Probucol inhibits oxidized-low density lipoprotein-induced adhesion of monocytes to endothelial cells \textit{in vitro}^1

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\textbf{KEY WORDS} probucol; LDL lipoproteins; adhesions; vascular endothelium; cultured cells; monocytes; P-selectin; E-selectin; intercellular adhesion molecule-1; vascular cell adhesion molecule-1

\textbf{ABSTRACT}

\textbf{AIM:} To investigate the mechanism by which probucol (PBC) affected adhesion of monocytes to human umbilical vein endothelial cells (HUVEC). \textbf{METHODS:} Effects of PBC on expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin, and E-selectin in human umbilical vein endothelial cells were examined. Moreover, the inhibitory effect of PBC were compared with that of monoclonal antibodies (mAbs) to ICAM-1, VCAM-1, P-selectin, and E-selectin on adhesion induced by oxidized-low density lipoprotein (Ox-LDL). \textbf{RESULTS:} PBC at 10 to 80 \textmu{}mol/L inhibited Ox-LDL-induced adhesion index from 16.7 % to 7.0 % \textbf{(P < 0.01)} and Ox-LDL-induced expression of ICAM-1 (75 %) and P-selectin (72 %). mAbs to ICAM-1 or P-selectin, when used alone, could only slightly reduce the adhesion of monocyte to HUVEC. When both monoclonal antibodies were used in combination, the adhesion was markedly inhibited from 16.7 % to 11.3 % \textbf{(P < 0.01)}, but the effect was still weaker than that of PBC (average 9.3 %). \textbf{CONCLUSION:} PBC exerts its inhibitory effect on the adhesion of monocyte to HUVEC by inhibiting the expression of ICAM-1 and P-selectin.

\textbf{INTRODUCTION}

It is an important event in the early stage of atherosclerosis and restenosis that monocytes adhere to the arterial endothelium, and accumulate in subendothelium. As an initial step, adhesion and transmigration of human blood monocytes to vascular endothelium can be induced by oxidized-low density lipoprotein (Ox-LDL) \textsuperscript{13}. It was proposed that Ox-LDL might stimulate the expression of adhesion molecules of endothelial cells (EC) and might cause the endothelial adhesion \textsuperscript{2,3,4}. Recently it was reported that Ox-LDL facilitated leukocyte adhesion to aortic intima\textsuperscript{41}. Studies in human umbilical vein endothelial cells (HUVEC) demonstrated the up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression could be induced by incubation with Ox-LDL or by long-term incubation with native LDL\textsuperscript{5,6,7}. Moreover, two endothelial adhesion proteins for monocytes have recently been described to be induced by minimally modified LDL\textsuperscript{7,8}. Evidence showed that LDL might be oxidized \textit{in vivo}. Macrophages can take up low density lipoprotein (LDL) at increased rate\textsuperscript{9,10}, and oxidized LDL even in the absence of metal ions\textsuperscript{11,12}. 

Probucol (PBC) may play a role in the prevention of atherogenesis\textsuperscript{13,14} and restenosis\textsuperscript{15-20}. It was also shown that PBC inhibited monocyte (MC) adhesion to vascular endothelium in the cholesterol-fed rabbit\textsuperscript{21}. Our previous data demonstrated that PBC protected EC against oxygen free radical damage\textsuperscript{22}, and inhibited Ox-LDL-induced adhesion of monocytes to EC \textit{in vitro}\textsuperscript{23}. However, the exact mechanism is not clear. Recently, some data showed that adhesion molecules were associated with atherosclerosis and early postangioplasty restenosis \textit{in vivo}. For example, expression of ICAM-1 in symptomatic versus asymptomatic human carotid atherosclerotic plaque is different. P, E-selectin-deficient and low-density lipoprotein receptor-deficient (LDLR / / P/E / / ) mice developed atherosclerosis lighter and slower than LDLR / / P/E
mice on atherogenic diet. The expression of VCAM-1 and ICAM-1 on the endothelium was upregulated in homozygous apolipoprotein E-deficient (ApoE−/−) mice; these mice developed complex lesions of atherosclerosis similar to those in humans. Circulating levels of soluble cell adhesion molecules are elevated in older men with uncomplicated essential hypertension, higher levels of E-selectin and ICAM-1 were observed in the patients with coronary heart disease (CHD) compared with the control subjects, and plasma soluble ICAM-1 levels increased persistently in early coronary restenosis after emergency angioplasty in patients with acute myocardial infarction (AMI). We hypothesize that PBC inhibits the adhesion of MC to EC in vitro by reducing the expression of adhesion molecule. The present work aimed at ascertaining the inhibitory effect of PBC on the adhesion of MC to HUVEC, and further to elucidate the mechanism of human peripheral blood MC adhesion to HUVEC.

