Monocyte chemoattractant protein-1 involvement in the α-tocopherol-induced reduction of atherosclerotic lesions in apolipoprotein E knockout mice

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We studied the effects of α-tocopheryl acetate supplementation on the development of fatty streaks and its ability to modulate the expression of monocyte chemoattractant protein (MCP)-1 in aortic lesions of apolipoprotein E knockout mice. For this purpose, 16-week-old apolipoprotein E knockout mice received α-tocopherol supplementation (800 mg/kg diet) for 6 weeks. After this time, total and lipoprotein cholesterol in the serum, hepatic tocopherol, aortic lesion area and MCP-1 (protein and mRNA) expression were analysed. Our present results showed that the dietary supplementation with α-tocopherol did not reduce serum cholesterol nor change lipoprotein profile, but it reduced the area of the aortic lesion by 55%. The reduction in the lesion size was correlated with the reduced expression of MCP-1 mRNA and protein, as detected by real-time quantitative polymerase chain reaction and immunohistochemistry respectively. In conclusion, the results obtained here are relevant to the study of atherosclerosis, as they correlate the effectiveness of vitamin E supplementation in inhibiting the plaque formation with diminished expression of MCP-1 at the aortic lesion.

Atherosclerosis: Apolipoprotein E knockout mice: α-Tocopherol: Monocyte chemoattractant protein-1: Antioxidant

In 1991, a workshop of the National Heart, Lung and Blood Institute discussed the oxidative hypothesis and the use of antioxidants in the prevention and treatment of atherosclerosis (Steinberg, 1992). As antioxidant vitamins have few adverse effects, trials with large doses of these vitamins were designed. Despite a clear rationale for its use, vitamin E supplementation has still an uncertain role in vascular disease.

Several reports have demonstrated the effect of vitamin E as an antioxidant, protecting the polyunsaturated fatty acids of the cellular membrane (Wefers & Sies, 1988). Its role in the prevention of oxidative damage extends to DNA, protein, carbohydrate and lipid (Wefers & Sies, 1988; Packer, 1991; Sies et al. 1992). As well as its antioxidant action, α-tocopherol (TOH) has precise cellular functions. Ricciarelli et al. (2002) recently reviewed the non-antioxidant role of this vitamin. They pointed out that, at the post-translational level, α-tocopherol inhibits protein kinase C and 5-lipoxygenase and activates protein phosphatase 2A and diacylglycerol kinase. Other genes (CD36, α-thiamin pyrophosphate, α-tropomysin and collagenase) are affected by TOH at the transcriptional level. TOH also induces inhibition of cell proliferation, platelet aggregation and expression of adhesion molecule, such as vascular cell adhesion molecule-1 and intercellular cell adhesion molecule-1, important components of formation and progression of atherosclerosis.

Besides the ‘oxidative hypothesis’ of atherosclerosis, the inflammatory hypothesis assumes that some pro-inflammatory molecules are involved in atherosclerosis. Circumstantial evidence supports a role for monocyte chemoattractant protein (MCP)-1 in early phases of atherosclerosis. This chemokine, chemotactic for monocytes and T lymphocytes, is a member of the β-chemokine subfamily secreted by various cell types present in the arterial wall, including endothelial cells, smooth muscle cells, fibroblasts and macrophages, in response to many physiologically relevant signals (Gu et al. 1998). Inducers of MCP-1 expression include minimally modified LDL and fluid shear. Increased expression of MCP-1 has been described in the arterial wall of hypercholesterolaemic rabbits (Libby et al. 1996; Gu et al. 1998) and human plaques (Nelken et al.

Abbreviations: Apo, apolipoprotein; CT, control; MCP, monocyte chemoattractant protein; PCR, polymerase chain reaction; TOH, α-tocopherol.

