Inhibitory Effect of δ-Tocotrienol, a HMG CoA Reductase Inhibitor, on Monocyte-Endothelial Cell Adhesion

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Summary We have previously shown that α-tocotrienol (α-T3), a vitamin E analogue and HMG CoA reductase (HMGR) inhibitor, markedly inhibited monocyte-endothelial cell adhesion, a process that was reversed with the addition of mevalonate intermediates involved in protein prenylation. Since δ-T3 and γ-T3 possess greater HMGR inhibition than α-T3, we postulated that these analogues might have a greater effect on protein prenylation, and thus on monocyte adhesion and endothelial adhesion molecule expression in comparison to α-T3. Hence, we pursued to investigate the effect of various analogues of tocotrienol (α, γ, δ) on monocytic cell adhesion and expression of adhesion molecules using a human umbilical vein endothelial cell-line, EA.hy926, as the model system. Relative to α-T3, δ-T3 displayed a more profound inhibitory effect on monocyte cell adherence using a 15 μmol/L concentration within 24h (δ: 42±5%; α: 26±8% vs. control). This inhibitory action was reversed by co-incubation with farnesol and geranylgeraniol, suggesting a role for prenylated proteins in the regulation of monocyte adhesion. To further evaluate the effect of tocotrienols on the vascular endothelium, we measured the surface expression of adhesion molecules. Compared to α-T3, δ-T3 markedly inhibited the expression of VCAM-1 (δ: 57±6%; α: 37±10% vs. control) and E-selection (δ: 36±3%; α: 18±6% vs. control) in TNF-α activated endothelial cells. The above result suggests that δ-T3 is a potent and effective agent for the reduction of cellular adhesion molecule expression and monocytic cell adhesion.

Key Words tocotrienol, leukocyte adherence, adhesion molecule, vitamin E, EA.hy926

Attempts to lower the production of adhesion molecules, namely vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin, have received wide attention in view of their role in atherogenesis (reviewed in Ref. 1). As a result, a number of agents, including hydroxymethylglutaryl coenzyme A reductase (HMGR) inhibitors, have been shown to lower the production of adhesion molecules in hypercholesterolemic subjects (2, 3). HMGR inhibitors, also known as statins, have been shown to lower monocyte-endothelial cell adhesion by inhibiting the expression of endothelial adhesion molecules, as well as their counterparts, the monocytic integrin receptors (4–11). The molecular basis for this effect has been ascribed to its ability to inhibit protein prenylation of small GTP binding proteins involved in the signaling pathway of adhesion molecule/integrin gene expression. Addition of mevalonate and its metabolites, farnesol and geranylgeraniol, to the culture media has confirmed the specific role of protein prenylation in regulating monocyte-endothelial cell adhesion. Thus, HMGR inhibitors offer a novel method of reducing atherogenesis independent of their cholesterol-lowering action.

Tocotrienol is a fat-soluble vitamin belonging to the vitamin E family (Fig. 1). As reflected in their structural similarity, tocotrienol and tocopherol are well recognized for their antioxidative effect. However, unlike α-tocopherol, tocotrienol has been shown to have an intrinsic hypocholesterolemic activity (12, 13). The favorable cholesterol-lowering effect of tocotrienol has been attributed to its down-regulation of the rate-limiting enzyme of the cholesterol biosynthetic pathway, HMGR (14). δ-T3, followed by γ-T3, was later identified to be more potent than α-T3 in suppressing cholesterol biosynthesis (12).

We have previously investigated the effects of α-T3 on monocytic cell adhesion and adhesion molecule ex-
Fig. 1. Structures of various analogues of tocopherol and tocotrienol. The four tocotrienols share a similar chromanol moiety with their corresponding tocopherols. However, while tocopherol has a saturated phytyl side-chain, tocotrienol has an unsaturated prenylated side-chain.

δ-Tocotrienol Inhibits Adhesion Molecules

Pression in TNF-α activated human umbilical vein endothelial cells (HUVEC) (15). We demonstrated that α-T3 is a potent inhibitor of monocyte adhesion relative to α-tocopherol. The addition of farnesol and geranylgeraniol, intermediates of the mevalonate pathway involved in protein prenylation, interestingly reversed this effect, suggesting that prenylated proteins may be involved in the adhesiveness of monocytes to HUVEC. Since tocotrienol is a unique vitamin E with an isoprenoid side chain, we postulated that this might have accounted for δ-T3’s superior activity over α-tocopherol. We were also able to observe a marked reduction in endothelial VCAM-1, ICAM-1 and E-selectin surface expression with α-T3 when compared to α-tocopherol. Since δ-T3 and γ-T3 possess greater HMG-R inhibition than α-T3 (12), we postulated that these analogues might have a greater effect on monocyte adhesion and adhesion molecule expression in comparison to α-T3. Hence, we investigated the effect of various analogues of tocotrienol (α, γ, and δ) on monocyte cell adhesion and expression of adhesion molecules using an established human umbilical vein endothelial cell-line, EA.hy926, as the model system.

