Effects of ethanol on the properties of platelets and endothelial cells in model experiments

Ksenija Stach, Anna-Isabelle Kälsch, Christel Weiß, Elif Elmas, Martin Borggrefe, Thorsten Kälsch

Abstract

AIM: To investigate effects of ethanol on activity markers of atherosclerosis in an in vitro endothelial cell model.

METHODS: After 24 h incubation with ethanol (0.0095%), human umbilical vein endothelial cells were stimulated for 1 h with lipopolysaccharide, and were then incubated in direct contact with activated platelets. Following this incubation, the expression of CD40L and CD62P on platelets, and the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), urokinase plasminogen activator receptor (upAR), and membrane-type 1 matrix metalloproteinase (MT1-MMP) on endothelial cells were measured by flow cytometry.

RESULTS: The increased expression of VCAM-1 and uPAR on endothelial cells by proinflammatory stimulation with activated platelets was significantly reduced through pre-incubation with ethanol (P < 0.05). Furthermore, platelets in direct contact with ethanol and with endothelial cells pre-incubated in ethanol showed a significant reduction in their CD40L expression (P < 0.05). Ethanol had no significant effect on ICAM-1 and MT1-MMP expression on endothelial cells.

CONCLUSION: Ethanol directly attenuates platelet activation and has significant endothelial cell-mediated effects on selected markers of atherosclerosis in vitro. These findings underline possible protective effects of ethanol on atherosclerosis.

© 2012 Baishideng. All rights reserved.

Key words: Platelets; Endothelial cells; Ethanol; Inflammation; Atherosclerosis

Peer reviewers: Ming-Jui Hung, MD, Cardiology Section, Department of Medicine, Chang Gung Memorial Hospital at Keelung, Chang Gung University College of Medicine, 222 Mai-Chin Road, Keelung City 20401, Taiwan, China; Yuri V Bobryshev, PhD, Associate Professor, School of Medical Sciences, Faculty of Medicine University of New South Wales, Kensington NSW 2052, Australia; Alberto Dominguez-Rodriguez, MD, PhD, FESC, Department of Cardiology, University Hospital of Canarias, Ofra s/n La Cuesta, La Laguna, E-38320, Tenerife, Spain

Stach K, Kälsch AI, Weiß C, Elmas E, Borggrefe M, Kälsch T. Effects of ethanol on the properties of platelets and endothelial cells in model experiments. World J Cardiol 2012; 4(6): 201-205 Available from: URL: http://www.wjgnet.com/1949-8462/full/v4/i6/201.htm DOI: http://dx.doi.org/10.4330/wjc.v4.i6.201

INTRODUCTION

Atherosclerosis is a progressive disease characterized by increased expression of proinflammatory markers and
interactions between endothelial cells and platelets\(^n\). Platelet activation leads to an increased expression of inflammatory factors that stimulate endothelial cells and recruit additional platelets\(^1,4\). CD62P and CD40L are expressed on activated platelets and are directly involved in the interaction of platelets with leukocytes and endothelial cells\(^6\). Stimulation of endothelial cells and interaction with platelets induces the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which initiate the recruitment of neutrophils, monocytes and lymphocytes\(^6\). In turn, these result in the expression and release of matrix-degrading proteinases, the matrix metalloproteinases (MMPs)\(^8\). In a complex cascade, the urokinase plasminogen activator receptor (uPAR) binds and activates urokinase-type plasminogen activator, and directly degrades various proteins of the extracellular matrix and promotes vascular inflammation.

Augmented and chronic alcohol consumption is accountable for a variety of organ damage including cardiomyopathy\(^9\), pancreatitis\(^10\) and chronic alcoholic cirrhosis of the liver\(^11\). In contrast, moderate alcohol consumption may reduce the risk of coronary artery disease and total mortality\(^12,13\). Therefore, a strict limitation of alcohol consumption (< 30 g/d for men and < 15 g/d for women) is recommended and reasonable. Cardioprotective effects of alcohol may be mediated by antioxidative properties, an increase in high-density lipoprotein (HDL) cholesterol, as well as anti-thrombotic and vasodilatory effects. However, the underlying causative mechanisms remain unclear.

The aim of this study was to investigate the effects of ethanol on the expression of established markers of atherosclerosis on endothelial cells and platelets in an \textit{in vitro} model of atherosclerosis.

