Conjugated Eicosapentaenoic Acid Inhibits Vascular Endothelial Growth Factor-Induced Angiogenesis by Suppressing the Migration of Human Umbilical Vein Endothelial Cells

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Introduction

Conjugated fatty acids form positional and geometrical isomers that contain several conjugated double bonds. Among such fatty acids, conjugated linoleic acid (CLA), which is mainly a mixture of 9Z11E- and 10E12Z-octadecadienoic acids, has various physiological properties that are associated with the conjugated double bond system. CLA is found naturally and is present at low levels in ruminant fats such as beef tallow and milk fat. In addition to CLA, many other conjugated fatty acids occur naturally in plant seeds and marine algae. The physiological properties of CLA, and we previously described the properties of these fatty acids. The physiological properties of CLA, and we previously described the properties of these fatty acids.

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Abstract

We have previously shown that conjugated eicosapentaenoic acid (CEPA), which is prepared by alkaline treatment of eicosapentaenoic acid and contains conjugated double bonds, suppresses tumor growth in vivo. In this earlier study, blood vessels were observed on the tumor surface in control mice, whereas in CEPA-treated mice, no such vessels were observed and the inner part of the tumor was discolored. These observations suggest that CEPA might suppress cancer cell growth through malnutrition due to a suppressive effect on tumor angiogenesis. In this study, the antiangiogenic effects of CEPA were investigated in vitro. CEPA at 5 μmol/L inhibited vascular endothelial growth factor (VEGF)-stimulated tube formation by human umbilical vein endothelial cells (HUVEC) and also inhibited VEGF-stimulated migration of HUVEC at a concentration of CEPA that suppressed tube formation but did not influence cell proliferation. The antiangiogenic mechanism of CEPA was investigated in vitro by measuring the secretion and expression of well-characterized angiogenic factors associated with cell migration, such as matrix metalloproteinases (MMP). CEPA at a concentration that suppressed tube formation inhibited secretion and mRNA expression of MMP2 and MMP9 in VEGF-stimulated HUVEC. Our findings suggest that CEPA has potential use as a therapeutic dietary supplement for minimizing tumor angiogenesis.


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2 Abbreviations used: CEPA, conjugated eicosapentaenoic acid; CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; ECM, extracellular matrix; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cell; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; WST-1, water-soluble tetrazolium salt.

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observed in mice that received EPA and CEPA; furthermore, the inner part of the tumor was discolored in the EPA- and CEPA-fed mice. These results suggest that tumor angiogenesis is suppressed by EPA, as also reported previously (28,29). The phenomena seen with EPA and CEPA might be due to suppression of growth of cancer cells through malnutrition and it is likely that EPA and CEPA have a suppressing effect on tumor angiogenesis. Therefore, we have investigated this possible antiangiogenic effect in vitro as a possible new physiological function of CEPA.

Antiangiogenic therapy was established as a strategy for cancer prevention (30), and we have described antiangiogenic properties of vitamin E (tocotrienol) (31). Angiogenesis involves a series of steps including endothelial cell activation and breakdown of the basement membrane, followed by migration, proliferation, and tube formation of endothelial cells (32). The purpose of this study was to obtain direct evidence for the effects of CEPA on the key steps of angiogenesis using human umbilical vein endothelial cells (HUVEC).

Materials and Methods

Materials. EPA (99% purity) and RPMI 1640 medium (containing 0.3 g/L l-glutamine and 2.0 g/L sodium bicarbonate) were obtained from Sigma. Fetal bovine serum (FBS) was purchased from Dainippon Pharmaceutical. Penicillin and streptomycin were products of Gibco BRL.

