A natural anti-inflammatory enone fatty acid inhibits angiogenesis by attenuating nuclear factor-κB signaling in vascular endothelial cells

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Abstract. An anti-inflammatory enone fatty acid, (E)-9-oxooctadec-10-enoic acid (C10), was previously isolated from red alga (Gracilaria verrucosa). Of the many cellular signaling pathways activated in response to the inflammatory stimulus, lipopolysaccharide, the extracellular signal-regulated kinase 1/2, the stress-activated protein kinase/Jun N-terminal kinase and the nuclear factor-κB pathways were specifically blocked by C10 in the macrophage-like cell line, RAW264.7. In this study, we investigated the anti-angiogenic and anti-inflammatory activities of C10 in endothelial cells. C10 only partially inhibited the proliferation of human cancer cell lines at relatively high concentrations of over 20 μg/ml. However, C10 inhibited the proliferation of RAW264.7 cells and human umbilical vein endothelial cells (HUVECs) with half-maximal inhibitory concentration (IC₅₀) values of 4-8 μg/ml. Both the proliferation and the migration of HUVECs induced by the vascular endothelial growth factor (VEGF) were markedly blocked by C10 with IC₅₀ values of 2-3 μg/ml. The activation of nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α, by tumor necrosis factor-α or VEGF in these cells was also blocked by C10. Furthermore, in an in vivo model of angiogenesis in the mouse cornea, the neovascularization induced by VEGF was markedly inhibited by C10. The processes involved in inflammatory signaling, angiogenesis, and the development of malignancy in cancer are closely related, suggesting that C10 could be a useful lead compound for the development of novel anti-angiogenic therapies for cancer.

Introduction

Angiogenesis is an essential process during the development and progression of a number of chronic inflammatory diseases including ocular diseases, rheumatoid arthritis, osteoarthritis, obesity, diabetes, psoriasis and Crohn's disease, as well as cancer. Furthermore, many molecules are known to have roles in both angiogenic and inflammatory pathways, including adhesion molecules, growth factors, angiogenic factors, reactive oxygen species, chemokines and cytokines (1,2). Angiogenesis and inflammation are now considered to be closely coupled processes, suggesting that the development of anti-inflammatory drugs could have dual applications, acting not only as therapeutics for inflammatory diseases but also as inhibitors of tumor angiogenesis.

The administration of inflammatory cytokines such as interleukin-1 (IL-1)-α/β, stimulates angiogenesis in mouse corneas by enhancing the production of pro-angiogenic factors, prostaglandins, proteinases and reactive oxygen species (3-7). In response to these inflammatory stimuli, infiltrating monocytes/macrophages in inflammatory foci or tumors are also activated to produce pro-angiogenic factors, extracellular matrix-degrading proteinases and prostaglandins, which provide cancer cells, vascular endothelial cells and other stromal cells with favorable conditions for angiogenesis, lymphangiogenesis, and tissue remodeling (8-10). We have previously reported that the depletion of infiltrating macrophages by treatment with liposome-encapsulated bisphosphonate can successfully suppress inflammatory angiogenesis and lymphangiogenesis (10-12). This suggests a critical role for activated monocytes/macrophages in the development of new blood and lymphatic vessels (2).

Dang *et al* isolated anti-inflammatory agents from red alga for the development of novel therapeutic drugs for inflammatory diseases (13). They showed that new prostaglandins and oxygenated fatty acids isolated from the red alga, Gracilaria verrucosa, inhibited the production of major mediators of inflammation, including nitric oxide (NO), IL-6
and TNF-α, in lipopolysaccharide (LPS)-activated RAW264.7 cells (13). In the present study, we investigated whether one of the anti-inflammatory agents extracted from the red alga, (E)-9-oxooctadec-10-enoic acid (C10), could inhibit angiogenesis in vitro and in vivo, and which inflammation-induced signaling pathways were involved in this anti-angiogenic activity. We discuss the possible role for nuclear factor-kB (NF-kB) in the anti-angiogenic activity of C10, and the possible development of novel anti-angiogenic drugs for cancer by targeting inflammatory signaling pathways.

Materials and methods

Animals. Male C57BL/6 mice, aged 6-10 weeks, were purchased from CLEA (Saga, Japan). All animal experiments were approved by the Committee on the Ethics of Animal Experiments at Kyushu University, Japan.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Walkersville, Inc. (Walkersville, MD) and cultured in endothelial cell basal medium-2 (EBM-2) supplemented with 2% fetal bovine serum (FBS). HeLa (human cervical cancer cell line), RAW264.7 (murine macrophage-like cell line) and MDA-MB231 (human breast cancer cell line) were purchased from ATCC (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. The cells were maintained under standard cell-culture conditions at 37°C and in 5% CO₂ in a humid environment.

