Osteosarcoma is an aggressive type of cancer primarily found in children and adolescents and current understanding of its etiology and treatments are rather limited. As a potential anti-cancer component, conjugated linoleic acid (CLA) has drawn significant attention in the last few decades. However, there is limited knowledge on CLA's effect on osteosarcoma. In this report, we tested effects of two major CLA isomers on growth of a canine osteosarcoma cell line. The results in this report suggest that the trans-10,cis-12 CLA isomer, but not the cis-9,trans-11 isomer, effectively inhibits canine osteosarcoma cell growth and induces apoptosis. Treatment with trans-10,cis-12 CLA increased expression of NSAID activated gene-1 (NAG-1), suggesting that NAG-1 may mediate trans-10,cis-12 CLA-induced apoptosis, via phosphoinositol-3-kinase (PI3K) dependent mechanism. The significance of this study is that CLA might be used as a dietary supplement in prevention and/or treatment of osteosarcoma. Journal of Nature and Science, 1(4):e67, 2015

Conjugated linoleic acid | CLA | osteosarcoma | NAG-1

Introduction

Osteosarcoma is one of the most aggressive types of cancer in children and adolescents (1). Overall, it comprises approximately 20% of all bone tumors including about 5% of pediatric tumors. On average, approximately 400 children and adolescents are diagnosed with osteosarcoma in the US each year, where the majority of them are in their early adolescence (1-3). It was suggested that osteosarcoma may be a disease of differentiation, particularly associated with impairment of osteoblastic differentiation, however, the current understanding of osteosarcoma etiology is relatively limited (2). To date, the survival rate for osteosarcoma patients is 70%, but 20% of all diagnosed osteosarcoma patients experience severe side effects from chemotherapy (2). Thus, there is a great need for understanding osteosarcoma to develop treatment as well as prevention strategies for osteosarcoma.

Conjugated linoleic acid (CLA) is a type of dietary fatty acid, which has been shown to have an anti-cancer effect in a number of studies (4). CLA is a geometric and positional isomer of linoleic acid, and was originally identified as an anti-cancer component from ground beef (5). Since then, CLA has drawn considerable attention due to its other beneficial effects, such as immune modulation, reduction of atherosclerosis, and reduction of body fat while enhancing lean body mass (6, 7). The anticancer properties of CLA have been reported in animal models, and it is suggested that CLA acts on all stages of cancer: initiation, promotion, progression, and metastasis by modulating eicosanoids production, interfering with cell signaling pathways, inhibiting DNA synthesis, promoting apoptosis, and modulating angiogenesis (8-17).

Currently the bioactivities of two isomers of CLA, the cis-9,trans-11 and the trans-10,cis-12, are the focus of much study. The cis-9,trans-11 CLA isomer, the major isomer present in food sources, derives naturally from rumen bacteria via biohydrogenation (18), or alternatively by Δ9 desaturation of trans-11 vaccenic acid in mamalian tissues (19, 20). On the other hand, the trans-10,cis-12 CLA isomer is present in relatively low amounts in natural sources. However, when CLA is prepared by chemical isomerization of linoleic acid from either pure or vegetable oils, it consists of approximately 40-45% trans-10,cis-12 CLA along with a similar amount of the cis-9,trans-11 CLA (21). It has been reported that these CLA as a mixture of these two isomers can have various biological effects, including effects on cancer suppression in a number of human cancer cell lines and animal models (6, 7, 22-26). There are studies using two separate CLA isomers in cancer studies; the trans-10,cis-12 CLA isomer is more effective than the cis-9,trans-11 isomer with regard to its anti-cancer effect, while others reported no differences between these two isomers or greater effects of the cis-9,trans-11 isomer (25, 27-32). Thus, the two CLA isomers may exert anti-cancer effects through different and/or independent mechanism(s) or depending on cell context, and understanding their exact roles in cancer prevention is necessary in order to determine the proper application of CLA.