Preparation of CEPA. In our previous study, CEPA was prepared by various methods, and we determined how to make CEPA with the strongest antitumor effect (26). In this study, CEPA was prepared from EPA by alkaline isomerization using the method previously reported (14,26,27). Potassium hydroxide at a concentration of 21% (wt/wt) in ethylene glycol was prepared and nitrogen gas was bubbled through the solution for 10 min. EPA (100 mg) was added to 10 mL of the 4 mol/L KOH solution in a test tube (100 mL vol). Nitrogen gas was again bubbled through the mixture, and then the tube was screw-capped and allowed to stand for 10 min at 180°C. The reaction mixture was cooled, 10 mL of methanol was added, and then the mixture was acidified to below pH 2 with 20 mL of 6 mol/L HCl. After dilution with 2 mL of distilled water, the conjugated fatty acids were extracted with 5 mL of hexane. The hexane extract was then washed with 3 mL of 30% methanol and 3 mL of distilled water before evaporation under a nitrogen gas stream. The CEPA concentrate was stored at −20°C after being purged with nitrogen gas. We confirmed the concentrate to be the EPA isomer (carbon number: 20, double bond number: 5) using GCMS and HPLC methods that have been reported (14,26,27). UV/VIS spectrophotometric analysis of CEPA was performed with a Shimadzu UV-2400PC; this confirmed the presence of conjugated fatty acids containing dienes (carbon number: 20, double bond number: 5) using GC/MS and HPLC (absorption at 235 nm), trienes (268 nm), tetraenes (315 nm), pentaenes (367 nm), hexaenes (375 nm). The CEPA used in the subsequent experiments consisted of 57.6% conjugated dienes, 34.5% conjugated trienes, 6.7% conjugated tetraenes, and 1.2% conjugated pentaenes. We prepared the CEPA carefully to avoid oxidation of conjugated fatty acids and confirmed that the fatty acids had not oxidized using a ferrous oxidation in xylene orange assay and a thiobarbituric acid test (12,27).

Cells and cell cultures. HUVEC were purchased from Iwaki. Cells were cultured in HuMedia-EG2 growth medium (Kurabo) and grown at 37°C in a humidified atmosphere of 5% CO2 in air. The HuMedia-EG2 medium consists of base medium (HuMedia-EB2) supplemented with 2% FBS, 0.5 mg/L human epidermal growth factor, 2 mg/L human basic fibroblast growth factor-B, 5 g/L insulin, 50 g/L gentamicin, and 50 mg/L amphoterocin B. Tightly confluent monolayers of HUVEC of passage 2 to 6 were used in the experiments.

Preparation of CEPA for cell cultures. A portion of 100 mmol/L CEPA and EPA in ethanol was placed in a sterile tube and solvent was removed using a N2 gas flux. The dried CEPA and EPA were dispersed in the culture medium (HuMedia-EG2) using sonication, followed by dilution with medium to achieve the desired final concentration. Media containing vascular endothelial growth factor (VEGF) and sample-free medium were prepared as controls for the study.

Tube formation. Angiogenesis assay was performed using an Angiogenesis kit (Kurabo) according to the manufacturer's instructions and the formation of capillary-like structures composed of HUVEC cocultured with human diploid fibroblasts was observed (31,33). The medium containing VEGF (final 10 μg/L; Kurabo) with various concentrations of CEPA and EPA was exchanged on d 4, 7, and 9, and the cells were washed and directly fixed with 70% ice-cold ethanol for 30 min in the wells after 11 d of culture. The fixed cells were serially incubated with 1% bovine serum albumin in buffer, mouse monoclonal antibody against human CD31 (Kurabo), alkaline phosphatase-conjugated goat anti-mouse IgG (Kurabo), nitro-blue tetrazolium chloride (NBT)/5-Bromo-4-Chloro-3’-Indolylphosphatase p-Toluidine salt (BCIP) (violet, Kurabo), and washed and photographed. The images were analyzed using Angiogenesis Image Analyzer software (Kurabo) to measure the gross area of CD31-positive tube (the area of endothelial tube) in culture. Data were shown by the percentage based on the area of endothelial tubes of untreated culture.

Migration of HUVEC in a wound closure model. HUVEC were cultured on 12-well plates (2 × 105 cells/well) in FBS-free HuMedia-EG2 medium. After 24 h, the cells were scratched with a yellow pipette tip to obtain a monolayer culture with space without cells (33,34). Media and dislodged cells were aspirated and fresh medium was added to the plates along with CEPA or EPA at various concentrations. After incubation at 37°C for 12 h, cell migration was observed using a phase contrast inverted microscope, and the width of the space without cells was measured in 4 randomly chosen fields.

