A Redox-Silent Analogue of Tocotrienol Inhibits Cobalt(II) Chloride-Induced VEGF Expression via Yes Signaling in Mesothelioma Cells

Ayami Sato,*a Nantiga Virgona,a Akira Ando,b Masako Ota,a and Tomohiro Yano*a,d

*Graduate School of Life Sciences, Toyo University; 1–1–1 Izumino, Itakura, Oura, Gunma 374–0193, Japan: and bFaculty of Pharmaceutical Sciences, Setsunan University; Hirakata, Osaka 573–0101, Japan.

Received October 27, 2013; accepted February 5, 2014

Vascular endothelial growth factor (VEGF) plays a crucial role in tumor angiogenesis and represents an attractive anticancer target. We have previously demonstrated that a redox-silent analogue of α-tocotrienol, 6-O-carboxypropyl-α-tocotrienol (T3E) exhibits potent anti-carcinogenic property in human malignant mesothelioma (MM) cells. However, inhibition of tumor growth by targeting VEGF pathway remains undetermined. In this study, we explored the inhibitory effect of T3E on the paracrine secretion of VEGF in MM cells under mimicked hypoxia by cobalt chloride (CoCl2). In this study we examine whether T3E can suppress the secretion of VEGF in MM cells exposed to mimic hypoxia by cobalt chloride (CoCl2). We found that CoCl2-induced hypoxia treatment leads to increased up-regulated hypoxia-inducible factor-2α (HIF-2α) and subsequently induced the secretion of VEGF in MM cells. This up-regulation activation mainly depended on the activation of Yes, a member of the Src family of kinases. Treatment of hypoxic MM cells with T3E effectively inhibited the secretion of VEGF. On the other hand, T3E inhibited CoCl2-induced gene expression of VEGF due to the inactivation of Yes/HIF-2α signaling. These data suggest that Yes/HIF-2α/VEGF could be a promising therapeutic target of T3E in MM cells.

Key words tocotrienol; redox-silent analogue; mesothelioma; hypoxia-inducible factor (HIF); Yes; vascular endothelial growth factor (VEGF)

Malignant mesothelioma (MM) is a highly aggressive cancer that arises from the mesothelial cell lining of serosal membranes and which is characterised by rapid progression, late metastases, and poor prognosis. Most common MM (90%) are of pleural origin, and approximately 80% of MM cases can be attributed to asbestos fiber exposure. Preclinical and clinical studies have identified inhibition of angiogenesis as a therapeutic strategy to achieve real progress in MM. Vascular endothelial growth factor (VEGF) is a potent angiogenesis inducer playing a critical role in tumor progression and whose expression of VEGF in cancer also promotes tumor growth and formation of invasive and metastatic cancers. The hypoxic microenvironments are also associated with alterations in signaling proteins including the Src family of kinases (SFK). Hypoxia-induced VEGF expression required Src activation and resulted in increased steady state levels of HIF-1α. The importance of Src function in response to VEGF has also been demonstrated in vivo. It has been shown that inactivation of SFK mediated VEGF expression in tumor cells and the extravasation of tumor cells from the vascular space. More recently, Yes, a member of SFK has been reported to be a central mediator of cell growth in MM cells. As the VEGF and HIF pathways are intertwined to promote angiogenesis and tumor growth, targeting Yes/HIF-2α/VEGF signal pathway may represent a viable anticancer therapeutic target for MM.

Tocotrienols, one of the vitamin E isoforms, has demonstrated inhibitory effect on tumor angiogenesis via regulation of angiogenic factors such as HIF-1α, and its downstream target VEGF. Our group has shown that a redox-silent analogue of α-tocotrienol, 6-O-carboxypropyl-α-tocotrienol (T3E), enhanced the anticancer effect of cytotoxic drugs by inhibiting the hypoxia adaptation. As we recently reported that T3E effectively suppresses the activation of SFK in tumor cells, it is possible that T3E acts as an effective anti-MM agent due to the inhibition of Yes/HIF-2α/VEGF signal pathway under hypoxia. The present study we report on...
the mechanistic effect of T3E on VEGF expression in MM induced by CoCl₂ as a hypoxia-mimetic agent.