Previously, it has been suggested that one of the main anti-cancer mediators of CLA is non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1) (12). Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of inflammatory disease. NAG-1 (also known as Growth Differentiation Factor-15, GDF-15) has been suggested as one of the main targets of NSAIDs and is a member of the transforming growth factor-β (TGF-β) super family, which plays a role in apoptosis, reduction of cell proliferation, and inhibition of cell cycle progression (33). It has been suggested that NAG-1 expression can be induced by several dietary bioactive compounds such as capsaicin (34), 6-gingerol (35), indole-3-carbolin (36), genistein (37), epigallocatechin gallate (38), tlemfanolic acid (39), and anti-cancer drugs such as peroxisome proliferator-activated receptor-γ (PPARγ) ligands (40). Previously, the trans-10,cis-12 CLA isomer, but not the cis-9,trans-11 isomer, has been linked to significant tumor suppressive effect via NAG-1 in HCT-116 human colon cancer cells (12). However, it is not known whether CLA induces NAG-1 expression in an osteosarcoma model. Thus, the objectives of this study were to determine the anti-cancer effects of the specific CLA isomers and to determine the involvement of NAG-1 as a mediator induced by CLA in the canine osteosarcoma cell CCL-183 model, which is known to develop osteosarcoma similar to human osteosarcoma (41, 42).

Materials and Methods

Materials: CCL-183 canine osteosarcoma cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD). The purity of linoleic acid was 99% (Nu-Chek Prep, Inc., Elysian, MN). The trans-10,cis-12 and cis-9,trans-11 CLA were provided by Natural Lipids (Hovdebygda, Norway). The trans-10,cis-12 CLA preparation was 94% pure, with 2% cis-9,trans-11 isomer and 3% other conjugated linoleic acid isomers. The cis-9,trans-11 CLA preparation was 90% pure, with 4% trans-10,cis-12 isomer, 2% other conjugated linoleic acid isomers, and 3% oleic acid.

Potential conflict of interest: Dr. Yeonhwa Park is one of the inventors of CLA use patents that are assigned to the Wisconsin Alumni Research Foundation. No other conflict of interest to report.

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Dulbecco’s Modified Eagle’s Medium (DMEM) and penicillin/ streptomycin mix were purchased from Sigma-Aldrich Co. (St. Louis, MO) and fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). Kinase inhibitors for p38 mitogen-activated protein kinase (MAPK, SB 203580), c-Jun N-terminal kinase (JNK, SP600125), p38 kinase (PKC, Go 6983), and protein kinase C (PKC, Go 6983) were purchased from Ascent Scientific (Princeton, NJ). Goat anti-GDF 15 antibody was from Abcam (Cambridge, MA) and horseradish peroxidase conjugated anti-goat IgG antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell culture: Canine osteosarcoma (CCL-183) cells were grown in DMEM with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 ºC under a humidified atmosphere of 95% air and 5% CO2. Medium was changed every other day until confluence. All fatty acids were incubated with fatty acid free albumin as previously described (43). Final concentrations of fatty acids were indicated in each figure. All treatments included 50 μM albumin. Kinase inhibitors were dissolved in dimethylsulfoxide (DMSO) and added at concentrations listed in Figure 4. The final concentration of DMSO was 0.05% for all treatments.

Cell viability assay: Cell viability was determined with a commercially available 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) based viability assay kit (Sigma-Aldrich Co.). Cells in 96-well plate were incubated for an hour at 37 ºC in a humidified atmosphere of 95% air and 5% CO2. Medium was changed every other day until confluence. All fatty acids were incubated with fatty acid free albumin as previously described (43). Final concentrations of fatty acids were indicated in each figure. All treatments included 50 μM albumin. Kinase inhibitors were dissolved in dimethylsulfoxide (DMSO) and added at concentrations listed in Figure 4. The final concentration of DMSO was 0.05% for all treatments.