Cell proliferation. HUVEC at 90% confluent growth were trypsinized, transferred to 96-well plates (2000 cells/well), and preincubated in HuMedia-EG2 medium for 24 h (33). The culture medium was then changed to medium only or to medium containing VEGF (10 μg/L) and CEPA at various concentrations and incubated for 72 h. At the end of this period, viable cell numbers were estimated using the water-soluble tetrazolium salt (WST-1) assay, as previously described (11). WST-1 is a tetrazolium salt that is converted into the soluble formazan salt by succinate-tetrazolium reductase of the respiratory chain of active mitochondria of proliferating viable cells. Briefly, 10 μL of WST-1 solution (Dojindo) was added to each well and incubated at 37°C for 3 h on a microplate reader (Model 350, Bio-Rad).

ELISA. HUVEC were cultured on 12-well culture plates (1 × 105 cells/well) in HuMedia-EG2 medium containing CEPA or EPA at various concentrations (33). After 24 h, aliquots of the conditioned medium were taken for measurement of the matrix metalloproteinases (MMP) levels using commercial ELISA kits obtained from Biotrak (Amersham-Pharmacia).

RNA extraction. For the real-time quantitative RT-PCR assay, total RNA was extracted from CEPA- or EPA-treated HUVEC (1 × 105 cells) using a commercial kit (RNAeasy Mini kit, Qiagen), as previously described (11). CEPA was prepared to a final concentration of 0 or 5 μmol/L in HuMedia-EG2 medium containing VEGF. Total RNA was eluted with 30 μL RNase-free water and stored at −80°C until use. The amount of total RNA was determined spectrophotometrically at 260 and 280 nm. RNA integrity was confirmed by visualizing intact 28S and 18S ribosomal RNA on a formaldehyde denaturing agarose gel.

Real-time quantitative RT-PCR assay. The expression levels of MMP2 and MMP9 mRNA in HUVEC 24 h after injection of CEPA and EPA were determined with a real-time PCR system (DNA Engine Opticon 2 system, MJ Research), which allows real-time quantitative detection of the PCR product by measuring the increase in fluorescence.
caused by binding of SYBR green to double-stranded DNA (11). The cDNA was made using a Ready-To-Go T-Primed First-Strand kit (Amersham Pharmacia Biotech) from the total RNA in HUVEC 24 h after injection of 0 or 5 μM CEPA. The cDNA was subjected to PCR amplification using a DyNamo SYBR Green qPCR kit (Finnzymes) and gene-specific primers for MMP2, MMP9, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer pairs used for MMP2 (NM_004530): Forward 5′-AGGCGACAAGAAGTGG-3′; Reverse 5′-ATTTGTTGCCGAGAAAGTG-3′; for MMP9 (NM_004994): Forward 5′-TGAGCAGGCGACAAGAAGTGG-3′; Reverse 5′-GCCATCAGCTGCTTAT-3′; for GAPDH (NM_002046): Forward 5′-CTGGGCAAGGTCATCCATG-3′; Reverse 5′-GGAAGGCATGCCAGTGAGC-3′. Real-time PCR was conducted under conditions suitable for the primers, as established in our previous study (11). The PCR conditions used were 95°C for 5 min, 95°C for 10 s, and 59°C for 50 s over 40 cycles for each gene. Melting curve analysis was performed following each reaction to confirm the presence of only a single reaction product. In addition, representative PCR products were electrophoresed on a 2.0% agarose gel to verify that only a single band was present. The threshold cycle represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected. The ratio between the GAPDH content in standard and test samples was used to normalize the results.

Statistical analysis. We performed statistical analysis using a 1-way ANOVA, followed by a Newman-Keuls test for multiple comparisons among several groups. A difference was considered significant at P < 0.05.

