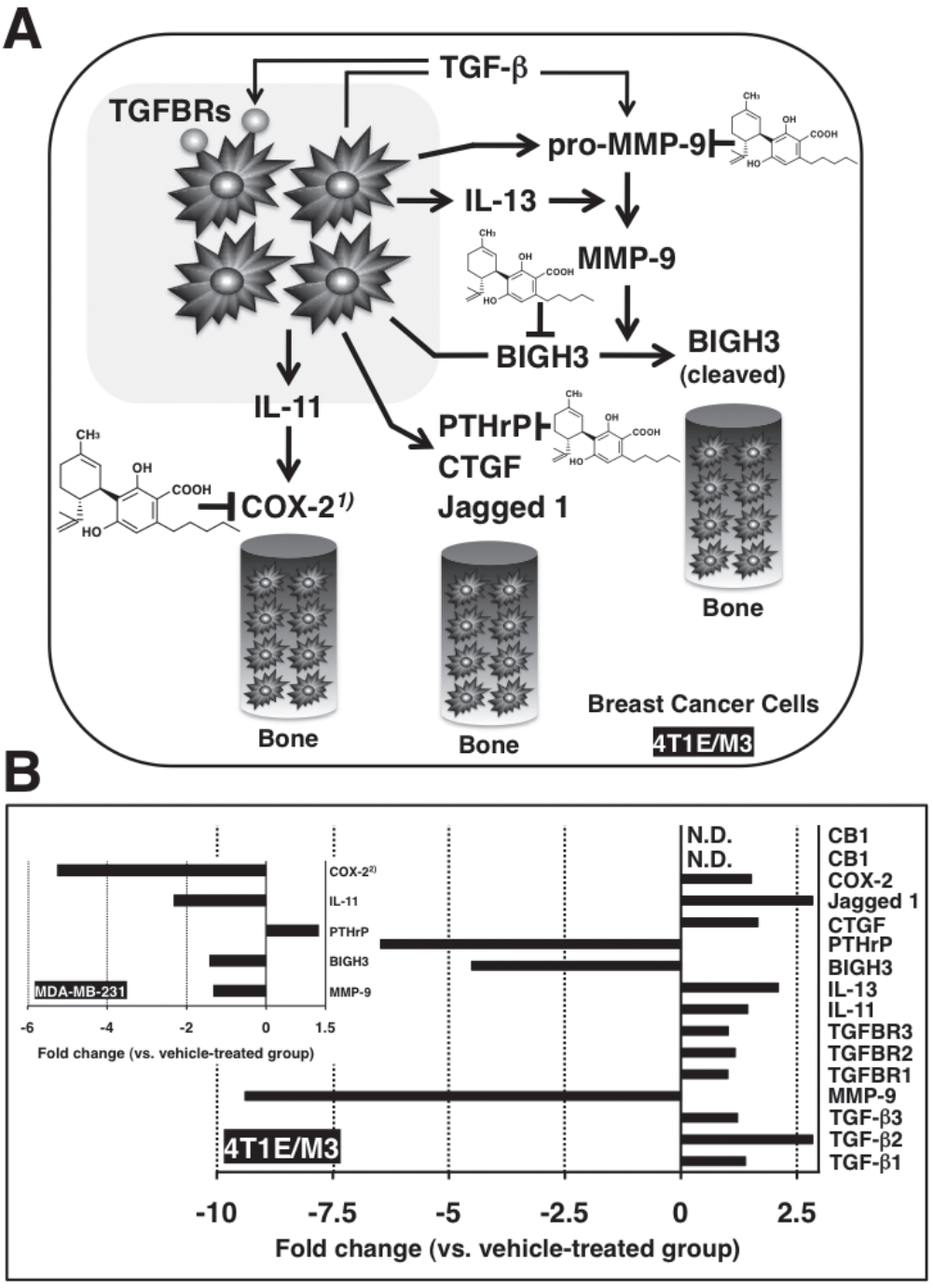

Fig. 3. Effects of CBDA on genes involved in breast cancer metastasis to bone. (A) Collective pathways of breast cancer metastasis to bone. CBDA inhibits the enzymatic activity of COX-2 or down-regulates genes (MMP-9, BIGH3, and PTHrP). [1)] The enzyme inhibition of COX-2 by CBDA has been reported previously (Takeda *et al*., 2008). (B) Results of the DNA microarray analysis. Data are expressed as a fold change vs. vehicle-treated groups. 4T1E/M3 cells were treated with vehicle or 25 μM CBDA for 48 hr, followed by the isolation of total RNA. (*Inset*) Results of the DNA microarray analysis of samples obtained from MDA-MB-231 cells. Cells were treated with vehicle or 25 μM CBDA for 48 hr, followed by the isolation of total RNA. [2)] The down-regulation of COX-2 in MDA-MB-231 cells treated with CBDA has been reported previously (Takeda *et al*., 2014).