Assessment of Apoptosis-FACS Analysis: Harvested cells were stained with a combination of fluoresceinated (FITC) annexin V and propidium iodide (PI) using Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Jose, CA) according to the manufacturer’s recommendations. Briefly, treated cells were stained with 5 μl of Annexin V-FITC and 5 μl of propidium iodide and incubated for 15 minutes at room temperature in the dark followed by flow cytometry. Flow cytometry was performed using LSRII and a FACS Vantage SE cell sorter (BD Bioscience, San Jose, CA).

TaqMan Real time PCR: mRNA expression level of NAG-1 was analyzed by using Step One Plus real time PCR machine (Applied Biosystems, Foster City, CA). Briefly, total RNA from cultured cells was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA) under RNase-free condition. Total RNA was reverse-transcribed using MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA). NAG-1 gene expression levels compared to control were determined by using eukaryotic 18S rRNA as an endogenous control. The TaqMan probe and primer sequences (5’-3’) used for canine NAG-1 were: “CCCGGACGCCCACACT” (probe), “CCCGGACGCCCACACT” (forward primer), and “CCACCGAGGCACAGT” (reverse primer).

Immunoblotting: Cells were washed with cold phosphate buffered saline and suspended in lysis radioimmuno precipitation assay (RIPA) buffer (50 mM Tris-HCl, 10 mM NaCl, 5 mM MgCl2, and 0.5% NP-40, pH 8.0, Boston Bioproducts Inc. Ashland, MA) supplemented 1% protease inhibitor cocktail (Sigma Aldrich) and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL) on ice for 30 min. Protein concentration of the cell lysate of each sample was determined by DC protein assay kit (BioRad, Hercules, CA). The lysate was separated in 10-14% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The blots were blocked with tris-buffered saline and tween 20 with 5% milk followed by overnight incubation with goat anti-GDF 15 antibody (1:1000) (Abcam). The blots were incubated for 1 hour at room temperature with horseradish peroxidase conjugated anti-goat IgG antibody (1:5000) (Santa Cruz Biotechnology Inc.). Detection was performed by enhanced chemiluminescence (GE Healthcicare, Piscataway, NJ). Glyceraldehyde-3-phosphate dehydrogenase expression was used as an internal control to normalize protein content. Blot image and results were quantified using Image J software.

Statistical Analysis: Statistical analysis was computed with SAS statistical software (Version 9.0; SAS institute Inc., Cary, NC). Data was analyzed by two-way analysis of variance (treatments and experiments). Mean separations were conducted with Duncan’s New Multiple Range Test (p<0.05).

Results

CLA inhibits osteosarcoma cell viability

To investigate whether CLAs affect proliferation of canine osteosarcoma cells. CCL-183 cells were treated with vehicle (BSA), linoleic acid and two CLA isomers (cis-9,trans-11 CLA or trans-10,cis-12 CLA) for 1-4 days. We used 50 μM because our previous data indicated that 50 μM of CLA effectively suppressed growth of human colorectal cancer cells and this concentration of CLA reflects the levels of CLA after supplementing 0.5% CLA for 4 weeks in rats or supplementing 0.8-3.2 g per day for 2 months in humans, which ranged from 23 to 200 μM (12, 44, 45). As shown in Figure 1, there was no difference in cell viability by all treatments at day 1 and 2. Both the cis-9,trans-11 and trans-10,cis-12 CLA isomers significantly reduced cell growth at day 3 compared to control and linoleic acid, while the trans-10,cis-12 isomer was more effective than the cis-9,trans-11 isomer. At day 4, similar trends were observed where linoleic acid treatment also reduced cell viability compared to control.