Results

The effect of CEPA on tubular morphogenesis of endothelial cells was first examined. A coculture of HUVEC with human fibroblasts incubated for 11 d with 10 μL/L VEGF showed an increase in the area of endothelial tubes, compared with cells incubated with medium only (Fig. 1). CEPA suppressed VEGF-induced tube formation (as measured by the area of endothelial tubes) in a dose-dependent manner (Fig. 1); this effect was significant at 5 μM CEPA and reached 29% suppression at 10 μM/L CEPA, compared with the culture without CEPA. EPA suppressed VEGF-induced tube formation significantly at 100 μM/L.

Next, the effect of CEPA on migration and proliferation of HUVEC was examined, because these properties are closely related to tubular morphogenesis. A denudation injury model was used to assess the impact of CEPA on endothelial cell migration. Monolayer cultures with spaces without cells were incubated with VEGF and CEPA or EPA, and the rate of closure over the following 12 h was determined. In cultures supplemented with VEGF (10 μM/L), endothelial cells migrated into the denuded area, covered the exposed surface, and reduced the uncovered area (Fig. 2). CEPA suppressed VEGF-induced migration in a dose-dependent manner (Fig. 2); this effect was significant at 5 μM/L CEPA and reached a level of 50% suppression at 10 μM/L compared with the culture with no CEPA. EPA did not influence the migration of HUVEC.

A WST-1 assay was used to assess the impact of CEPA on endothelial cell proliferation. HUVEC were cultured in the presence or absence of VEGF and the ratio of viable cells was evaluated 72 h after addition of CEPA or EPA (Fig. 3). For cells cultured without VEGF, CEPA suppressed cell proliferation significantly at 80 μM/L, but the cells remained viable. EPA suppressed cell proliferation significantly at 150 μM/L. In cultures supplemented with VEGF (10 μM/L), CEPA suppressed cell proliferation significantly at 40 μM/L, and cell proliferation induced by VEGF was almost completely reversed at this concentration of CEPA. EPA suppressed VEGF-induced cell proliferation significantly at 100 μM/L. These results show that CEPA suppressed the proliferation of HUVEC, but the concentration of CEPA required to suppress cell proliferation was very high compared with that required to suppress tube formation and cell migration.

We next evaluated the mechanism of angiogenesis inhibition of CEPA in vitro by measuring the secretion of well-characterized angiogenic factors, especially those associated with cell migration such as MMP. ELISA for MMP2 and MMP9 showed that...
The effects of CEPA on HUVEC proliferation. HUVEC were incubated in medium with several concentrations of CEPA (0–80 μmol/L) or EPA (0–150 μmol/L) for 72 h (A), or in medium containing VEGF (10 μg/L) with several concentrations of CEPA (0–40 μmol/L) or EPA (0–100 μmol/L) for 72 h (B). The number of viable cells was assessed in a WST-1 assay. Values are shown as means ± SD, n = 6. Means without a common letter differ, P < 0.05. --, No addition; +, addition.

CEPA at the concentration that suppressed tube formation caused downregulation of VEGF-induced MMP2 and MMP9 secretion from HUVEC (Fig. 4). In contrast, EPA did not affect MMP2 and MMP9 secretion from HUVEC. To examine the effect of CEPA on MMP2 and MMP9 secretion in greater detail, the expression levels of MMP2 and MMP9 mRNA in HUVEC were measured using a real-time quantitative RT-PCR assay; these data showed that CEPA at the concentration that suppressed tube formation caused downregulation of VEGF-induced MMP2 and MMP9 mRNA expression in HUVEC (Fig. 4).

**Discussion**

Studies of the physiological activities of CEPA have been limited to antitumor and antiobesity effects (14,15,26,27), but here we have demonstrated for the first time that CEPA exerts antiangiogenic effects in vitro in endothelial cells. In the experiments reported here, we have measured the proliferation, migration, and tube formation of HUVEC under a variety of culture conditions, using the known antiangiogenic effect of EPA as a positive control (27,28).

