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Excessive retinol intake exacerbates choroidal neovascularization through upregulated vascular endothelial growth factor in retinal pigment epithelium in mice

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ABSTRACT

As a part of the visual cycle, all-*trans*-retinol (all-*trans*-ROL), the major form of vitamin A in circulating blood, is transported to the retinal pigment epithelium (RPE). All-*trans*-ROL is essential for normal retina function. However, recent researches have shown that excessive retinol intake can cause increase of all-*trans*-retinal. This can lead to the accumulation of lipofuscin, which is important in the pathogenesis of retina degeneration disease, such as dry type age-related macular degeneration (AMD). Since there are few reports regarding the involvement of all-*trans*-ROL in exudative AMD, we investigated the effects of all-*trans*-ROL in vitro and in vivo. We evaluated vascular endothelial growth factor (VEGF) expression in ARPE-19 cells and THP-1 cells after all-*trans*-ROL treatment using ELISA and real-time RT-PCR. In-vitro tube formation assay was performed with HUVEC cells using the conditioned medium (CM) obtained from ARPE-19 cells treated with all-*trans*-ROL. Transcriptional activity of retinoic acid receptor (RAR) was evaluated using luciferase assay. In mice, VEGF expressions were investigated in the retina and RPE/choroid after three weeks of excessive oral retinol intake. Laser-induced choroidal neovascularization (CNV) models were evaluated after they were fed with various doses of retinol. VEGF mRNA expression and VEGF production were significantly increased in all-*trans*-ROL treated ARPE-19 cells, which were inhibited by an RAR antagonist LE540. In contrast, there were no significant changes in VEGF production in THP-1 cells. Transcriptional activity of RAR was upregulated by all-*trans*-ROL treatment in ARPE-19 cells. The CM, obtained from ARPE-19 cells treated with all-*trans*-ROL, induced more capillary-like tube formation than cells treated with control vehicles. In vivo, the high retinol diet group has increased VEGF expression in the RPE/choroid and larger lesion size was induced. Our results suggest that all-*trans*-ROL is a pro-angiogenic factor. Excessive retinoid intake may be a potential risk factor for exudative AMD.

1. Introduction

Vitamin A derivatives include retinol (the alcohol form), retinal (the aldehyde form) and retinoic acid (the acid form). Vitamin A derivatives have diverse functions. The alcohol and aldehyde forms are generally considered to be major metabolites of the visual cycle, and the acid form is the biologically active metabolite. All-*trans*-retinol (all-*trans*-ROL), the major serum retinoid, circulates in the blood as a tertiary complex bound to retinol binding protein (RBP) and transthyretin (TTR), and is taken into the retinal pigment epithelium (RPE) to participate in the visual cycle (Kawaguchi et al., 2007; Newcomer and Ong, 2000). All-*trans*-ROL is transformed into a chromophore, 11-*cis*-retinal in the RPE cells, which is delivered to the photoreceptor cells and used to form photopigments with opsins. After visual signal transduction, all-*trans*-retinal (all-*trans*-RAL) released from opsins is converted into all-*trans*-ROL for the next cycle (Travis et al., 2007; Wang and Kefalov, 2011).

All-*trans* retinoic acid (atRA), another active metabolite of retinol, regulates the expression of over 500 genes and an unknown number of noncoding RNAs (Balmer and Blomhoff, 2002; Cawley et al., 2004). It is reported that atRA induces VEGF expression via retinoic acid receptor (RAR) in some cell lines, including the human RPE cell line, and is pro-angiogenic (Akiyama et al., 2005; Iriyama et al., 2008).

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Retinol cannot be synthesized by humans, and therefore must be absorbed as either retinol/retinyl esters from animal products or provitamin A carotenoids, such as β-carotene contained in plant products. The digested forms of vitamin A are absorbed in the intestines and stored in the liver (Thurnham and Northrop-Clewes, 1999). Carotenoids are a group of pigments that perform several important physiological functions. β-carotene, the most important carotenoid in the human diet, is proposed to have inherent antioxidant capabilities and prevent oxidative damage to cells (Amengual et al., 2011). However, the association between provitamin A and age-related macular degeneration (AMD) is still under debate. In the Eye Disease Case-Control Study, investigators found that a high dietary intake of carotenoids was associated with a lower risk for advanced AMD (Seddon et al., 1994). However, the Blue Mountains Eye study group found that there was no association between serum β-carotene and AMD (Smith et al., 1997). A study of smokers in Finland found that supplementation with β-carotene did not appear to be protective against AMD (Teikari et al., 1998). Recently, the Age-Related Eye Disease Study 2 (AREDS2) reported that there was no apparent effect of β-carotene elimination from the AREDS formulation (antioxidant vitamins C and E, β-carotene, and zinc) on the progression to advanced AMD (Group, 2013).