Figure 1. Effects of conjugated linoleic acid treatments on osteosarcoma cell viability. CCL-183 osteosarcoma cells were treated with bovine serum albumin (control, white bars), linoleic acid (50 μM, black bars), cis-9,trans-11 CLA (50 μM, lined bars), or trans-10,cis-12 CLA (50 μM, gray bars) for 1-4 days. Numbers are mean ± S. E. (n=9, collected from 3 independent experiments). Means with different letters are significantly different at each time point (p<0.05).
Effects of conjugated linoleic acid (CLA) on apoptosis in osteosarcoma. (a) Flow cytometry results (b) percentages of cells in apoptosis (Upper and lower right quadrants in (a)). CCL-183 osteosarcoma cells were treated with bovine serum albumin (control), linoleic acid (50 μM), cis-9,trans-11 CLA (50 μM), or trans-10,cis-12 CLA (50 μM) for 3 days. (b) Numbers are mean ± S. E. (n=3). Means with different letters are significantly different at p<0.05.

Effects of conjugated linoleic acid (CLA) on NAG-1 Expression. CCL-183 osteosarcoma cells were treated with bovine serum albumin (control), linoleic acid (LA), cis-9,trans-11 CLA, or trans-10,cis-12 CLA at 25, 50, or 100 μM for 3 days. Numbers are mean ± S.E. (n=12). Means with different letters are significantly different at p<0.05.

Determination of NAG-1 induction by CLA

To determine if CLA-induced apoptosis is associated with NAG-1 overexpression, we determined NAG-1 expression, using various concentrations of CLAs along with linoleic acid (Figure 3). While there was no induction of NAG-1 by linoleic acid at all concentrations tested, 100 μM cis-9,trans-11 CLA significantly induced NAG-1 expression. The trans-10,cis-12 CLA significantly induced NAG-1 expression at all concentrations tested, 2, 3, and 4-fold in 25, 50, and 100 μM, respectively, compared to control.

Determination of CLA’s mechanism of controlling NAG-1

To investigate the degree of mediation of NAG-1 induced by the trans-10,cis-12 CLA isomer as well as determination of the principle apoptotic pathway via NAG-1, four of the representative apoptotic pathways protein kinase: (I) p38 MAPK [SB 203580] ; (II) JNK [SP 600125] ; (III) PI3K [Ly 294002] and (IV) PKC [Go 6983] were chosen and tested for the influence of NAG-1 expression as shown in Figure 4. There was dose-dependent regulation of NAG-1 protein expression by LY294002, PI3K inhibitor, suggesting that NAG-1 expression in osteosarcomas was dependent on PI3K. However, no effects of the other three protein kinase inhibitors were observed (Fig. 4).

To verify the effect of the trans-10,cis-12 CLA isomer on the anti-apoptotic pathway, we co-treated these cells with LY 294002 and CLA. The trans-10,cis-12 CLA isomer significantly induced higher NAG-1 expression than control (BSA), whereas co-treatment with LY 294002 and the trans-10,cis-12 CLA isomer failed to increase the expression level of NAG-1 (Fig.5). This indicates that the apoptotic effect of the trans-10,cis-12 CLA isomer is mediated by an PI3K dependent pathway.
CLA has been reported to inhibit cancer cell growth by interfering with the cell cycle and inducing apoptosis (29, 46). Some reported greater efficacy of the trans-10,cis-12 CLA isomer than the cis-9,trans-11 CLA isomer with regard to anti-cancer effects (25, 27-32, 47). The only known effects of CLA on osteosarcoma were reported by Kim et al. (48), using the human osteosarcoma model, MG-63 (48). In this report, all CLA isomers tested (trans-10,cis-12; cis-9,trans-11; and trans,trans) showed significant growth inhibition, but the most effective isomer was the trans,trans CLA. Although we didn't test the trans,trans CLA isomer in this study, the results from Kim et al. (48) showed that the effect of trans-10,cis-12 CLA on growth was greater (although not significantly) than the cis-9,trans-11 isomer, which is consistent with our observation. In addition, Kim et al. (48) reported that CLA effectively inhibited the growth of human osteosarcoma cells, via increasing apoptosis through reduction of Bcl-2 expression and induction of Bax protein expression.