CEPA inhibited formation of capillary-like networks by HUVEC (Fig. 1) and moderately inhibited proliferation and migration of HUVEC in a dose-dependent manner (Figs. 2 and 3). However, the CEPA concentration required for suppression of cell proliferation was very high compared with that required for suppression of tube formation and cell migration; this suggests that the main effect of CEPA is suppression of migration and tube formation by HUVEC. EPA also showed an antiangiogenic effect but only at 20 times the concentration at which CEPA suppressed tube formation (Fig. 1). EPA suppressed cell proliferation at the same concentration required for suppression of tube formation but did not influence cell migration (Figs. 2 and 3); these data suggest that the main effects of EPA are suppression of cell proliferation and tube formation by HUVEC. Overall, the results show that CEPA has an antiangiogenic effect that is much stronger than that of EPA, and that the antiangiogenic effects of CEPA and EPA occur through different mechanisms. This suggests that the conjugated double bond in CEPA is important for antiangiogenesis; in this context, it is of note that CLA exerts an antiangiogenic effect, whereas linoleic acid does not do so (35,36).

The mechanism of angiogenesis inhibition by CEPA in vitro was investigated by measuring the secretion and expression of angiogenic factors such as MMP. Degradation of subendothelial basement membrane and surrounding extracellular matrix (ECM) is an initial step in the angiogenic process (37). Following matrix breakdown, endothelial cells migrate and proliferate to form new vessels. Therefore, proteolytic enzyme activity for degrading ECM is essential in angiogenesis, and the MMP are a family of Zn²⁺-dependent endopeptidases that digest a variety of ECM components (38). Endothelial cells express various MMP, including MMP2 (gelatinase A) and MMP9 (gelatinase B) (39). Although the contribution of MMP to angiogenesis has been attributed primarily to their ability to break down the ECM, they may also promote migration of endothelial cells. The most direct and compelling evidence for this conclusion was that MMP inhibitors, both synthetic and endogenous, inhibit angiogenic responses, including cell migration both in vitro and in vivo (40,41). In this study, MMP2 and 9 were selected based on a report that MMP2 and 9 have especially important roles in angiogenesis (42). Given these important roles of MMP in angiogenesis, we measured the concentration of MMP2 and MMP9 in conditioned medium of HUVEC and found that secretion of MMP2 and MMP9 was inhibited significantly when the cells were exposed to CEPA (Fig. 4A,B). This result was reflected in the corresponding decrease in MMP2 and MMP9 mRNA levels (Fig. 4C, D). Therefore, CEPA suppresses cell migration through modifications at the gene expression level, leading to suppression of tube formation by HUVEC in vitro. This antiangiogenic effect is likely to be related to tumor suppression by CEPA (27) and may also be related to the antiobesity effect of CEPA (15), because angiogenesis has been associated with an increase of white adipose tissue (36).

Screening for compounds with antiangiogenic properties is currently an active area of research: curcumin (43), flavonoids (44,45), selenium (46), N-acetylcysteine (47), vitamin D3 (48), tocochromenols (31), and several fatty acids, including EPA, in dietary supplements (28,29,35,36) have all been shown to inhibit...
angiogenesis in vitro and/or in vivo. We note that CEPA was prepared from EPA by alkaline isomerization and this procedure leads to a mixture of isomers with conjugated double bonds. Therefore, the exact compound that exerts the antiangiogenic effect of CEPA is unknown. However, this effect was considerably stronger than that of EPA, suggesting that the number of conjugated double bonds may be of importance; further structure-function studies of conjugated fatty acids are needed to resolve this issue. Nonetheless, we were able to show that CEPA significantly inhibited VEGF-induced migration and tube formation of HUVEC. CLA made by alkaline isomerization of safflower oil and corn oil is sold as a health food supplement (49). When humans (~70 kg) ate 3–6 g CLA/d for several months, there were few effects (49). We previously fed mice 2.5 mg CLA/d for 28 d, which is equivalent to a human dose of 5 g/d (27). In these mice, the plasma CEPA concentration after 28 d was ~10 μmol/L. In Figure 1, 5 μmol/L CEPA showed the antiangiogenic effect. Even if CEPA is taken, the effect of CEPA can be expected. The use of CEPA in foods and dietary supplements is likely to be beneficial; however, the safety of CEPA must be confirmed in long-term studies and clinical trials, as have been conducted using CLA.

Literature Cited