**MATERIALS AND METHODS**

**Co-incubation of platelets with ethanol pre-incubated endothelial cells**

Human umbilical vein endothelial cells (HUVECs) were prepared as previously described\(^3,4\) and cultured in endothelial cell basal medium (PromoCell) containing 2% fetal calf serum [1 μg/mL hydrocortisone (HC-500); 0.4% endothelial cell growth supplement (ECGS/H-2); 0.1 ng/mL epidermal growth factor (hEGF-0.05); 1 ng/mL basic fibroblast growth factor (hFGF-0.5); 5 ng/mL amphotericin and 50 μg/mL gentamicin]. The confluent endothelial cells were added to 12-well plates and incubated for 24 h with ethanol 0.0095% (Merck, Hohenbrunn, Germany). Platelets were prepared from blood of healthy probands as previously described\(^6,7\). Washed platelets were stimulated for 30 min with thrombin (0.5 U/mL) and lipopolysaccharide (LPS) (1000 ng/mL). Before co-incubation experiments, thrombin activity was antagonized by hirudin (5 U/mL). Pretreated platelets (final concentration 2 × 10^9/mL) were added to confluent endothelial monolayers with and without ethanol.

After 60-min co-incubation under cell culture conditions, all platelets were removed by gentle washing, which was confirmed by light microscopy. After an additional 6 h of incubation of the endothelial cells, supernatant was aspirated, centrifuged at 2000 g and stored at -80 °C\(^16\). Following this incubation, the expression of activity markers on platelets, as well as that on endothelial cells were measured by flow cytometry.

**Flow cytometric analysis**

Flow cytometric analysis of platelets and endothelial cells was performed by gating in forward and side scatter. Platelets were gated back for determination of the expression of CD40L and CD62P. For the analysis of platelets, 100 μL of each sample were stained for 15 min at room temperature with 10 μL aliquots of mouse anti-human CD40L-FITC antibodies (BD Pharmingen, Heidelberg, Germany) and mouse anti-human CD62P-PE antibodies (Beckman-Coulter, Krefeld, Germany). Endothelial cells were gated back for determination of the surface expression of ICAM-1, VCAM-1, uPAR and membrane type 1 matrix MMP (MT1-MMP). For the analysis of endothelial cells, 100 μL of each sample were stained for 15 min at room temperature with 10 μL aliquots of anti-human CD54 PE-Cy5 (ICAM-1 from BD Pharmingen, Heidelberg, Germany), anti-human CD106-FITC (VCAM-1 from R&D Systems, Inc., Wiesbaden, Germany), anti-human CD87-FITC (uPAR from American diagnostica inc, Stanford, United Kingdom), anti-human MT1-MMP (Ab-1) Mouse mAb (114-IF2) (Merck Chemicals Ltd., Nottingham, United Kingdom). Corresponding isotypes (Beckman Coulter, Marseille, France) were used as a control. All flow cytometric analysis was performed on an EPICS XL-MCL analyzer (Beckman Coulter, Krefeld, Germany) equipped with an argon laser tuned at 488 nm. Mean fluorescence intensity was measured and all FACS data is expressed as MFI in this manuscript. Statistical analysis was performed using SAS release 9.2 (SAS Institute Inc. Cary NC, United States). Numerical data were expressed as mean ± SD. A Student \(t\) test was applied as a parametric test. A two-tailed probability value < 0.05 was considered significant.

**RESULTS**

**Effects of pre-incubation with ethanol on endothelial cell surface markers**

HUVEC pre-incubation with ethanol resulted in decreased surface expression of VCAM-1 and uPAR under proinflammatory stimulation with LPS and by direct endothelial contact with activated platelets compared to HUVEC without ethanol. Pre-incubation with ethanol...
CD40L expression compared to platelets incubated with endothelial cells showed a significant reduction in their direct contact with ethanol-pre-incubated platelets (Figure 1B). Ethanol had no significant effects on untreated endothelial cells. Surface expression of CD40L on thrombin-stimulated platelets after 1 h in direct contact with HUVECs pre-incubated with ethanol significantly decreased from 2.15 ± 0.19 to 1.98 ± 0.16 (P = 0.004) and, when stimulated with thrombin and LPS, significantly decreased from 2.20 ± 0.17 to 1.98 ± 0.14 (P = 0.03) (Figure 2A). Ethanol had no significant effects on CD62P on platelets after endothelial cell contact (Figure 2B). To discriminate between effects mediated by pre-treated endothelial cells and possible direct effects of ethanol on platelets, platelets were directly incubated with ethanol alone for 1 h. Here, ethanol incubation significantly decreased CD40L expression on thrombin-stimulated platelets from 2.31 ± 0.17 to 1.92 ± 0.26 (P = 0.01) (Figure 3A). Surface expression of CD62P on thrombin-stimulated platelets after 1 h incubation with ethanol was significantly reduced from 3.56 ± 2.17 to 1.83 ± 0.76 (P = 0.03) (Figure 3B).