Since NAG-1 was found to be a pro-apoptotic/anti-tumorigenic gene in several types of cancer cell model (49-51), it may serve as a marker gene of apoptosis that is targeted by CLA, particularly for the trans-10,cis-12 isomer. Among major signaling pathways for apoptosis, previously it was reported that PI3K has been positively linked with NAG-1 expression (52). PI3K is a lipid kinase which converts phosphatidylinositol-4, 5 bisphosphate (PI(4,5)P2) to phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3). Akt protein (also known as Protein Kinase B) translocates to the cell membrane and interacts with PI(3, 4, 5)P3 for further activation. The activated Akt controls fundamental cellular processes as well as the regulation of cell proliferation (53). Most significantly, gene amplification of the Akt gene is commonly found in a variety of cancers, which increases the chance of activation of Akt protein for further cell proliferation (54).

Our current results that trans-10, cis-12 CLA regulates NAG-1 by the PI3K mediated pathway is consistent with Yamaguchi et al.'s (52) report in the HCT-116 human colon cancer model. The same authors identified that the inhibition of PI3K-Akt results in the activation of glycogen synthase kinase 3 (GSK-3), which plays a role in the control of cellular response to DNA damage. The activation of GSK-3 enhanced NAG-1 expression in HCT-116 colon cancer cells as well as increased apoptosis. There are two other reports with regard to CLA's involvement in the PI3K-Akt pathway in human colon and breast cancer cells (22, 24). However, others reported that CLA inhibits proliferation or increases apoptosis by modulating PKC (31) in human prostate cancer cells, by modulating p21WAF1/CIP1 (p53 dependent), bcl-2 (p53 independent), ERK1/2, and Bak in human breast cancer cells (55, 56), by down-regulation of APC/β-catenin pathways (57), or by increasing reactive oxygen species and a subsequent ER stress in human colon cancer cells (47).

Compared to extensive evidence for CLA's anticancer effects in animal studies, only a small number of human studies involving CLA and cancer have been reported. Most of these are focused on CLA and breast cancer, where dietary CLA is the primarily focus (4). The first was by Knekt et al. (58), a cohort study for a 25-year follow up period reported a significant inverse relationship between milk consumption and breast cancer incidence. This suggests a protective effect of milk consumption, potentially associated with CLA. There are additional studies on the effects of CLA on breast cancer where inconsistencies were observed (59-64). Additionally, Larsson et al. (65) reported an inverse correlation between CLA and colorectal cancer incidence in women. These publications used food questionnaires or dietary occurring CLA, therefore limited to accessing the cis-9,trans-11 CLA isomer, thus conclusions cannot be made for trans-10,cis-12 CLA. Moreover, Mohammadzadeh et al. (66) reported that supplementing CLA during chemo-radiotherapy in rectal cancer patients significantly lessened matrix metalloproteinase type 2 and 9, which are important markers of angiogenesis and tumor metastasis. However, controlled trials with CLA supplementation are necessary in order to determine the mechanisms of CLA's action and specific effects associated with cancer, particularly for osteosarcoma.
We selected the canine osteosarcoma cell line since it is not only known to develop osteosarcoma similar to human osteosarcoma but also certain breeds of canines are prone to developing osteosarcoma (41, 42). The primary treatment for canine osteosarcoma is amputation and chemotherapy, however, it is likely that affected dogs will die within a year along with increased risk of metastasis (67-69). The significance of this study is that CLA can serve as a prevention and/or treatment option for canine osteosarcoma using a dietary supplement approach. In addition, the canine osteosarcoma model can be a very important way to evaluate CLA for osteosarcoma prevention and/or treatment prior to human clinical trials due to faster cancer development in canines than humans.

In summary, the results in this report suggest that the trans-10,cis-12 CLA isomer effectively inhibits canine osteosarcoma cell growth and induces apoptosis, potentially by a NAG-1 via PI3K mediated mechanism.

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