Pro-atherogenic effects of probucol in apo E-KO mice may be mediated through alterations in immune system: Parallel alterations in gene expression in the aorta and liver

Zuyuan Xu, Nazila Azordegan, Zhaohui Zhao, Khuong Le, Rgia A. Othman, Mohammed H. Moghadasian*

Department of Human Nutritional Sciences, University of Manitoba, and Canadian Center for Agri-food Research in Health and Medicine, St. Boniface Hospital Research Center, 351 Tache Avenue, Winnipeg, MB, Canada R2H 2A6

ARTICLE INFO

ABSTRACT

Objective: To establish underlying molecular mechanisms of pro-atherogenic effects of probucol in apo E-KO mice.

Methods: Affymetrix Gene Chip System, GenMAPP/MAPPFinder software and real-time PCR techniques were used to identify alterations in gene expression and biological pathways in the liver and aorta of both male apo E-KO and male wild-type mice treated with or without probucol (1%, w/w) for 18 weeks. Plasma levels of lipids, cytokines, liver function test, and the extent of atherosclerosis and liver histology were examined.

Results and conclusions: Probucol treatment paradoxically reduced plasma cholesterol levels, increased plasma cytokine levels and atherogenesis in apo E-KO mice. Three hundred and sixty genes/transcripts and 110 biological processes were significantly differentially expressed in the liver of probucol-treated apo E-KO mice. The response to biotic stimulus, immune response and inflammatory response were the most prominent processes expressed in the liver. The expression of 60 of these genes involved in immune response including inflammatory responses, antigen presentation, humoral immune response, immune cell activation, innate immune response, and regulation of immune response was over-expressed. Many of these genes were also over-expressed in the aorta of probucol-treated apo E-KO mice. Such effects of probucol were not observed in the liver and aorta of wild-type mice. A significant interaction between apo E deficiency and probucol treatment was observed. Histological examinations showed a significant infiltration of inflammatory cells in the liver of probucol-treated apo E-KO mice, but not in C57BL/6 mice. These findings suggest that probucol-induced atherogenesis may be mediated through a pro-inflammatory state.

1. Introduction

Several human and animal studies reported anti-atherosclerotic effects of probucol [1,2]. In contrast to these studies, probucol paradoxically promoted atherogenesis in apo E-KO mice [3,4]. These findings suggest that factors other than hypercholesterolemia and decreased antioxidant status may be involved in the development of atherosclerotic plaques. Several modifiable and non-modifiable risk factors have been identified for atherosclerosis [5,6]. Among them, elevated LDL-cholesterol levels, and its oxidized forms as well as a pro-inflammatory state may play a major role. In this regard, LDL-cholesterol-lowering and strong antioxidant properties of probucol [7–9] may theoretically prevent atherosclerosis in models other than apo E-KO mice.

Our goal was to identify potential molecular mechanisms involved in pro-atherogenic effects of probucol in apo E-KO mice. Both apo E-KO mice and wild-type counterparts were treated with probucol and the expression of a number of genes was studied in both liver and aorta of all groups of mice. Similarly, plasma lipid levels and histological examinations of aortic roots and liver tissues were performed to understand the associations between gene expression and biochemical and histological alterations after probucol treatment.

2. Materials and methods

2.1. Animals and diets

Twelve male 4-week-old apo E-KO mice and twelve male wild-type counterparts (C57BL/6) were purchased from the Jackson Laboratory. After a 10-day adaptation period, they were assigned to four groups, including apo E-KO control ($n = 6$), apo E-

---

KO probucol-treated ($n = 6$), C57BL/6 control ($n = 6$) and C57BL/6 probucol-treated ($n = 6$) mice as previously described [3,8]. All groups of animals received a diet containing 9% (w/w) fat and 0.15% (w/w) cholesterol with (treated groups) or without (control groups) 1% (w/w) probucol for 18 weeks as previously described [3]. At sacrifice, the hearts and liver specimens were harvested and fixed for histological examinations; thoracic aorta was collected, trimmed and stored in RNAlater (Ambion, Inc., Austin, TX) for RNA isolation. Specimens of liver tissue was also collected and stored in RNAlater. All animal protocols were approved by the Animal Care Committee at the University of Manitoba.

2.2. Affymetrix oligonucleotide microarrays assay and data analysis

Total RNA of liver was extracted and purified as previously described [10]. Four liver RNA samples were made by pooling six samples from each apo E-KO mice groups and used for microarray assays (4 chips each group) at the Microarray Facility of the Hospital for Sick Children (Toronto, Canada). The Affymetrix mouse gene 1.0 ST array was used (www.affymetrix.com). Microarray data normalization was performed as previously described [10–12]. We downloaded gene annotation file for mouse gene 1.0 ST array from Affymetrix (www.affymetrix.com). After removing all genes without gene names from the file, we mapped the genes with the gene annotation information, such as Gene Symbol and Gene Ontology (GO) to the normalized data. This resulted in 21,220 genes' annotations and their normalized data.