Western blot analysis. The cells were rinsed with ice-cold phosphate-buffered saline and lysed in 50 mM Tris-HCl pH 7.5, 350 mM NaCl, 1% octylphenoxypolyethoxyethanol (NP40), 50 mM NaF, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM sodium vanadate and 5 mM ethylenediaminetetracetic acid (EDTA). The nuclear fraction was separated and lysed in sodium vanadate and 5 mM ethylenediaminetetracetic acid (E)-9-oxooctadec-10-enoic acid (C10), could inhibit angiogenesis and TNF-α expression in RAW264.7 cells and inhibited the activity of nuclear factor-kB (NF-kB) in the anti-angiogenic activity of C10, and the possible development of novel anti-angiogenic drugs for cancer by targeting inflammatory signaling pathways.

Migration assay. Migration assays were performed using 24-well chambers with 8-µm polycarbonate filters coated with 1.33 µg/ml fibronectin, as described previously (9). Briefly, 1.5x10⁵ HUVECs in EBM-2 containing 0.3% FBS were seeded in the inner chamber with increasing concentrations of C10 from 0 to 10 µg/ml. The outer chambers were filled with the same medium, with or without 50 ng/ml VEGF. After incubation for 7 h at 37°C, the cells that had migrated under the filter were counted manually by microscopic examination.

Corneal micropocket assay in mice. A corneal micropocket assay was used to assess the effect of 4 µg C10 on corneal neovascularization in response to 0.3 µl hydron pellets (Sigma-Aldrich) containing 200 ng human VEGF, implanted in the corneas of mice, as described previously (12). Seven days after implantation, the formation of corneal vessels was photographed and recorded using Viewfinder 3.0 (Pixera) with standardized illumination and contrast, saved to disk, and quantified using the National Institutes of Health image analysis software package as described previously (14).
Figure 1. C10 and its effect on RAW264.7 cells. (A) Chemical structure of C10. (B) Western blots using protein extracts from RAW264.7 cells cultured in serum-free medium, pre-treated with C10 for 1 h, and then stimulated with 1 μg/ml LPS for 30 min. Protein extracts were separated by 10% SDS-PAGE, transferred to membranes, and probed with the indicated antibodies. (C) Quantification of the labeling of phosphorylated ERK1/2 (p-ERK1/2), p-SAPK/JNK, and p-IκBα, shown in (B), using ImageJ software.

Figure 2. Effect of C10 on the proliferation of cancer cell lines, a macrophage-like cell line and vascular endothelial cells. (A) HeLa. (B) MDA-MB231. (C) RAW264.7. (D) HUVECs. Cells were seeded in 24-well plates, grown with or without C10 for 48 h, and then counted using a Coulter counter. Data are the means of triplicate cultures ± standard deviation (SD). *P<0.05.
confluence, the medium was replaced with medium containing 0.3% serum. HUVECs were pre-treated with C10 for 3 h, followed by treatment with TNF-α for 24 h at which time the conditioned medium was analyzed by ELISAs to determine the concentrations of CXCL1/GRO-α and CXCL5/ENA-78, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Results

C10 inhibits the activation of ERK1/2, JNK and IκBα in LPS-stimulated RAW264.7 cells. The chemical structure of the anti-inflammatory compound, C10, is shown in Fig. 1A. Dang et al reported that C10 inhibited the expression of IL-6, TNF-α and NO in RAW264.7 cells (13). We initially assessed the effect of C10 on the activation of other cell signaling pathways that are known to be closely associated with inflammation and cell proliferation in RAW264.7 cells. The potent inflammatory compound LPS induced marked phosphorylation of ERK1/2, SAPK/JNK and IκBα, but no activation of p38 (Fig. 1B). Treatment with C10 markedly inhibited the phosphorylation of ERK1/2, SAPK/JNK and IκBα after stimulation with LPS (Fig. 1B and C) under our experimental conditions.

C10 inhibits cell proliferation in macrophages and vascular endothelial cells. We compared the anti-proliferative effects of C10 on cancer cell lines (HeLa and MDA-MB231), a macrophage-like cell line (RAW264.7), and HUVECs in culture under basal conditions. The proliferation of the two human cancer cell lines was blocked by 10-30% in the presence of 20 μg/ml C10 (Fig. 2A and B). In contrast, the proliferation of RAW264.7 and HUVECs was inhibited by >50% in the presence of 6-7.5 μg/ml C10 (Fig. 2C and D). The proliferation of vascular endothelial cells and macrophage-like cells therefore showed relatively higher sensitivity to the inhibitory effect of C10 than the cancer cell lines.