A toxic vitamin A-based fluorophore such as N-retinylidene-N-retinylethanolamine (A2E) present within lipofuscin is produced by condensation of all-*trans*-RAL with phosphatidylethanolamine in photoreceptor outer segments. A2E accumulations have been implicated in the death of RPE cells and causing dry AMD and Stargardt disease (Mata et al., 2000; Radu et al., 2005). Recent studies reported that reduction in serum vitamin A or inhibition of retinol uptake can arrest accumulation of toxic retinal fluorophores such as A2E (Radu et al., 2008; Sparrow et al., 2003). From these results, the possibility of a new treatment for retinal disease is being explored, which reduces serum retinol and inhibits ocular all-*trans*-ROL uptake to slow down the visual cycle (Dobri et al., 2013; Radu et al., 2008). However, another study demonstrated that a high-dose course of oral retinol increased the rate of rod-mediated dark adaptation in older adults with normal retinal aging or early age-related maculopathy, which are caused by a localized vitamin A deficiency in the RPE and Bruch's membrane (Owsley et al., 2006), suggesting that excessive intake of retinol is beneficial to retinal functions at least in some circumstances.

Considering the aforementioned diverse biological functions of vitamin A, we attempted to determine the pro-angiogenic effects of all-*trans*-ROL in several human cell lines, and found that VEGF expression was upregulated by all-*trans*-ROL treatment specifically in ARPE-19 cell line via retinoic acid receptor (RAR) activation, at least in part. The data in vivo also showed that laser-induced choroidal neovascularization (CNV) was exacerbated by excessive retinol intake. Together, these results suggest that retinol itself upregulates VEGF expression in RPE cells, and is pro-angiogenic in the posterior eye segment.

2. Materials and methods

2.1. Materials

All-*trans*-ROL was purchased from Sigma Chemical Co (St. Louis, MO). atRA and LE540, an RAR antagonist, were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). All reagents were dissolved in ethanol, and ethanol was used as a vehicle control in each experiment. GAL-4DBD containing the MH100-tkLuc reporter plasmid, and p-CMX-Gal-RARα, the GAL-fused expression vector, were described previously (Iriyama et al., 2008). FITC-conjugated concanavalin A was purchased from Vector Laboratories (Burlingame, CA).

2.2. Cell culture and ELISA analysis

Human retinal pigment epithelial cells (ARPE-19) and human monocytic cells (THP-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). ARPE-19 cells were seeded in laminin-coated transwell filters (Corning Costar, 0.4 μm pores, 12 mm inner diameter, NY), and cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (PS) at 37 °C in 5% CO_2 for 4–6 weeks to form a polarized differentiated monolayer. The apical side corresponded to the retinal facing side of RPE, and the basal side corresponded to the RPE/choroidal side (Dunn et al., 1996; Kannan et al., 2006; Sonoda et al., 2009). The supernatants secreted from the basal side of ARPE-19 cells were used for the ELISA analysis. THP-1 cells were cultured in RPMI 1640 supplemented with 10% FBS and 1% PS in 24-well plates at 37 °C in 5% CO_2.

All-*trans*-ROL was delivered at concentrations of 10^{-11}, 10^{-10} and

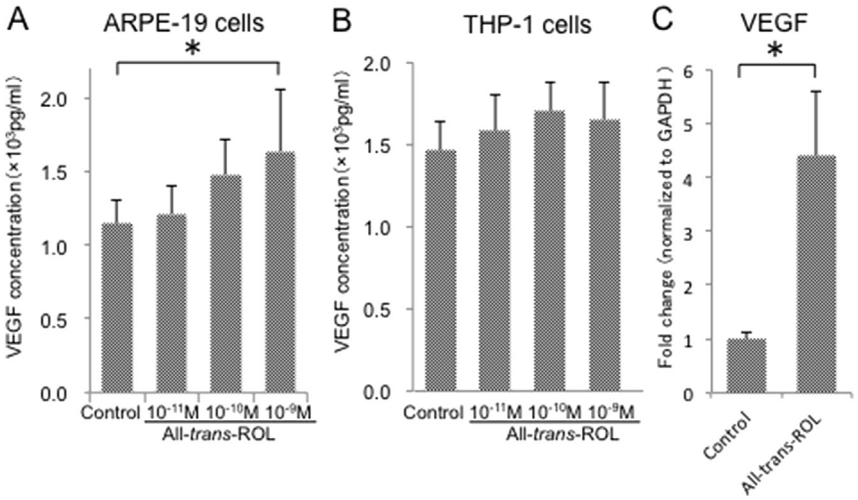

Fig. 1. VEGF expressions with all-*trans*-ROL treatment in ARPE-19 cells and THP-1 cells. (A) VEGF production of the culture medium increased in a dose-dependent manner in the basal side of ARPE-19 cells after 24 h of all-*trans*-ROL stimulation. (B) There were no significant changes in VEGF production between controls and retinol treatment groups in THP-1 cells. (C) VEGF mRNA level was also increased in the treatment of ARPE-19 cells with 10^{-9} M all-*trans*-ROL. *$P < 0.05$.