MATERIALS AND METHODS

Human mesothelioma H2452 cell line (ATCC, Rockville, MS, U.S.A.) were routinely grown in RPMI1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 10 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (Hepes), 50 IU/mL penicillin, and 50 µg/mL streptomycin (culture medium) at 37°C in a humidified atmosphere with 5% CO₂. For experiments, exponentially growing cells were used. Cells were plated on culture plates and cultured for 24 h to permit the cells to adhere. After attachment, the cells were cultured in RPMI1640 supplemented with 2% FBS (culture medium) containing each reagent, and each assay was performed. The cells were pretreated in culture medium containing cobalt chloride (CoCl₂) for 6 h to produce a mimic hypoxia condition, and subsequently treated with CoCl₂ and T3E for each time described in figure legends as mentioned below. Treatment of short interfering RNA (siRNA) was performed for 24 h before CoCl₂ treatment as described below. After the treatment, cell viability was determined with Cell Proliferation Assay Kit using WST-1 reagent (Roche Japan, Tokyo, Japan).

A siRNA targeting human Yes (Cat. No. SI02223935) and non-specific siRNA (NsssiRNA) were designed and synthesized by QIAGEN Japan (Tokyo, Japan). siRNA was transfected into the cells by using RNAiFect Transfection Reagent (Invitrogen, Carlsbad, CA, U.S.A.) as previously reported. At 24 h after the transfection, the cells were then incubated for 24 h under mimic hypoxic condition, and subsequently each assay was performed according to each method.

Total RNA was isolated by using SV Total RNA Isolation System (Promega, Madison, WI, U.S.A.) and cDNA was synthesized as previously described. Reverse transcription (RT) real-time polymerase chain reaction (PCR) was performed by using an ABI Prism 7000 Sequence Detection System (Applied Biosystems Japan Ltd., Tokyo, Japan) and SYBR Premix Ex Taq™ (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The primers used were as follows; ribosomal protein CL32 (PRL32), Accession No. (NM_000994), sense (nucleotides 77–96), antisense (nucleotides 229–199); VEGF, Accession No. (NM_001025368), sense (nucleotides 1163–1182), antisense (nucleotides 1390–1370). Immunoblot analysis was performed as previously described. Briefly, cell lysate was prepared in Cell Lysis/Extraction Reagent (Sigma, St. Louis, MO, U.S.A.) including phosphatase inhibitor cocktail, phosphatase inhibitor cocktail2, and protease inhibitor cocktail, and 10 µg total protein extract from each sample was loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes. The blots were incubated with each antibody. Each immunoreactive band was detected using the ECL system (Amersham, Buckinghamshire, U.K.) and a cooled CCD camera-linked Cool Saver system (Atto, Osaka Japan). Molecular sizing was done using Rainbow MW marker (Amersham). Protein concentrations were determined using DC Protein Assay System (Bio-Rad, Hercules, CA, U.S.A.).

After CoCl₂ treatment for 6 h, the cells were changed to a serum free medium with CoCl₂ with or without T3E for 24 h.

Fig. 1. Effect of CoCl₂ Treatment on Cell Viability, the Expression of HIF-2α Protein and VEGF mRNA in H2452 Cells

(A) Dose-dependent changes of cell viability in the cells treated with CoCl₂ for 24 h. Cell viability was determined by WST-1 assay, and each column indicated the mean and vertical lines indicated S.D. (N=5). (B) HIF-2α protein level in the cells treated with CoCl₂ for 24 h was estimated by immunoblot analysis, and α-tubulin was used to confirm equal protein level loading among each lane. The bar graph shows the ratio of HIF-2α protein density to α-tubulin protein density. Each column indicated the mean and vertical lines indicated S.D. (N=3). (C) Dose-dependent alteration of VEGF mRNA level in the cells treated with CoCl₂ for 24 h. VEGF mRNA level was determined by RT-real time PCR, and each column indicated the mean and vertical lines indicated S.D. (N=5). *Significant difference from 0 µM treated-group.
The cultured supernatants were collected and centrifuged. The VEGF protein levels in the supernatants were quantified by ELISA (Biosource, Camerillo, CA, U.S.A.).

Data were analyzed by one-way ANOVA followed by Student’s t-test or Dunnett’s multiple-range test. The p-values of 0.05 or less were considered significant.

RESULTS

Effect of CoCl₂ on Cell Viability, and the Expression of HIF-2α Protein and VEGF mRNA in H2452 Cells

To estimate the effect of CoCl₂ on growth of H2452 cells, the cell viability was determined using the WST-1 assay. The cells were treated with each concentration (50, 75, 100 µM) of CoCl₂ for 24h. As shown in Fig. 1A, cell viability showed a decreased tendency in a dose-dependent manner. However, the decrease was only statistically significant in the 100 µM-treated group compared to control group. The inhibitory effect of CoCl₂ (100 µM) on cell viability was due to induction of apoptosis but not cell cycle arrest, and other treatment doses did not show any apoptosis (data not shown). In contrast, HIF-2α level in H2452 cells was increased in a dose-dependent manner (Fig. 1B). Similarly, VEGF mRNA level in the cells was elevated by CoCl₂, but the increase reached a plateau in the 75 µM-treated group (Fig. 1C). These results suggested that 75 µM treatment of CoCl₂ was adequate to stabilize HIF-2α and induce VEGF with less toxicity compared to other treatment groups.