C10 inhibits cell proliferation, migration, and tube formation in HUVECs in vitro and angiogenesis in mouse corneas in vivo

Figure 3. Effects of C10 on the proliferation and migration of HUVECs stimulated by VEGF. (A) Proliferation of HUVECs seeded in 24-well plates and incubated with or without 20 ng/ml VEGF in the presence of increasing doses of C10 for 48 h. Cells were counted using a Coulter counter. Data are the means of triplicate cultures ± SD. *P<0.05. (B) Migration of HUVECs seeded in the inner chamber of migration chambers with or without C10, and in the outer chamber with medium with or without 50 ng/ml VEGF. After incubation for 7 h at 37˚C, the migrated cells were counted. Data are the means of triplicate cultures ± SD. *P<0.05. The images are typical of migrated cells.
in response to VEGF. The proliferation of HUVECs was inhibited by C10 in basal culture conditions with 2% serum (Fig. 2D). We then investigated the effects of C10 on the proliferation and migration of HUVECs induced by the potent angiogenic factor, VEGF. After incubation for 7 h, the capillary-like structures in each well were photographed and quantified. Data are the means of triplicate cultures ± SD. *P<0.05. (B) C10 inhibited corneal neovascularization, stimulated by 200 ng VEGF, in mouse corneas implanted with hydron pellets. Neovascularization was photographed and quantified after 7 days, in the region around the implants. In certain cases, 4 μg C10 was administered with the VEGF in the hydron pellets. *Significant difference (P<0.01), n=7 for VEGF alone and n=8 for VEGF plus C10.

C10 inhibits NF-κB activation by VEGF in HUVECs. The VEGF-dependent proliferation and migration of vascular endothelial cells was highly susceptible to the inhibition by C10. Moreover, the three main signaling pathways activated in LPS-stimulated RAW264.7 cells are known to be NF-κB, ERK1/2 and SAPK/JNK. We therefore investigated whether these signaling pathways could also be affected by C10 in VEGF-stimulated vascular endothelial cells. In HUVECs, we found that although VEGFR2, ERK1/2 and Akt were phosphorylated in response to VEGF, this process was not affected by C10 (Fig. 5A). VEGF also induced IκB phosphorylation and this was blocked by C10 in a dose-dependent manner (Fig. 5A and B). The nuclear translocation of p65...
and p50 was increased when HUVECs were stimulated with VEGF, and this was only slightly, but not significantly, blocked by C10 (Fig. 5C). However, the expression of the IKK family members, IKKα, IKKβ and IKKγ, which regulate the phosphorylation of IκBα, was not blocked by C10 (Fig. 5A).

C10 inhibits NF-κB activation and the expression of certain CXC chemokines induced by TNF-α in endothelial cells. We investigated whether the TNF-α-induced activation of NF-κB in endothelial cells could be suppressed by C10. The IκBα activation was suppressed by 5-7.5 μg/ml C10 in TNF-α-stimulated endothelial cells (Fig. 6A and B). However, the expression of the IKK family member proteins was not inhibited by C10 at concentrations of up to 7.5 μg/ml (Fig. 6A). The nuclear translocation of p65 and p50 was increased when HUVECs were stimulated with TNF-α and this was blocked by C10 (Fig. 6C).

Inflammatory cytokines such as IL-1α/β and TNF-α, are known to enhance the expression of angiogenic CXC chemokines, such as CXCL1/GRO-α and CXCL5/ENA-78, in many cancer and vascular endothelial cells, as well as monocytes/macrophages (2). We investigated the effect of C10 on the expression of these CXC chemokines in TNF-α-stimulated HUVECs. The expression of the CXC chemokines, CXCL1/GRO-α and CXCL5/ENA-78 was markedly enhanced by TNF-α, and this enhanced chemokine expression was almost completely inhibited by treatment with 5 μg/ml C10 (Fig. 6D).

**Discussion**

An important development in anti-cancer therapy has been the introduction of drugs targeting tumor angiogenesis, such as the VEGF-specific neutralizing antibody, bevacizumab, and small inhibitory molecules, such as sorafenib and sunitinib, which act as multikinase inhibitors of VEGFR and other growth factor receptors (15-20). It can be speculated that there are also other types of anti-angiogenic drugs for treating cancer with different mechanisms of action from those that target VEGF.