MATERIALS AND METHODS

Culture of endothelial cells: HUVEC were obtained from human umbilical cord veins by digestion with a-chymotrypsin and cultured in flasks with medium 199 plus 20% fetal calf serum (FCS). Cell purity was assessed by morphology and factor III staining. After 1 week EC grew to confluence in 95% air plus 5% CO2 and were passaged using 0.05% trypsin and 0.02% edetic acid. The 4th and 5th passage of HUVEC (5 x 10^6 L−1) were seeded in 24-well plates for 16 h before experiments. When the passaged cells had grown to confluence, fresh medium was provided before they were then transferred to the control media (M199 + 20% FCS) or to the medium containing PBC 10, 20, 40, and 80 μmol/L + Ox-LDL (100 mg/L) for the designed time periods. PBC was dissolved in ethanol and kept as a stock solution. All the EC (treated or untreated) were prepared for adhesion assay and for the determination of adhesion molecule expression. The viability of cells was always examined morphologically using an inverted microscope and by the Trypan blue dye exclusion test in 4 to 24 h after probucol treatment. Medium 199, trypsin, and FCS were purchased from Gibco.

Preparation of monocytes and oxidation of LDL: Monocytes and native LDL were isolated as described previously. The amounts and concentrations of LDL were expressed in terms of protein. The edetic acid-free LDL was incubated in PBS containing CuSO4, 10 μmol/L for 16 h at 37°C. Oxidation was stopped by addition of edetic acid at a final concentration of 0.24 μmol/L. Then it was dialyzed in PBS containing 0.01% edetic acid (w/v). The extent of LDL oxidation was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS, malondialdehyde).

Adhesion assay: Inhibition of monocytic adhesion to Ox-LDL-stimulated HUVEC: The adhesion of the monocytes to Ox-LDL-stimulated HUVEC was evaluated by protein content as described. The treated HUVEC monolayers were rinsed three times with M199 before addition of monocytes. Monocyte suspension 1 mL (containing 6.5 x 10^6 L−1 in M199 + 2% FCS) was added to each well of the 24-well culture plates. An additional 1 mL of the monocyte suspension was also obtained for protein determination. The culture plates were then incubated for 30 min at 37°C in 5% CO2 plus 95% air. After incubation, the wells were gently rinsed twice with PBS containing Ca2+ and Mg2+ to remove nonadherent cells. HUVEC and adherent monocytes were then dissolved in 1 mL of NaOH 0.2 mol/L for subsequent protein analysis using Bio-Rad assays. The adhesion of monocytes was estimated by comparing the amount of protein in wells containing endothelial cells and monocytes minus the amount of protein in wells containing EC alone, divided by the amount of protein in 1 mL of monocyte cell suspension. The adhesion was also estimated by directly counting of non-adherent monocytes collected from each well and the number of cells in 1 mL of monocyte suspension that was not added onto the endothelium. The adhesion index was calculated by the number of cells in 1 mL monocyte suspension minus the number of nonadherent monocytes collected, divided by the number of cells in 1 mL of monocyte suspension. Some Ox-LDL-stimulated HUVEC were treated with PBC (10, 20, 40, and 80 μmol/L) or saturating amounts (10 mg/L, determined by our assay) of monoclonal antibodies to the ICAM-1, VCAM-1, E-selectin, P-selectin, or combinations for 30 min, respectively, before addition of monocytes. Blocking agents were present during coincubation. Adhesion indexes were determined from eight wells.
performed in triplicate. IgG, mAbs of the ICAM-1, VCAM-1, E-selectin, and P-selectin were purchased from DAKO Co, Denmark.