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1991; Takeya et al. 1993). Moreover, studies using MCP-1-deficient mice have suggested a correlation between MCP-1 expression, monocyte infiltration and the development of atherosclerotic lesions (Aiello et al. 1999). In this regard, both LDL-specific and cell-specific antioxidant actions may lead to decreased adhesion molecule and MCP-1 expression. As a consequence of the latter effects, decreased foam cell deposition, protection of vascular endothelium function, and decreased atherogenesis would be expected (Parthasarathy et al. 1998).

Apolipoprotein (Apo) E-deficient mice develop lesions very similar to those observed in human subjects (Nakashima et al. 1994; Reddick et al. 1994) and may be used as a tool in the study of the effects of vitamin E. In the present work, we investigated the effects of TOH supplementation on the size of aortic atherosclerotic lesions in ApoE knockout mice, focusing on the presence and expression of MCP-1. Our present results demonstrated that dietary supplementation with TOH was efficient in reducing expression of MCP-1, which correlated with smaller lesion area in aortas from the experimental group.

**Methods**

**Animals and diets**

Forty-three 16-week-old ApoE knockout mice, obtained from Jackson Laboratories, Bar Harbor, ME, USA and maintained at the animal facilities of the Laboratory of Nutrition and Gnotobiology (Universidade Federal de Minas Gerais, Brazil), were used. Ten C57BL/6, mice fed chow diet were used as a wild-type control (CT) group for MCP-1 analyses. The animals were maintained in collective cages (maximum five animals per cage) in an appropriate room with controlled temperature and with a 12 h light–dark cycle. The animals had free access to water and food. The experimental protocol was accepted by the Animal Care Committee of the Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais. The CT diet was based on the AIN-93G diet (Reeves et al. 1993) and was composed of (g/kg): starch 610, casein 200, cellulose 50, soya bean oil 70, vitamin E-free vitamin mixture 10, mineral mixture 50, choline 10. Acetate-o-tocopheryl (Sigma Chemical Co., St Louis, MO, USA) was added to the control (40 mg/kg diet) and supplemented group (800 mg/kg diet). Diets were kept in a dark room at 4°C.

**Experimental protocol**

Before starting the experimental diet at 16 weeks of age, eleven animals were killed for the evaluation of the presence of lesions at the aortic root. The remaining animals were then divided into CT (n 15) and TOH (n 17) groups and fed on the CT or TOH-supplemented diet respectively for 6 weeks. Food intake and body weight were measured weekly. The experiment was continued until mice reached 22 weeks of age, when the animals were killed under anaesthesia. Blood was drawn from the axillary region and serum was separated by centrifugation at 2500 rpm for 15 min. Samples of serum and liver were gassed with N2 (to eliminate O2) and frozen at ~20°C for the determination of total cholesterol and vitamin E.

Serum lipoproteins were separated on fast protein liquid chromatography, as described by Fazio et al. (1997). Forty fractions of 500 μg each were collected. A sample of 100 μl was used for cholesterol determination by the enzymatic method adapted for a ninety-six-well microplate.

Hepatic lipids were extracted by the technique of Folch et al. (1957). Total cholesterol in serum and in the lipid extract of liver were determined by cholesterol oxidase enzymatic method (Allain et al. 1974) using a commercial kit (Katal, Belo Horizonte, Brazil).

The extraction and the determination of TOH in serum and liver were carried out according to the technique of Ueda & Igarashi (1990).

**Histological analysis**. The heart and proximal section of the aorta were removed from animals and cleaned of adventitious tissue. The top half of the hearts was obtained under stereoscopic observation and fixed by immersion in paraformaldehyde (40 ml/l) in 0.1 M-PBS at room temperature. Specimens were routinely processed for paraffin embedding. The entire specimen was analysed (250 sections per mouse). The aortic root area was identified by the proximal presence of aortic valve leaflets. Every consecutive 5 μm thick section throughout the aortic root area (300 μm, sixty sections per mouse) was taken for analysis and stained with haematoxylin and eosin, according to Paigen et al. (1987). Of every five sections, one was kept for morphometrical analyses using a computerized image analyser (KS 300 program; Karl Zeiss, Berlin, Germany) attached to a micro-camera and microscope. The total lesion area of each animal represents the sum of lesion areas obtained from the ten sections analysed.

