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A redox-silent analogue of tocotrienol inhibits hypoxic adaptation of lung cancer cells

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Abstract

We have previously reported that a redox-silent analogue of α-tocotrienol (T3), 6-*O*-carboxypropyl-α-tocotrienol (T3E) shows more potential anti-carcinogenic property than T3 in a lung cancer cell (A549 cell). However, the mechanisms by which T3E exerts its potential anti-carcinogenic effect is still unclear. As tumor malignancy is associated with hypoxia adaptation, in this study, we examined whether T3E could suppress survival and invasion in A549 cells under hypoxia. Hypoxia treatment drastically-induced activation of the protein tyrosine kinase, Src, and its regulated signaling required for hypoxia adaptation of A549 tumor cells. The survival and invasion capacity of the tumor cells under hypoxia was suppressed by T3E via the inactivation of Src. More specifically, T3E-dependent inhibition of Src-induced Akt activation contributed to suppression of cell survival under hypoxia, and the reduction of fibrinolytic factors such as plasminogen activator-1(PAI-1) via the decrease of hypoxia-inducible factor-2α by T3E led to inhibition of hypoxic invasion. Overall these results suggest that T3E suppresses hypoxia adaptation of A549 cells by the inhibition in hypoxia-induced activation of Src signaling.

Keywords: 6-*O*-carboxypropyl-α-tocotrienol; Redox-silent analogue; Hypoxia adaptation; Src; Hypoxia-inducible factor-2α; Plasminogen activator-1; A549 cell

Although hypoxia is toxic to both tumor cells and normal cells, the tumor cells undergo genetic and adaptive changes that allow them to survive and proliferate in hypoxic environments, leading to aggressive tumor behavior [1]. Hypoxic tumor cells are also considered to be resistant to most anti-cancer agents for several reasons: for example, hypoxia selects for cells that have lost sensitivity to p53-mediated apoptosis, which might lessen sensitivity to some anti-cancer agents [2]. Thus, inhibition of hypoxic adaptation in tumor cells may contribute to effective negative growth control of tumor cells. For survival, invasion and metastasis of tumor cells under hypoxia, it is required for the hypoxic tumor cells to adapt to an anaerobic environment by transcriptional induction of genes involved in glycolysis, haematopoiesis, angiogenesis, apoptosis and tissue invasion [3]. The majority of the genes required for hypoxia adaptation are regulated by hypoxia-inducible factor (HIF), which is composed of two subunits: a hypoxia-inducible HIF-α subunit and a constitutive expressed HIF-β subunit [4]. In recent studies, the activation of the protein tyrosine kinase, c-Src rapidly increases following hypoxia, and the activated Src induces the expression and stabilization of HIF [5,6]. These reports suggest that c-Src is a promising target to regulate the hypoxia adaptation of tumor cells.

Vitamin E (tocotrienols and tocopherols), a fat soluble vitamin, is well known for its cellular antioxidant and lipid

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lowering properties [7]. The two isoforms of vitamin E share the same aromatic chromanol 'head' but differ in their side chains. Tocopherols posses saturated phytyl side chain while tocotrienols have an unsaturated isoprenoid chain along their 'tails'. Due to the structural difference of the two compounds, tocotrienols have more potential physiological functions than tocopherols such as anti-carcinogenic effect [8].

In our previous study, α-tocopherol (T), a representative tocopherol, is inactive for the negative growth control of cancer cells; while in contrast an ether derivative of T, 6-*O*-carboxypropyl-α-tocopherol (TE), a redox-silent analogue of T, is a potent growth inhibitor against cancer cells *in vitro* and *in vivo* [9]. Similarly, an ether derivative of α-tocotrienol (T3), 6-*O*-carboxypropyl-α-tocotorienol (T3E), has cytotoxicity against a lung adenocarcinoma cell line (A549) *in vitro* much stronger than that of T3 or TE [10]. However, the reason why T3E has a potential anti-carcinogenic effect is still unclear at present. In recent studies, it is shown that T3 has protective effects against several toxins-induced neurodamages and ischemia-reperfusion-mediated cardiac dysfunction via inhibition of c-Src activation, maybe due to a non-antioxidant property of T3 [11]. Collectively, it seems to be possible that the potential anti-carcinogenic effect of T3E depends on the suppression of Src activation in cancer cells. In order to address this issue, this study was undertaken.