Expression of adhesion molecule in cultured HUVEC Expression of ICAM-1, VCAM-1, P-selectin, and E-selectin on HUVEC membrane were all assayed as described by Li et al.22(ELISA kits from Gene Co Ltd). Briefly, 0.1 mL of 0.25% collagenase-1 was added to each well of HUVEC plates pretreated with Ox-LDL or Ox-LDL + PBC, and incubated for 15 min at 37°C. Then the digested HUVEC of each well were centrifuged at 300 × g for 10 min and the HUVEC pellet was resuspended with PBS containing 0.05% Tween-20. The suspension was centrifuged at 300 × g for 10 min again. The pellet was re-suspended with PBS (pH 7.4), containing 1% FCS at a cell concentration of 3 × 10^6 L^-1. Standard IgG 0.1 mL (serial diluted concentrations) or HUVEC was added to each well of microtiter plates coated with IgG of ICAM-1, VCAM-1, P-selectin, or E-selectin, followed by addition of 0.1 mL of HRP-GAM to each well and incubated for 30 min. Then 0.05 mL of H_2SO_4 3 mol/L was added to each well. After incubation at room temperature for 10 min the absorbance at 492 nm was measured with a V_max kinetic microplate reader interfaced to softmax software. ICAM-1, VCAM-1, P-selectin, or E-selectin antigen levels in mg/L were obtained from a standard curve (absorbance at 492 nm) constructed using serial dilutions of standard ICAM-1, VCAM-1, P-selectin, or E-selectin, and evaluated by protein content.

Statistics All data were expressed as $\bar{x} \pm s$. The difference between multiple groups was tested by analysis of variance followed by Newman-Keuls test. Comparison between two groups was made by unpaired t-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Ox-LDL on adhesion of MC to HUVEC After incubated for 24 h at 37°C, about (3.7 ± 0.8)% of human peripheral blood MC adhered to HUVEC without treatment of Ox-LDL. Monocyte adhesion to HUVEC was enhanced by preincubation of HUVEC with Ox-LDL for 24 h, in a concentration-dependent manner (Fig 1). Adhesion was enhanced 2.1-fold to 7.8% ± 1.1% by Ox-LDL 10 mg/L ($P < 0.05$) and 4.4-fold to 16.0% ± 2.3% by Ox-LDL 100 mg/L ($P < 0.01$ vs control (0 mg/L)).

Fig 1. Effect of Ox-LDL on adhesion of monocytes to HUVEC. $n = 5$ in control. $n = 8$ in other groups. $\bar{x} \pm s$. $P < 0.01$ vs control (0 mg/L).

Ox-LDL 100 mg/L ($P < 0.01$ vs control untreated by Ox-LDL), but only 3.1-fold to 10.4% ± 1.5% by Ox-LDL 200 mg/L. Thus Ox-LDL 100 mg/L was used as the standard concentration in all subsequent experiments to induce adhesion molecule. The adhesion index of MC-HUVEC varied with the time of exposure of the HUVEC to Ox-LDL (Fig 2). The initial increase in adhesion appeared 6 h after exposure to Ox-LDL (100 mg/L). Ox-LDL enhanced the MC-HUVEC adhesion from 6 h to 24 h and then leveled it off. These results were different from our previous data in quality. The main cause might be that our previous material was pig aortic endothelial cell while in this study we used HUVEC.