A second strategy for the quantification of atherosclerotic lesions was performed at the same anatomical level for both groups (sampled lesion area), measuring one selected section 180–200 μm distal to the beginning of the aortic valve. This method has already been used by other authors to measure and compare aortic atherosclerotic lesions (Tangirala et al. 1995; Boisvert et al. 1999). The measurements of the three largest lesions of each mouse were used to give the average of the group. Morphometrical analyses were performed using the average of three repeated measurements of the lesions from each animal.

**Immunohistochemistry**. Tissue sections were deparaffinized with xylene and dehydrated with graded ethanol. Serial 5 μm sections, obtained in representative areas and previously stained by haematoxylin and eosin, were chosen. Endogenous peroxidase activity was quenched by incubating the sections in H2O2 (300 ml/l) for 30 min at room temperature. After washing, sections were blocked with goat serum (50 ml/l PBS) for 30 min. Anti-MCP-1 (rabbit anti-mouse sera, a kind gift from Dr N. W. Lukacs, University of Michigan) (5 ml/l PBS) was applied to the sections and incubated for 18 h at 4°C in a humidified chamber. The sections were washed, incubated with pre-diluted biotinylated secondary antibody (CA93013; Dako Corporation, Carpinteria, CA, USA) for 1 h and then washed again. The sections were incubated for 30 min with a peroxidase-labelled avidin–biotin complex (CA93013; Dako Corporation), washed, colour developed

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with diaminobenzidine and counterstained in haematoxylin. The percentage of MCP-1 positive cells was calculated by counting the total and MCP-1 positive inflammatory cells observed in ten different slides per mouse (five × 40 fields per slide) and five mice per group. The average percentage per animal was used to perform the statistical analyses.

**Measurement of monocyte chemoattractant protein-1 expression by real-time quantitative polymerase chain reaction**

Fresh tissue samples were prepared using TRIzol reagent (GIBCO BRL, Rockville, MD, USA). Total RNA was extracted from a region of aorta stretching from the aortic arch until the kidney artery (CT n 7, TOH n 9, wild-type n 3) and cDNA prepared by reverse transcription. Briefly, RNA was measured spectrophotometrically and 1 µg RNA was reverse transcribed using 25 U M-MLV Reverse Transcriptase (Promega Corp, Madison, WI, USA) in 12.5 µl reaction mixture containing 250 µM-dNTP (Promega Corp.), 50 mM-Tris-HCl (pH 8.3), 75 mM-KCl, 3 mM-MgCl2, 10 mM-1,4-dithiothreitol, 10 U RNAsin (GIBCO BRL), 7.5 µM-oligo dT15 (GIBCO BRL). The mixtures were incubated for 5 min at 95°C, 5 min at 4°C and 5 min at 25°C; at this step 25 U reverse transcriptase was added to each sample and the reaction mixture was incubated for 60 min at 37°C. The temperature was then elevated to 95°C for 5 min and cooled again at 4°C for 5 min. The cDNA products were diluted up to 300 µl with sterile bi-distilled water.

Real-time reverse transcription–polymerase chain reactions (PCR) were performed in the ABI-Prism 7900HT Sequence detection system (Perkin Elmer/Applied Biosystems, Foster City, CA, USA). Reaction conditions were programmed using the software SDS 2.0 on Windows NT 4.0 (Applied Biosystems, Foster City, CA, USA), linked directly to the sequence detector.

PCR amplifications were carried out as previously described (Overbergh et al. 1998). Briefly, the reactions were performed in a total volume of 25 µl containing 2.5 µl sample cDNA, 50 mM-KCl, 10 mM-Tris–HCl (pH 8–3), 10 mM-EDTA, 60 mM-passive reference 1, 200 nmol each primer/l, 500 µM-dATP, 500 µM-2'-deoxyctydine 5'-triphosphate, 500 µM-dGTP, 1000 µM-2'-deoxyuridine 5'-triphosphate, 3.5 mM-MgCl2, 0.5 U AmpErase Uracil N-Glycosylase (Perkin Elmer/Applied Biosystems). Each reaction contained 100 nm corresponding detection probe. PCR amplification of each sample was performed in triplicate wells, using the following conditions: 2 min at 50°C and 10 min at 95°C, followed by a total of forty-five two-temperature cycles (15 s at 95°C and 60 s at 60°C).