Assessment of differential expression genes of microarray data was performed using an empirical Bayes (EB) method [13] and Benjamini and Hochberg's algorithm [14]. Tests were considered to be significant, if the adjusted *p*-value was <0.1, nominally controlling the expected false discovery rate to no more than 10%. Biological pathway analysis was performed by mapping significantly expressed genes to the Gene Ontology database (www.geneontology.org) using GenMAPP (Gene Map Annotator and Pathway Profiler), as previously described [15,16].

2.3. Analyses of gene expression in liver and aorta using real-time PCR (RT-PCR)

Total RNA in the aorta was isolated using RNeasy Mini Kit (Qiagen) following the instruction with slight modification. Methods for liver RNA isolation and purification were explained above (Section 2.2). The purified total RNA from all of four groups' liver and aorta specimens ($n = 6$ in each group) was reverse transcribed with Oligo(dT) and random primer (Invitrogen) using SuperScript II reverse transcriptase (Invitrogen) to generate cDNA. RT-PCR analyses were performed as previous described [10]. The data were analyzed using 7500 system sequence detection software (version 1.3.1; Applied Biosystems) to determine the relative quantitative gene expression.

2.4. Plasma cholesterol, cytokines and liver function-related enzyme activity analyses

Total plasma cholesterol was measured at baseline and at the end of the study using standard enzymatic methods [3,8]. The pooled plasma samples ($n = 3$ for each apo E-KO mice group) were used to assay plasma cytokines level using Mouse G Series Cytokine Antibody Array II (RayBitech Inc., Norcross GA) as previously described [17]. For liver function test, plasma samples ($n = 4$ pooled samples per group) were sent to the biochemistry department of St. Boniface General Hospital (Manitoba, Canada) to test the activity of alkaline phosphatase (ALK) and gamma-glutamyltranspeptidase (GGT).

2.5. Aortic atherosclerosis and liver histological observation

Routine light microscopy examinations were performed on sections from aortic roots and liver specimens; we have extensively described these methods elsewhere [3,8]. The liver inflammation scoring system [18] and the aortic root inflammation scoring system [19] were used to evaluate the degree of inflammatory cell infiltration in liver and aorta, respectively.

2.6. Statistical analysis

For the analysis of microarray data, an empirical Bayes method [13] was used to determine statistically significant changes in the expression of genes given the adjusted-p-value < 0.1, nominally controlling the expected false discovery rate to no more than 10%, as previously described [13,14]. One way ANOVA (SPSS software) followed by the Tukey test was also used to determine significant changes in the expression of genes following RT-PCR procedures among four experimental groups.

3. Results

3.1. Body weight, plasma total cholesterol, cytokine and hepatic-related enzyme activity

Treatment with probucol did not result in any statistically significant differences in body weight among the four groups of mice. However, plasma total cholesterol concentrations were significantly decreased in the probucol-treated apo E-KO mice and C57BL/6 mice at week 18, as compared to corresponding controls. The extent of reduction in the mean plasma total cholesterol levels was 38% (308.5 ± 55.6 mg/dl vs. 492.2 ± 63.7 mg/dl, $p < 0.001$) in the apo E-KO mice and 74% (16.2 ± 2.5 mg/dl vs. 61.2 ± 9.3 mg/dl, $p < 0.001$) in the C57BL/6 mice.

Data about plasma cytokine concentrations are shown in supplemental data 1. The level of IL (interleukin)-6, IL-12p70, IL-17, IFN (interferon)-γ, MCP (monocyte chemoattractant protein)-1, MCP-5, and TARC (thymus and activation-regulated chemokine) were significantly higher in the probucol-treated apo E-KO mice as compared to those in the control apo E-KO mice. The activities of ALK and GGT were comparable between the probucol-treated and control apo E-KO mice (supplemental data 1).

3.2. Aortic atherosclerosis in apo E-KO mice

Histological examinations revealed advanced atherosclerosis in the probucol-treated apo E-KO mice, the observations are similar to our previous observations [3,8] (supplemental data 2). The atherosclerotic lesion size was $0.65 \pm 0.02 \times 10^6\ \mu m^2$ in the probucol-treated apo E-KO mice, while it was $0.44 \pm 0.07 \times 10^6\ \mu m^2$ in the control group. Similarly, the aortic root inflammation score in probucol-treated apo E-KO mice was higher than that in the control group (score of 3 and 2, respectively). None of C57BL/6 mice developed atherosclerosis.

3.3. Microarray data analysis: general features of gene expression profile in liver of probucol-treated apo E-KO mice

Using an empirical Bayes method and the Benjamini and Hochberg's algorithm as mentioned above, 360 genes and transcripts (accounting for 1.69% in all 21,220 probe sets) were identified that they had significantly differential expression in the liver of