Inhibition of monocyte adhesion to Ox-LDL-stimulated HUVEC by PBC Treatment of EC with Ox-LDL (100 mg/L) for 24 h resulted in a 4.4-fold
increase in the percent of adherent MC (P < 0.01 vs untreated EC group, Fig 1). Pretreatment of EC with Ox-LDL + PBC for 24 h inhibited Ox-LDL-induced MC adhesion to HUVEC in a concentration-dependent manner. The effective threshold concentration of PBC was 10 μmol/L. The difference of adhesion index between PBC 10 and 80 μmol/L was significant. The average inhibitory rate of PBC on Ox-LDL-induced adhesion was almost 55% (Fig 3). Thus PBC 40 μmol/L was used as the standard concentration in all subsequent experiments. Some Ox-LDL-stimulated HUVEC were preincubated with MC and different mAbs (10 mg/L) to ICAM-1, VCAM-1, E-selectin, or P-selectin. As a result, monoclonal antibody to ICAM-1 and P-selectin but not VCAM-1 or E-selectin slightly reduced MC adhesion to HUVEC, however, combinations of mAbs to ICAM-1 with mAbs to P-selectin markedly inhibited monocyte adhesion to Ox-LDL-stimulated HUVEC, but the inhibitory effect was weaker than PBC.

**Effect of Ox-LDL and/or PBC on adhesion molecule expression in HUVEC.** Cytokine-inducible molecules VCAM-1 and E-selectin were not expressed on untreated HUVEC and not induced by treatment with Ox-LDL (100 mg/L) for 24 h. In contrast, ICAM-1 and P-selectin were expressed at low levels on untreated HUVEC and up-regulated by stimulation with Ox-LDL for 24 h. The baseline expression of ICAM-1 and P-selectin on untreated HUVEC membrane were (67±8) and (41±6) μg · g⁻¹ protein respectively (Tab 1). After 24 h of exposure to Ox-LDL (100 mg/L), expressions of ICAM-1 and P-selectin were increased significantly ((143±17) and (237±41) μg · g⁻¹ protein respectively, P < 0.05). The expression of ICAM-1 and P-selectin in HUVEC pretreated with PBC (40 μmol/L) was (86±10) μg · g⁻¹ protein, and (96±23) μg · g⁻¹ protein respectively.

**DISCUSSION.**

Under normal circumstances the endothelium is a nonadhesive surface that actively inhibits cell-cell interactions with blood elements such as leukocytes or platelets. The pathogenesis of vascular diseases is characterized by perturbations in cell-cell and cell-matrix interactions that predispose vascular tissue to thrombosis, inflammation, and atherosclerotic lesion formation. The influx of inflammatory cells into the vessel wall is a multistep process involving sequential interactions between adhesion molecules from the selectin family (for example, P-selectin), chemokines (for example,
MCP-1), adhesion molecules from the integrin family (for example, VCAM-1) as well as chemotaxants [for example, platelet activating factor (PAF)], and cytokines (for example, interleukin-1).

Ferns et al.\(^{23}\) showed the inhibitory effect of PBC on the adhesion of MC to EC in cholesterol-fed rabbits. However, the exact mechanism of PBC to inhibit monocyte adhesion to EC and whether this effect is mediated by attenuation of the expression of adhesion molecules is not clear. Our results showed that treatment of HUVEC with Ox-LDL enhanced the adhesion of human peripheral blood monocytes in a concentration-dependent and time-related manner (Fig. 1, 2), increased the expression of ICAM-1 and P-selectin but not VCAM-1 and E-selectin. Adhesion index was decreased by Ox-LDL 200 mg/L relative to Ox-LDL 100 mg/L, which might be the reason that Ox-LDL 200 mg/L showed a little cytotoxicity (not shown). Expression of VCAM-1 in our results was different from that reported by Cominacini et al.\(^{29}\), that the expression of VCAM-1 was increased. The difference needs further study, whether the pretreatment of LDL with antioxidants such as vitamin E and probucol may protect oxidation of LDL in different degree. In addition, our results showed PBC inhibited the Ox-LDL-enhanced adhesion of MC to EC in a concentration-dependent manner in vitro, and the expression of ICAM-1 and P-selectin, partly different from that reported by Kaneko et al.\(^{30}\), which probucol did not affect the expression of ICAM-1, but down-regulated E-selectin expression. These may be caused by different conditions. Kaneko et al. used lipopolysaccharide (LPS) as stimulus. LPS induced expression of different adhesive molecules with that of oxidative LDL. Moreover, our results showed that pretreatment of Ox-LDL-stimulated HUVEC with different mAbs (10 mg/L), mAbs to ICAM-1 or P-selectin slightly reduced monocye binding to treated HUVEC respectively. Both mAbs to ICAM-1 and P-selectin inhibited 17% adhesion of MC to EC, but lower than that of PBC (55%). All these suggested that the enhanced endothelial adhesiveness was associated in part with an up-regulation of ICAM-1 expression but not of VCAM-1 or E-selectin expression, and requires additional ligands, possibly carbohydrate-decorated, heparin-like structures, eg, endothelial proteoglycans\(^{23}\). The inhibitory effect of PBC on MC-EC adhesion was not only associated with inhibitory expression of ICAM-1 and P-selectin, but also with other effects, which needs further study.