Primers and fluorogenic probes (purchased from Applied Biosystems) for the target genes are listed below and have been described previously (Giulietti et al. 2001): GAPDH forward TCACCACCATGGAGAAGGC, reverse GCTAAGCACTTGTGCTGTGGA, TaqMan Probe ATGCCCCCATGGTTGTATGGGT; MCP-1 forward CTTCTGGGCTGCTGTTCA, reverse CCAGGCCTACTCATATTGGGATCA, TaqMan Probe CTCAGCCAGATCGAGTTAACGCCCC.

**Statistical analysis**

The data were initially analysed using Komogorov–Smirnov test to verify their symmetry. As all data had normal distribution, t test and ANOVA were used to compare two and three independent groups respectively, with the level of significance set at P<0.05.

**Results**

Table 1 shows that TOH did not affect food intake and body weight. Since food intake was the same for both groups, TOH intake is expected to be higher in the TOH-supplemented compared with CT group. The higher levels of TOH in serum and liver in TOH group were confirmed by HPLC analysis (Table 1). The TOH supplementation had no effect on total cholesterol levels in serum (Table 1) or on its distribution among the different lipoprotein fractions (results not shown). Similar to the findings in serum, there were no differences in the hepatic concentrations of cholesterol (Table 1), suggesting that differences in the size of atherosclerotic lesions could not be due to differences in the levels of cholesterol in liver or blood.

Histological observation of the aorta before the supplementation with TOH (at 16 weeks of age) showed initial fatty streak lesions in animals fed on CT (results not shown). Quantitative histological analysis of an aortic lesion is shown in Fig. 1. Both the average of the largest lesion (Fig. 1(A)) and total lesion (Fig. 1(B)) areas was smaller at week 16 than at 22 in CT groups (P<0.049 and 0.029 respectively). However, no statistical increase in lesion area was detected after 6 weeks of TOH supplementation when CT at week 16 and TOH at week 22 were compared (Fig. 1(A and B)). The TOH supplementation was able to prevent the development of lesion area at week 22 when compared with the non-supplemented group at the same week (P<0.05). Besides the lesion size, CT animals showed more advanced lesions with intimal thickening, necrotic core and deposition of inflammatory and smooth muscle cells between the endothelium and foam cells when compared with the TOH group (Fig. 2).

**Table 1.** Final body weight, and serum cholesterol and α-tocopherol levels in mice fed on control diet supplemented with 29.5 mg or 588 mg α-tocopherol/kg diet* (Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Diet...</th>
<th>CT</th>
<th>TOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables</td>
<td>n</td>
<td>Mean</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>15</td>
<td>19.3</td>
</tr>
<tr>
<td>Serum TOH (mg/l)†</td>
<td>2</td>
<td>4.92</td>
</tr>
<tr>
<td>Liver TOH (mg/mg)</td>
<td>5</td>
<td>5.81</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/l)</td>
<td>15</td>
<td>6.7</td>
</tr>
<tr>
<td>Week 22</td>
<td>15</td>
<td>12.4</td>
</tr>
<tr>
<td>Liver cholesterol (mg/g)</td>
<td>10</td>
<td>8.4</td>
</tr>
</tbody>
</table>

* CT, control; TOH, α-tocopherol; nd, not detected.
† The values refer to the concentrations in two different pools of serum.
When the expression of MCP-1 protein was evaluated (Fig. 3), mononuclear cells positive for MCP-1 were seen in the lesion area of the aorta in the CT group (Fig. 3(A)). Only sparse positive cells were observed in tissues of animals supplemented with TOH (Fig. 3(B)). Analysis of immunostained cells (positive cells (n)/total cells (n) × 100) showed that the % MCP-1-positive mononuclear cells was significantly greater in the CT group compared with the TOH and wild-type (C57Bl/6 mice) groups (Fig. 3(C)). To determine whether MCP-1 mRNA expression was also altered after TOH treatment, MCP-1 mRNA was assessed in the distal and proximal aorta by real-time quantitative PCR (Fig. 4). The results are in agreement to those seen after immunostaining of lesions in the aortic valve: there was a significant reduction in MCP-1 expression after TOH supplementation (Fig. 3(A)).