MATERIALS AND METHODS

Chemicals and reagents. Pure tocotrienols (α, γ, and δ) were provided by the Malaysian Palm Oil Board (MPOB). CellTiter 96® AQ™ One Solution Cell Proliferation MTS assay was obtained from Promega (Madison, WI, USA). Monoclonal antibodies against ICAM-1 and E-selectin were purchased from Southern Biotechnology (Birmingham, AL, USA), whereas monoclonal VCAM-1 antibody was obtained from Genzyme Diagnostics (Cambridge, MA, USA). Human recombinant tumor necrosis factor (TNF)-α was purchased from Calbiochem (La Jolla, CA, USA). Immortalized human umbilical vein endothelial cell-line (EA.hy926) was provided by Dr. Cora-Jean S. Edgell (University of North Carolina, Chapel Hill, NC, USA). Monocytic THP-1 cells were from the American Type Culture Collection (Rockville, MD, USA). Collagen (type I), Rose-Bengal, heparin, endothelial cell growth supplement, trans-farnesol and trans-geranylgeraniol were from Sigma Chemical Co. (St. Louis, MO, USA). DMEM media, RPMI-1640 media, fetal bovine serum (FBS) and all other tissue culture supplies were purchased from Invitrogen Life Technologies Inc. (Grand Island, NY, USA).

Cell culture. Stock solutions of tocotrienols (α, γ, and δ) were freshly prepared in 95% ethanol and preserved at −25°C for no longer than 3 wk. Immediately before use, the stock solutions were diluted in culture medium to give a final ethanol concentration of 0.1% (v/v). Preparations were found to be pure by HPLC (data not shown). EA.hy926 endothelial cells were maintained in DMEM supplemented with 10% FBS, 0.1 mmol/L sodium hypoxanthine and 16 μmol/L thymidine at 37°C, 5% CO2 and 95% air. This endothelial cell-line was derived from HUVEC by fusion with a relatively undifferentiated A549/8 cell line that had been characterized to possess normal human endothelial cell properties (16, 17). In respect to monocyte en-
endothelial cell adhesion, this cell-line has been shown to respond similarly to HUVEC when stimulated with TNF-α (18). Upon reaching confluence, EA.hy926 grown in 96-well microplates were pretreated with various tocotrienol analogues for 20 h prior to stimulation with TNF-α (100 U/mL) for 4 h. Untreated control cells received 0.1% (v/v) 95% ethanol without tocotrienol. The human monocytic cell-line, THP-1, was grown in RPMI-1640 media supplemented with 10% FBS. Cells were split according to standard procedures.

Cell-based ELISA. To measure the surface levels of ICAM-1, VCAM-1 and E-selectin expressed on EA.hy926 endothelial cell monolayers, a modified cell-based enzyme-linked immunosorbent assay (ELISA) was used as previously described (15). The degree of enzymatic turnover of the substrate, p-NPP (p-nitrophenyl phosphate), was determined by absorbance measurement at 405 nm with a ThermoMax microplate reader ( Molecular Devices, Sunnyvale, CA, USA). Absorbance readings in the absence of primary antibody served as the blank value.

Cell adherence assay. Adherence of monocytic cells to EA.hy926 endothelial cells was carried out by the Rose Bengal method as previously described (15). This method has been shown to compare well with the isotopic method (19, 20). It eliminates the need of pre-labeling monocytes, thus reducing the possibility of monocyte activation. THP-1 monocytes (6 x 10^4 cells/mL) were used in this assay. Monocyte-endothelial cell adhesion was then calculated from the difference in absorbencies at 570 nm between wells that contained monocytes and endothelial cells and wells that contained only endothelial cells.

Statistical analysis. Statistical differences were analyzed using Student’s t test with the level of significance set at 0.05. All data are expressed as mean±SE.

RESULTS

δ-T3 markedly reduces monocyte-endothelial cell adhesion

Initially, cell viability and proliferation were verified by the trypsin blue exclusion technique and MTS viability assay, respectively. Both methods revealed 15 μmol/L as an optimal concentration without cytotoxicity or decrease in cell proliferation (data not shown). This concentration was subsequently used in all experiments. Although, this concentration is considered to be at a higher concentration than that found in human plasma (21, 22), our concentration may still have physiological relevance. Hayes et al. (21) reported that tocotrienol was transported by chylomicrons and would disappear quickly from the plasma during chylomicron clearance. Since lipoprotein lipase on the surface of vascular endothelial cells has the ability to transfer vitamin E (23), it is probable that tocotrienol reaches the endothelium in substantial amounts even though plasma concentrations may not reflect this. In support of this theory, large amounts of tocotrienols were shown to accumulate in the skin of rats and mice after supplementation of a tocotrienol-rich diet, despite the fact that little was found in the plasma (24).