We performed a DNA microarray analysis to investigate the genes regulated by CBDA in 4T1E/M3 cells under the condition of 25 μM CBDA for 48 hr. Of the genes analyzed in 4T1E/M3 cells, CBDA increased the expression of 307 genes (> 2.5-fold) and decreased the expression of 639 genes (< 0.4-fold). We focused on the fourteen genes that were previously shown to be mutually involved in the bone metastasis of breast cancer (Yin *et al*., 1999; Buijs *et al*., 2012; Kim *et al*., 2012): TGF-β1, TGF-β2, TGF-β3, MMP-9, TGF-β receptor 1 (TGFBR1), TGF-β receptor 2 (TGFBR2), TGF-β receptor 3 (TGFBR3), interleukin 11 (IL-11), IL-13, BIGH3, PTHrP, connective tissue growth factor (CTGF), Jagged 1, and COX-2. The expression of MMP-9, BIGH3, and PTHrP was down-regulated by CBDA by 0.11-fold, 0.22-fold, and 0.15-fold, respectively, while that of TGF-β2 and Jagged 1 was co-upregulated by 2.84-fold (Fig. 3B).

COX-2 is expressed in approximately 40% of human invasive breast cancers (Singh *et al*., 2007; Holmes *et al*., 2011), and its expression is known to be positively regulated by IL-11, coupled with enhanced breast tumor metastasis to bone (Sotiriou *et al*., 2001; Yoshinaka *et al*., 2006; Singh *et al*., 2007). Thus, one strategy to reduce the risk of breast cancer metastasis to bone is to utilize COX-2 inhibitors. The DNA microarray data in Fig. 3B showed that the expression of IL-11 and COX-2 was modestly stimulated (1.66- and 1.69-fold, respectively); however, inverted modulation by CBDA occurred in MDA-MB-231 cells (Fig. 3B, *inset*) (*See* Takeda *et al*., 2014). We previously identified CBDA as a selective COX-2 inhibitor (Takeda *et al*., 2008); therefore, it may abrogate the signaling of COX-2-mediated bone metastasis in an animal model treated with 4T1E/M3 cells through the inhibition of enzyme activity (*see* Fig. 3A).

Previous studies demonstrated that TGF-β played an important role in the development of breast cancer metastasis to bone through BIGH3, PTHrP, CTGF, and Jagged 1 (Yin *et al*., 1999; Rahim *et al*., 2014). TGF-β was found to be secreted from bone (osteoclasts) as well as breast cancer cells, and then interacted with TGFBRs expressed in breast cancers to activate its signaling (Rahim *et al*., 2014). In addition, after the activation of TGF-β signaling, the expression of MMP-9 was stimulated (*i.e.*, pro-MMP-9 production), and subsequently finally activated by IL-13 to exhibit full catalytic activity. BIGH3 was thereafter cleaved by the activated MMP-9 enzyme, leading to increased cell invasion (*i.e.*, MMP-9) via IL-13, and BIGH3 was thereafter cleaved by the activated enzyme, leading to increases in cell invasion (Pivetta *et al*., 2011; Gomes *et al*., 2012). Although the expression of Jagged 1, which is up-regulated by TGF-β, was strongly stimulated by CBDA, that of other genes activated by TGF-β-mediated signaling was decreased. Since TGF-β is functionally active, CBDA may interfere with the interaction between TGF-β with TGFBRs, resulting in the significant suppression of BIGH3 and PTHrP in 4T1E/M3 cells. Studies on this point are ongoing. An mRNA analysis of cannabinoid receptors (CB1 and CB2) in 4T1E/M3 cells revealed that the basal expression of CB1/CB2 was very low or undetectable (Figs. 2 *inset* and 3B), indicating that the involvement of these receptors in the CBDA-mediated modulation of these genes is low.

In summary, the results of the present study suggest that, in addition to the phenomenon already observed in MDA-MB-231 cells, CBDA down-regulated the expression of the genes participating in the bone metastasis of 4T1E/M3 cells, thereby potentially abrogating metastasis. Further studies are needed to demonstrate the biological effects of CBDA in an *in vivo* model using BALB/c mice injected with 4T1E/M3 cells.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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