Angiogenesis is known to be closely coupled with inflammation in many types of tumors (2,21,22). Therefore, a compound that targets inflammatory tumor-stromal responses could be useful for the development of novel anti-angiogenic therapies. The current study describes the novel finding that a natural enone fatty acid inhibits VEGF-driven angiogenesis in vitro. Furthermore, VEGF-driven angiogenesis in mouse
corneas in vivo was also markedly blocked by C10. Angiogenesis is thus highly susceptible to inhibition by C10 both in vitro and in vivo.

To elucidate the mechanism by which C10 suppresses angiogenesis induced by VEGF and/or TNF-α, we examined the possible involvement of the NF-κB pathway. We show that the NF-κB signaling pathway is activated in vascular endothelial cells stimulated with VEGF or TNF-α, and that the activation by both factors is suppressed by the enone fatty acid. The expression of angiogenic CXC chemokines was also blocked by C10. On the whole, our results show that this natural enone fatty acid can modulate angiogenesis in vascular endothelial cells in two ways: Firstly, by inhibiting the VEGF-induced activation of NF-κB, and secondly, by inhibiting the TNF-α-induced activation of NF-κB, which results in the suppression of the expression of CXCL1/GRO-α and CXCL5/ENA-78, possibly through NF-κB (Fig. 7).

NF-κB is known to play a role in VEGF-induced angiogenesis (23). The VEGF-induced activation of IkBα and the nuclear translocation of p65 and p50 were slightly, but not significantly blocked by C10. As an inducer of angiogenesis, VEGF is known to activate phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and ERK1/2 through VEGFR2 in many vascular endothelial cells (24). However, we did not observe any apparent inhibition of VEGFR2, Akt and ERK1/2 by C10 in the endothelial cells stimulated by VEGF. The phosphorylation of IκBα is regulated by the IKK complex, which comprises of IKKα, IKKβ and IKKγ (25,26). However, there were no marked changes in the expression of the IKK complex proteins after treatment with C10. At present, the mechanism by which the VEGF-induced phosphorylation of IkBα is blocked by this drug, and also how the C10-induced attenuation of NF-κB signaling stimulated by VEGF is involved, remains unclear. However, in our experiments, VEGF-induced angiogenesis could be, at least in part, attributed to the activation of the NF-κB pathway. In contrast, TNF-α markedly enhanced the expression of the CXC chemokines, CXCL1/GRO-α and CXCL5/ENA-78, which
Macrophages are classified into two groups: M1 macrophages, which mediate immunity, inflammation and tumor suppression, and M2 macrophages, which mediate matrix remodeling, tissue repair, angiogenesis and tumor progression (29-32). Tumor-associated macrophages (TAMs) generally have M2-specific characteristics. We previously showed that depleting activated macrophages and TAMs can reduce angiogenesis or lymphangiogenesis and tumor growth, which is closely associated with inflammatory stimuli (10-12,33), suggesting that natural anti-inflammatory compounds can suppress not only inflammatory signaling but also VEGF-induced angiogenesis. Dang et al developed anti-inflammatory enone fatty acids, including C10, by screening for the inhibition of NO, TNF-α and IL-6 production in stimulated macrophages (13). The enone fatty acid induced apoptosis and inhibited DNA synthesis in human cancer cells (34). The enone fatty acid analogues, including C10, not only down-regulated the expression of inducible nitric oxide synthase (iNOS), TNF-α and IL-6 in LPS-stimulated macrophages, but also blocked various inflammation- and apoptosis-related signaling pathways, such as the Janus kinase (Jak)/signal transducer and activator of transcription 1 (Stat1) and NF-κB pathways. In contrast, these compounds did not inhibit PI3K/mitogen-activated protein (MAP) signaling (35). In the present study, we show that C10 inhibits ERK, JNK and NF-κB signaling in macrophages stimulated by LPS. In addition to the two pathways that promote inflammatory angiogenesis, which can be modulated by C10, we speculate that tumor-associated macrophages and neutrophils are also involved in promoting angiogenesis occurring in response to inflammatory stimuli, and that this could be susceptible to the inhibition by C10.

In conclusion, we show here that the anti-inflammatory enone fatty acid, C10, derived from red alga, blocks both angiogenesis and NF-κB activation in vascular endothelial cells stimulated by VEGF. This compound also inhibits NF-κB signaling, and the expression of the angiogenic CXC chemokines, CXCL1/GRO-α and CXCL5/ENA-78, in vascular endothelial cells stimulated by TNF-α (Fig. 7). C10 inhibited VEGF-induced angiogenesis both in vitro and in vivo, and also inhibited inflammation-induced angiogenesis signaling pathways. This compound could therefore be useful for the development of further inhibitors of tumor angiogenesis, which occurs in close association with inflammation.

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