T3E Inhibits CoCl₂-Mediated Activation of Yes/HIF-2α/VEGF Signaling in H2452 Cells

First, we estimated the effects of different doses of T3E on cell viability in H2452 cells under presence and absence of CoCl₂ to determine an ad-

---

Fig. 2. Effect of T3E Treatment on Cell Viability, the Activation of Yes Signaling and VEGF Secretion in H2452 Cells Treated with CoCl₂

The cells were treated with T3E (10, 20 µM) for 4h and subsequently treated with CoCl₂ (75 µM) and T3E (10, 20 µM). After the treatment, each parameter was determined. (A) Dose-dependent changes of T3E in cell viability of the cells under CoCl₂ (+) or (−). Cell viability was determined by WST-1 assay, and each column indicated the mean and vertical lines indicated S.D. (N=5). (B) T3E inhibited CoCl₂-induced elevation of VEGF mRNA level in the cells (N=3). The mRNA level was determined by RT-real time PCR. (C) T3E suppressed CoCl₂-stimulated secretion of VEGF in the cells. The secretion of VEGF was determined by ELISA, and each column indicated the mean and vertical lines indicated S.D. (N=5). (D) T3E inactivated CoCl₂-stimulated Yes signaling in the cells. Each molecule in Yes signaling was determined by immunoblot analysis, and α-tubulin was used to confirm equal protein level loading among each lane. The bar graphs show the ratio of Yes phosphoprotein density to total Yes protein density and that of HIF-2α protein density to α-tubulin protein density, respectively. Yes-p, phosphorylated form of Yes; total Yes, un-phosphorylated and phosphorylated form of Yes. Each column indicated the mean and vertical lines indicated S.D. (N=3). * Significant difference from control group.
equate treatment dose of the agent. As shown in Fig. 2A, only 20 µM treatment of T3E significantly reduced cell viability compared to control under both the presence and absence of CoCl2. Next, we checked the effects of different doses of T3E on the expression of VEGF mRNA and secretion of VEGF protein under both the presence and absence of CoCl2. The CoCl2-increased expression of VEGF mRNA and secretion of VEGF protein were mostly suppressed by only the 20 µM treatment of T3E with a significant difference, whereas but the other lower doses of T3E did not affect the VEGF level. Additionally, all treatment doses of T3E had no influence on the expression of VEGF mRNA and secretion of VEGF protein under the absence of CoCl2 (Figs. 2B, C). Thus, these results indicated that the effect of T3E on VEGF level in H2452 cells under the presence but not absence of CoCl2 (Figs. 2B, C). Finally, to investigate if T3E could inhibit the secretion of VEGF from H2452 cells treated with CoCl2 via the inactivation of Yes, we investigated the effect of T3E on Yes and HIF-2α, signal molecules located in the upstream of VEGF. As shown in Fig. 2D, CoCl2-induced activation of Yes and stabilization of HIF-2α were almost cancelled by 20 µM treatment of T3E, demonstrating that T3E inhibited the secretion of VEGF via the suppression of CoCl2-mediated activation Yes/HIF-2α signaling in H2452 cells. In order to confirm this, knockdown of Yes signaling by the use of siRNA was carried out to ascertain the effect on HIF-2α and VEGF levels in CoCl2-treated H2452 cells. As shown in Figs. 3A–C, under the silencing of Yes by siRNA treatment, HIF-2α protein, VEGF mRNA and secretion were all reduced in the CoCl2-treated cells. These results completely supported the above speculation.