To characterize the relative effect of the tocotrienol analogues on monocytic cell adherence, we analyzed the adhesion of monocytic THP-1 cells to TNF-α stimulated EA.hy926. Pretreatment of cells with either 15 μmol/L of α, γ and δ-T3 for 20 h prior to stimulation with TNF-α all displayed significant inhibitory effects on monocytic cell adherence (26±8%, 27±4% and 42±5%, respectively, n=3, p<0.05 vs. control). Notably, δ-T3, as illustrated in Fig. 2, exerted a more profound inhibitory action on monocytic cell adhesion when compared to α- and γ-T3.

Farnesol and geranylgeraniol reverse δ-T3’s effect on monocyte-endothelial cell adhesion

Prenylated proteins have been shown to play a role in the signaling pathway involved in monocyte adhesion. Evidence supporting this mechanism of action has been derived from studies using HMG CoA reductase inhibitors known to interfere with protein prenylation (5-10). The addition of mevalonate and mevalonate intermediates and complete reversal of the HMG CoA reductase inhibitor effects confirmed the role for protein prenylation in these studies. To evaluate the role of protein prenylation with tocotrienol, we investigated whether or not δ-T3, an HMG CoA reductase inhibitor, in the presence of mevalonate intermediates can reverse the inhibitory effect of δ-T3 on monocytic cell adherence. To test this hypothesis, δ-T3-treated EA.hy926 were co-incubated with 5 or 10 μmol/L of farnesol (FOH) or geranylgeraniol (GGOH) for 20 h followed by a 4-h incubation with TNF-α. A preliminary study showed that a minimum of 5 μmol/L FOH and GGOH was needed to rescue EA.hy926 cells from the inhibitory effects of tocotrienol on monocyte adhesion (data not shown). As shown in Fig. 3, both FOH and GGOH were shown to reverse the inhibitory effect of δ-T3 on monocyte adherence, suggesting that prenylated proteins may be involved in the adherence of monocytes to endothelial cells under our experimental condition. A control experiment showed that neither FOH nor GGOH had an effect on TNFα-stimulated
Δ-Tocotrienol Inhibits Adhesion Molecules

Fig. 3. Effects of Δ-tocotrienol and mevalonate intermediates on monocyte adhesion. EA.hy926 cells were grown and treated with Δ-T3 and co-incubated with 5 or 10 μmol/L farnesol or geranylgeraniol followed by TNF-α activation for an additional 4 h. After treatment, cells were subjected to the monocytic cell adhesion assay. Data are expressed as a percentage of control. Values represent mean±SE of three independent experiments performed in triplicate. *p<0.05 versus control.

Fig. 4. Effects of tocotrienol analogues on adhesion molecule expression. EA.hy926 cells were pretreated with 15 μmol/L of α, γ and Δ-T3 for 20 h, washed and changed to growth media supplemented with TNF-α for 4 h. After stimulation, a modified cell-based ELISA was carried out to measure the surface expression of ICAM-1, VCAM-1 and E-selectin. Results are expressed as a percentage of control. Values represent mean±SE based on three independent experiments performed in triplicate. * p<0.05 versus control. 1 p<0.05 versus Δ-T3.

Adhesion molecule expression is markedly inhibited by Δ-tocotrienol

To further evaluate the relative effect of the tocotrienol analogues on the vascular endothelium, comparative analyses of α-T3, γ-T3 and Δ-T3 on endothelial expression of VCAM-1, ICAM-1 and E-selectin were performed. EA.hy926 cells were pretreated with 15 μmol/L of tocotrienols for 20 h before stimulation with TNF-α. As depicted in Fig. 4, all three analogues failed to significantly inhibit ICAM-1 expression; whereas all significantly reduced VCAM-1 and E-selectin expression to varying degrees compared to the untreated control cells (VCAM-1: α-37±6%, γ-38±8%, Δ-57±6%; E-selectin: α-18±6%, γ-31±10%, Δ-36±3%; n=3, p<0.05 vs. untreated control). Among the various analogues, Δ-T3 displayed a superior effect for reducing VCAM-1 expression, while both Δ-T3 and γ-T3 were more potent than α-T3 in inhibiting E-selectin expression.