The results of real-time PCR showed that expression of MCP-1 in the aorta of the TOH group and wild-type group was similar. However, MCP-1 protein, as assessed by immunohistochemistry, was higher in TOH compared with the wild-type group. The reason for these apparently divergent results is that the analyses were done in different sites of the aorta: total RNA was extracted from the proximal and distal aorta and immunohistochemistry was performed at lesions in the aortic valve. Therefore, the differences between ApoE knockout (supplemented or not with TOH) and wild-type animals will be more pronounced in the lesion area than in the total proximal and distal aorta, where the number of non-stimulated cells is higher than in the lesion area.

Discussion

Observational studies indicated that the consumption of vitamin E reduces atherosclerosis progression (Rimm et al. 1993; Hodis et al. 1995; Azen et al. 1996). However, randomized controlled trials have had conflicting results regarding the effects of this vitamin on atherosclerosis (Stephens et al. 1996; GISSI-Prevenzione Investigators, 1999; Boaz et al. 2000; Salonen et al. 2000; The Heart Outcomes Prevention Evaluation Study Investigators, 2000; Brown et al. 2001; Collaborative Group of the Primary Prevention Group, 2001; Iuliano et al. 2001; Lonn et al. 2001; Hodis et al. 2002; Howard et al. 2002).
Several recent comments and reviews have addressed this subject, but there is still no plausible explanation for these conflicting results (McCall & Frei, 1999; Stocker, 1999; Chisolm & Steinberg, 2000; Steinberg, 2000; Landmesser & Harrison, 2001; Heinecke, 2001; Witztum & Steinberg, 2001; Brown et al. 2002; Devaraj et al. 2002; Huang et al. 2002; Rilley & Stouffer, 2002; Steinberg & Witztum, 2002). Against this conflicting background, it is important to identify the components of atherosclerosis that can be modulated by tocopherols. Knowledge about the molecular mechanism of action of vitamin E will help to elucidate the situations where vitamin E supplementation could be advantageous. Our present study addressed this question and showed that, at least in the initial stages, TOH supplementation was able to impair atherosclerotic development through a mechanism that involved reduction of MCP-1 production.

Although atherosclerosis is a complex human disease, ApoE knockout mice develop lesions very similar to those found in human subjects and are, thus, a suitable model for studies of atherosclerosis (Virmani et al. 2000). According to Hayek et al. (1994), atherosclerosis in ApoE knockout animals is related to the increase of lipid peroxidation in plasma, especially of lipoproteins.

As in trials with human subjects, there are conflicting reports on the ability of TOH to protect against atherosclerosis (Phonpanichrasamee et al. 1990; Williams et al. 1992; Kayden & Traber, 1993; Nakashima et al. 1994; Crawford et al. 1998; Munday et al. 1998; Parthasarathy et al. 1998; Asmis & Jelk, 2000; Tsimikas et al. 2000; Peluzio et al. 2001; Upston et al. 2002). This divergence may be explained by differences in dietary composition, dose of vitamin administered and the degree of pre-existing lesions. We previously showed the effect of TOH (400 mg/kg atherogenic diet) in reducing aortic lesions in ApoE knockout mice supplemented from 4 to 10 weeks old, when fatty streaks are absent or very sparse (Peluzio et al. 2001). In the present study, the supplementation with TOH was also efficient when initiated after the establishment of the initial aortic lesion and maintained until intermediate stages, suggesting the protective effect of this vitamin during the establishment of fatty streaks.