DISCUSSION

Under hypoxia condition, angiogenesis in solid tumors is a main determinant of malignancy.9) Tumor neovascularization depends on the production of specific angiogenic factors. Of the factors, VEGF is a main factor closely related to angiogenesis and metastasis in several solid tumors. Actually, under hypoxia condition, HIF-1α induced the overexpression of VEGF, and this event leads to tumor progression and poor clinical outcome.9) Thus, HIF-1α and VEGF have been considered promising molecular targets for solid tumor treatment.18) Also, HIF-1α acts as a main transcription factor to induce VEGF under hypoxia condition.15) However, we could detect HIF-2α but not HIF-1α in H2452 cells under our hypoxia condition. Additionally, of the HIF subtypes, HIF-2α is strongly associated with adaptation of cancer cells during prolonged hypoxia,18) and target genes in HIF-2α are almost the same as wth those in HIF-1α.19) Taken together, it seems that HIF-2α mainly contributes to VEGF overexpression in H2452 cells. With respect to molecule inducing stabilization of HIF-2α under hypoxia, the activation of a main member of SFK, c-Src plays a central role in several types of cancer such as lung cancer cells.19) The phosphorylation of HIF-2α by molecules located in the downstream of the SFK, such as phosphoinositide 3-kinase (PI3K)/AKT finally leads to the stabilization and activation of the HIF.19) However, it is well known that the expressed pattern of SFK is different among each cancer.20) Moreover, we have reported that Yes but not c-Src is a central role in determining malignancy of MM cells.19) Overall, it is considered that the activation of Yes/HIF-2α by CoCl2 treat-
ment is associated with VEGF overexpression in H2452 cells. As previously reported, T3 has potential physiological activities, such as anti-cancer activity, irrespective of its antioxidant activity.\(^2\)\(^3\) In order to clarify this notion, we have synthesized a redox-silent analogue of T3, T3E and reported that the anti-cancer activity of this analogue is much more potent than that of T3 in MM cells.\(^2\)\(^3\) Thus, the current finding completely supports the notion. However, with respect to a possible mechanism on anti-cancer effect of T3, multiple target molecules have been reported.\(^2\)\(^3\) Of these target molecules, SFK could be promising. Of the subtypes of SFK, only the activation of c-Src during the development of neurodamaes has been inhibited by T3 irrespective of its antioxidant property.\(^2\)\(^3\) Also, we have observed that T3E but not T3 strongly inhibited the activation of c-Src in lung cancer cells.\(^10\) These reports led us to hypothesize that T3E can inhibit secretion of VEGF and subsequent angiogenesis driven by the factor in MM cells via the inactivation of Yes/HIF-2α/VEGF signaling under a hypoxia condition. Actually, we observed that T3E suppressed the secretion of VEGF in H2452 cells due to the inhibition of CoCl\(_2\)-induced activation of Yes/HIF-2α/VEGF signaling. In detail, the scheme is as follows. T3E directly suppresses the activation of Yes caused by CoCl\(_2\) treatment, and this inactivation induces the instabilization of HIF-2α via the reduction of its phosphorylation level. Since the stabilization leads to a decrease of transactivation activity of HIF-2α for VEGF, finally, the secretion of the VEGF is inhibited. With respect to the influence of T3E on VEGF-mediated angiogenesis, we can speculate a beneficial effect of T3E on an angiogenesis based on the following report and the present data.

In an angiogenesis model in vivo, T3 significantly inhibits the angiogenetic activity induced by VEGF,\(^2\)\(^3\) and our present data clearly demonstrated that the CoCl\(_2\)-caused VEGF secretion in H2452 cells was drastically suppressed by T3E. The effect of T3E observed in this study is similar with that of T3 reported in gastric cancer cells treated with CoCl\(_2\).\(^16\) Furthermore, secreted VEGF from tumor cells binds to specific trans-membrane receptors such as VEGF receptor 1 on endothelial cells and consequently initiates a number of key endothelial angiogenic responses, leading to the development of tumor tissues.\(^2\)\(^4\) Since a previous study has shown that T3 suppresses the VEGF-induced angiogenesis in vivo,\(^2\)\(^3\) T3E may also have a similar effect on the event. If so, T3E may have dual inhibitory effects on angiogenesis in tumors, that is, inhibition of both VEGF secretion from tumor cells and VEGF-driven angiogenic responses in endothelial cells. Collectively, our findings provide positive evidence that T3E can inhibit VEGF-driven angiogenesis in MM cells under hypoxia by blocking Yes/HIF-2α/VEGF signaling, leading to a speculation that T3E acts as a potential anti-MM agent.

In conclusion, our present study for the first time showed that T3E remarkably inhibited HIF-2α accumulation and VEGF secretion by a mimic hypoxia (CoCl\(_2\))-induced Yes activation in H2452 cells. Thus, T3E might be a useful candidate reagent for treating MM, due to blocking Yes/HIF-2α/VEGF signaling. T3E should be investigated by further in vivo study.

**Acknowledgment** This study was supported by a research Grant (Inoue Enryo Memorial Foundation) from Toyo University.

---

**REFERENCES**