Materials and methods

Reagents. All culture and chemicals were purchased from Gibco BRL (Tokyo, Japan) and Sigma (St. Louis, MO), unless otherwise indicated. LY 294002 (a phosphatidylinositol 3-kinase (PI3K) inhibitor) was purchased from Calbiochem–Novabiochem (La Jolla, CA, USA). T3 was purchased from Tama Biochemicals (Tokyo, Japan). Antibodies against α-tubulin and HIF-2α were obtained from BD Transduction Laboratories, Inc. (Franklin Lake, NJ) and Novus Biologicals, Inc. (Littleton, CO), respectively. Other antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

T3E synthesis. T3E was synthesized from T3 according to a previously reported procedure [12]. The purity of T3E was confirmed by GC–MS, ^{1}H-NMR, ^{13}C-NMR, and IR.

Cell culture and treatment. A549 cells (Riken cell bank, Saitama, Japan) and NIH 3T3 cells (ATCC, MA) were routinely grown in DMEM supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin, and 50 μg/ml streptomycin (culture medium) at 37 °C in a humidified atmosphere with 5% CO_2. 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 DMEM supplemented with 2% FCS containing each reagent, and each assay was performed. The cells were pretreated with each agent (T3E, T3, and LY294002) for 4 h and subsequently cultured under a hypoxic condition for each time described in figure legends as mentioned below. Treatment of Short interfering RNA (siRNA) was performed as described in siRNA treatment.

Exposure to hypoxia. A hypoxia condition (1% O_2, 5% CO_2) was produced using the AnaeroPack system (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan), according to the manufacturer's instruction. When the cells were co-treated with siRNA and T3E, instead of the AnaeroPack system, cobalt chloride ($CoCl_2$) was used to mimic hypoxia condition.

Cell growth and apoptosis analysis. Cell growth was determined with Cell Proliferation Assay Kit using WST-1 reagent (Roche Japan, Tokyo, Japan), and apoptosis was determined by a Becton Dickinson FACScan.

Preparation and transfection of short interfering RNA (siRNA). A siRNA targeting human c-Src (Cat. No. SI02663038), the signal transducer and activator 3 (Stat3) (Cat. No. SI02662338), HIF-2α Cat. No. SI02223928) and non-specific siRNA (NssiRNA) were designed and synthesized by QIAGEN. siRNA was transfected into A549 cells by using RNAiFect Transfection Reagent (Invitrogen) as previously reported [13]. At 12 h (Stat3 and HIF-2α) or 24 h (c-Src) after the transfection, the cells were then incubated for each time indicated in figure legends under hypoxic condition, and subsequently each assay was performed according to each method.

Isolation of total RNA and real-time PCR. Total RNA was isolated by using SV Total RNA Isolation System (Promega, Madison, WI, USA) and cDNA was synthesized as previously described [14]. Real-time 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); type-1 plasminogen activator inhibitor (PAI-1), Accession No. (NM_000602), sense (nucleotides 887–905), antisense (nucleotides 1012–991).

Immunoblotting. Immunoblot analysis was performed as previously described [10]. Briefly, cell lysate was prepared in Cell Lysis/Extraction Reagent (Sigma) including phosphatase inhibitor cocktail1, phosphatase inhibitor cocktail2, and protease inhibitor cocktail, and 10 μg total protein extract from each sample was loaded onto 10% 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) 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, USA).

In vitro invasion assay. Invasion assay in a Matrigel invasion chamber (Becton Dickinson, Bedford, MA) was performed as described previously [15]. Cells were treated with siRNA for 12 h (HIF-2α or 24 h (c-Src). After that, the cells to be estimated were placed in the upper compartment in culture medium, and FBS-free medium was placed in the lower compartment. The cells were allowed to attach and spread for 12 h, and then, 3T3 conditioned medium as a chemoattractant were placed in the bottom compartment. In case of T3E and T3, after allowing the cells to attach and spread, pretreatment of each agent was performed for 4 h, and subsequently, the chemoattractant was added. After 24 h of incubation under hypoxic condition, the cells on top of the filter were scraped off and discarded. The remaining cells were fixed, stained, and counted using light microscopy (OLYMPUS, Tokyo, Japan). In parallel experiments, cells from the same lines were treated identically in 24-well plates. These cells were harvested, stained with trypan blue, and counted. The number of cells that had invaded was normalized to analyze the effects on cell viability. Ten random fields were chosen from each group and counted.

Statistical analysis. Data were analyzed by one-way ANOVA followed by Student's *t*-test or Dunnett's multiple-range test. *P* values of 0.05 or less were considered significant.

Results and discussion

Hypoxia adaptation is a critical factor to determine malignancy of tumor cells, and the activation of c-Src is a key event to support the hypoxic adaptation of the tumor cells [1,6]. In a recent report, T3 inhibited the activation of c-Src during the development of neurodamages irrespective of the antioxidant property [11]. These reports suggest that any potential anti-carcinogenic effect of T3E depends on