Praticò et al. (1998) reported a lesion area 2.5-times smaller in the TOH-supplemented group (2000 mg/kg diet) compared with CT group. We obtained the same reduction administering lower doses of vitamin E for a shorter time. On the other hand, vitamin E deficiency caused by disruption of the TOH transfer protein gene

Fig. 2. Histopathological analysis of aorta of apolipoprotein E knockout mice fed on control diet (A and C) or control diet supplemented with 800 mg α-tocopherol/kg diet (B and D). (C), (D), details of slides showing a small proportion of the wall area thickened by necrotic degeneration (*) and thin fibrous cap (→). Original magnification: (A) and (B) × 58; (C) and (D) × 256. For details of diets and procedures, see p. 4.
increased the severity of atherosclerotic lesion in ApoE knockout mice (Terasawa et al. 2000).

Studies using mice that overexpress MCP-1 have shown variable results with regard to monocyte infiltration (Phoonpanichrasamee et al. 1990; Munday et al. 1998). In the same way, in ApoE knockout mice the additional deficiency of CCR2 gene, despite producing hypercholesterolaemia, reduced tissue macrophage and atherosclerosis formation (Boring et al. 1998). Thus, a causal relationship between accumulation and modification of LDL and monocyte recruitment to the arterial wall seems to exist in the initial stages of atherosclerotic lesion development (Frei, 1999).

Attraction and adhesion of immune cells to the vascular endothelium and subsequent transmigration are important steps in the initiation and development of the inflammatory process associated with atherosclerosis. Oxidized lipoproteins may modulate the expression of adhesion molecules and stimulate monocyte recruitment and monocyte-endothelial interactions via chemokines such as MCP-1. Vitamin E could exert its protective effects against cardiovascular diseases by protecting LDL against oxidative modification, leading to decreased adhesion molecule and MCP-1 expression and foam cell formation (Djahansouzi et al. 2001). Alternatively, the vitamin could act directly on gene expression, reducing the expression of this chemokine.

Although there are some in vitro studies showing the influence of TOH supplementation on the production of MCP-1 (Wu et al. 1999), we provide in vivo evidence that the expression of MCP-1 is increased in aortic lesion in ApoE knockout animals and that TOH supplementation decreases its expression.

Fig. 3. The immunostained sections (A and B) of aortic lesions of apolipoprotein E knockout mice fed on control (CT) diet or CT diet supplemented with 800 mg α-tocopherol (TOH)/kg diet. (A), monocyte chemoattractant protein (MCP)-1 has increased expression in aortic lesions mice fed on CT diet. (B), TOH-supplemented mice have little expression of MCP-1 protein by macrophages. macrophages positive for MCP-1 protein; macrophages negative for MCP-1 protein. Original magnification × 990. (C), quantitative analysis of immunostained tissue sections showing % MCP-1 positive mononuclear cells. The % MCP-1 positive cells was calculated by counting the average of total and the MCP-1 positive inflammatory cells observed in ten different slides per mouse (five × 40 fields per slide). The percentage of positive cells was 70·5 for CT group, 16·7 for TOH-supplemented group and 3·2 for wild-type (C57BL/6) mice. Values are means for five mice per group with their standard errors shown by vertical bars. Mean value was significantly different from that of the CT group: *P<0·0001. Mean value was significantly different from that of the wild-type group: †P<0·001. For details of diets and procedures see p. 4.

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Our present results are in agreement with the hypothesis that one of the favourable effects of TOH is changing the expression of some important chemotactic molecules (as MCP-1). In this way, it can be hypothesized that vitamin E could be efficient in reducing fatty streak formation by reducing cell migration to the lesion site. In human trials, this positive action could be masked by other associated risk factors, making TOH supplementation ineffective in some of the published studies, mainly in secondary prevention.

In conclusion, the results obtained here are relevant to the study of atherosclerosis, as they correlate the effectiveness of vitamin E supplementation with a mechanism that implicates macrophage-active chemokines in the genesis of the early phase of atherosclerosis.

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