DISCUSSION

The signaling pathway that leads to increased monocyctic cell adhesion is not fully understood. However, it is generally recognized that several signaling pathways, including protein kinase C (PKC), mitogen activated protein kinase (MAPK) and protein prenylation, in concert with the generation of reactive oxygen species (ROS) as the second messenger, lead to the activation of transcription factors, including NF-κB and activator protein-1 (AP-1). Since ROS is central to the pathway, a number of studies have indicated that antioxidants, including α-tocopherol, represent potential inhibitors of monocyctic cell adherence (25–27). Additionally, the involvement of prenylated proteins in the signaling pathway and the use of HMGR inhibitors represented other potential inhibitors of monocyctic cell adherence (4–11). While some studies have shown an inhibitory effect on monocyte adhesion by HMGR inhibitors via adhesion molecule expression (7–10), others have found the integrin receptors to be involved (4–6).

In a previous study in our laboratory, α-T3, a unique vitamin E with antioxidative and hypcholesterolemic properties, was shown to be a very potent agent relative to α-T in reducing monocyctic cell adhesion (15). This is consistent with the findings that α-T3 possesses greater antioxidant activity than α-T (28), and thus α-T3 may have greater capability in blocking ROS generation and the subsequent expression of adhesion molecules/integrin receptors. Furthermore, tocotrienol, unlike tocopherol, is an HMGR inhibitor and has the potential to inhibit protein prenylation and the subsequent expression of adhesion molecules/integrin receptors. Indeed, our laboratory has shown that α-T3 inhibited monocyctic cell adhesion, a process that was reversed by co-incubation with mevalonate intermediates, confirming the role for prenylated proteins in the regulation of monocyctic adhesion (15). Hence, tocotrienols may inhibit monocyte adhesion at various points in the signaling pathway including protein prenylation and ROS generation.

Tocotrienol, unlike tocopherol, possesses the ability to inhibit HMGR activity. The mechanism appears to involve a post-transcriptional suppression of HMGR (29). When Pearce et al. (12) investigated the structure-activity relationship that regulate cholesterol biosynthesis, they demonstrated the importance of tocotrienol side-chain unsaturation. They also ascertained that tocotrienol lacking 5-methyl substitution was more potent than α-T3 in suppressing HMGR activity (15). This is consistent with these observations, Δ-T3 (8-methyl) was found to be the most potent cholesterol inhibitor among the natural tocotrienol, followed by γ-T3 (7,8-dimethyl) and α-T3 (5,7,8-trimethyl). Qureshi et al. (30) even showed that the didesmethyl-T3 derivative (no methyl groups) delivered a higher level of HMGR suppression, unsur-
passed by the natural tocotrienol, confirming a structure-activity relationship. Since δ-T3 inhibits HMGR activity to a greater extent than α-T3, we speculated that δ-T3, followed by γ-T3, would cause greater inhibition on protein prenylation than α-T3, and thus on monocyte adhesion and adhesion molecule expression.

Our results demonstrated that δ-T3 displayed a more profound inhibitory effect on monocyte cell adhesion when compared to α- and γ-T3 and exerted a similar effect with VCAM-1. The fact that α-T3 and γ-T3 showed indistinguishable effects on monocyte adhesion may be due to the fact that VCAM-1, and not E-selectin, may be the adhesion molecule primarily responsible for monocyte adhesion in our system.

To delineate the mechanisms through which tocotrienol may be acting on monocyte-endothelial cell adhesion, we studied protein prenylation as a target point based on our previous findings with α-T3 (15). As described in our report, soluble intermediates of the mevalonate pathway and precursors of protein prenylation (i.e., farnesol and geranylgeraniol) added to the culture medium reversed monocyctic cell adherence in δ-T3-treated endothelial cells. This suggests that, with TNF-α stimulation, prenylated proteins are involved in the adherence of monocytes to endothelial cells.

To further evaluate the effects of tocotrienol analogues on the vascular endothelium, we measured the surface expression of adhesion molecules. Our data showed that δ-T3 markedly inhibited the expression of VCAM-1 in TNF-α-activated endothelial cells within 24 h when compared to α-T3 and γ-T3. In regards to E-selectin, δ-T3 and γ-T3 showed significant and unequivocal effects when compared to α-T3. The fact that we did not necessarily see a staircase effect (i.e., δ-T3 > γ-T3 > α-T3), in particular with respect to γ-T3, remains inexplicable at this point in time. However, the fact that δ-T3 consistently showed a greater effect over α-T3 suggests a structure-function relationship that may be partly dependent on HMGR activity.

In summary, we have shown that δ-T3 had a profound inhibitory effect on monocytes adherent relative to α-T3. This observation may be due in part to the prenylation signaling pathway. However, we cannot determine, at this point, if this effect is being exerted on adhesion molecules or on the integrin receptors. Further studies must be undertaken to determine which molecules are involved. Our findings provide the groundwork necessary to achieve a more comprehensive understanding of the complex, but beneficial, effects of tocotrienol in the prevention of atherosclerosis.

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δ-Tocotrienol Inhibits Adhesion Molecules