The adhesion of monocytes to vessel wall endothelial cells may play an important role in atherogenesis\(^{11}\). Increased data suggest that Ox-LDL may be involved in the pathogenesis of atherosclerosis\(^{1, 14}\). Our results showed that Ox-LDL could induce the expression of several adhesion molecules on endothelial cells, such as ICAM-1 and P-selectin, and could increase the adhesion of monocytes to endothelial cells, which were inhibited by probucol. Moreover, we found that effects of PBC on the adhesion of monocytes to endothelial cells were more than that of both mAbs to ICAM-1 and P-selectin. Taken together, PBC inhibits Ox-LDL-stimulated adhesion of monocytes to HUVEC not only by reducing ICAM-1 and P-selectin expression, but also including other mechanisms, which are still unclear, and need further study.

REFERENCES

7. Calderon TM, Factor SM, Hatcher VB, Berliner JA, Berman JW. An endothelial cell adhesion protein for monocytes recognized by monoclonal antibody 1G7. Expression in vivo in inflamed human vessels and atherosclerotic human and Watanabe rabbit vessels. Lab Invest 1994; 70; 386 - 40}


13 Carew TE, Schwenke DC, Steinberg D. Antithrombogenic effect of probucol unrelated to its hypocholesterolemic effect; evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. Proc Natl Acad Sci USA 1987; 84: 7225–9.

14 Schwartz CJ. The probucol experience; a review of the past and a look at the future. Am J Cardiol 1990; 62: 1B–5B.


丙丁酚抑制体外氧化低密度脂蛋白诱导的单核细胞对内皮细胞的粘附

关键词：丙丁酚；LDL脂蛋白类；粘附；血管内皮；培养的细胞；单核细胞；P选择素；E选择素；细胞间粘附分子-1；血管细胞粘附分子-1

目的：研究丙丁酚抑制体外氧化低密度脂蛋白诱导的单核细胞对内皮细胞的粘附机制。方法：采用酶联免疫吸附检测丙丁酚对内皮细胞粘附分子的影响，细胞间粘附分子-1（ICAM-1），血管细胞粘附分子-1
（VCAM-1）、P. 选择素和 E. 选择素表达的影响。对比分析丙丁酚和上述粘附分子克隆抗体抑制氧化低密度脂蛋白诱导的单核细胞对内皮细胞的粘附作用。 结果：丙丁酚浓度依赖性抑制氧化低密度脂蛋白诱导的单核细胞对内皮细胞的粘附，丙丁酚浓度从 10 μmol/L 增加到 80 μmol/L，单核细胞对内皮细胞的粘附从 16.7% 降低至 7.0% （P < 0.01），同时 ICAM-1 和 P. 选择素表达分别被抑制 75% 和 72% （P < 0.05），而对 VCAM-1 和 E. 选择素表达无明显作用。 独立使用 ICAM-1 和 P. 选择素单克隆抗体只能微弱抑制单核细胞对内皮细胞的粘附，两者合用则可显著抑制，粘附指数为 11.3%（P < 0.05）。 但仍然高于丙丁酚保护的粘附指数 9.3%。 结论：丙丁酚通过多途径抑制氧化低密度脂蛋白诱导的单核细胞对内皮细胞的粘附，其中包括抑制 ICAM-1 和 P. 选择素的表达。