DISCUSSION

Several prospective clinical studies suggest an inverse correlation between moderate alcohol consumption and coronary artery disease. In a prospective study, Rimm et al. [19] studied alcohol intake and prevalence of coronary artery disease in 51,591 males and reported that increasing alcohol intake was inversely correlated with coronary artery disease incidence (P < 0.001). Previous experimental...
Stach K et al. Ethanol: Friend or foe in atherosclerosis?

Figure 3 Direct effects of ethanol on surface expression of CD40L (A) and CD62P (B) on platelets. PLT: Platelets; Thr: Thrombin; LPS: Lipopolysaccharide; NS: Not significant.

studies investigated possible cardioprotective mechanisms of alcohol. Linn et al\[20\] and Goldberg et al\[21\] described in a representative sample of the United States adult population, that moderate alcohol consumption (one or two drinks per day) increased circulating levels of HDL by 12% on average. Gil-Bernabe et al\[22\] compared low- and high-dose treatment with ethanol in a mouse model and showed less dilatation of the aorta and less atherosclerotic plaques in mice treated with low-dose ethanol. Authors discussed the possible mechanism as an increased secretion of stromal cell-derived factor-1 (SDF-1) with subsequent enhanced mobilization of progenitor cells. Although the antiinflammatory signalling mechanisms of ethanol in detail still are unknown, they may be result from an increase in SDF-1 concentration in peripheral blood or an enhancement of the activity of endothelial nitric oxide synthase.

The present study provides further mechanistic insights and evidence for atheroprotective effects of ethanol by demonstrating significantly reduced expression of VCAM-1 and uPAR on endothelial cells after pre-incubation with ethanol and stimulation with activated platelets. VCAM-1 and uPAR are well established atherosclerotic markers and play an important role in the development of cardiovascular diseases. Furthermore this study investigated direct and endothelial cell-mediated effects of ethanol on platelets, and provides evidence that ethanol has significant attenuating effects on CD40L and CD62P expression on platelets. Since the aim of this study was to investigate effects of moderate alcohol consumption in an in vitro endothelial cell model, the experimental condition of alcohol applied in our study (alcohol concentration of 0.0095%) was equivalent to a human blood alcohol concentration of 0.1%. This dose of ethanol therefore is consistent with moderate alcohol consumption in real life. The present study was not designed to assess different ethanol concentrations in vitro. The possibility of different effects of different concentrations of ethanol on endothelial and platelet functions therefore cannot be excluded.

In conclusion, the findings of the present study underline possible protective effects of ethanol on atherosclerosis. Ethanol significantly decreased the expression proatherogenic markers on endothelial cells and has direct inhibitory effects on platelets. Further studies are warranted to confirm our results and to expand scientific knowledge about atheroprotective effects of ethanol on endothelial cells and platelets.

**COMMENTS**

**Background**

Moderate alcohol consumption may reduce the risk of coronary artery disease and total mortality. This study investigated the effects of ethanol on the expression of established markers of atherosclerosis on endothelial cells and platelets in an in vitro model of atherosclerosis.

**Research frontiers**

Ethanol, at a moderate concentration equivalent to a human blood alcohol concentration of 0.1%, significantly reduced the expression of vascular cell adhesion molecule-1 and urokinase plasminogen activator receptor, on endothelial cells after pre-incubation with ethanol and stimulation with activated platelets

**Innovations and breakthroughs**

Ethanol can significantly decrease the expression proatherogenic markers on endothelial cells and has direct inhibitory effects on platelets.

**Applications**

The study underlined possible protective effects of ethanol on atherosclerosis.

**Peer review**

This is a brief article regarding the possible mechanisms of ethanol on endothelial cells and platelets. This manuscript provides some insights in understanding the effect of ethanol on vessels.

**REFERENCES**

1. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002; 105: 1135-1143
2. Lusis AJ. Atherosclerosis. Nature 2000; 407: 233-241
3. Kalsch T, Nguyen XD, Elmas E, Grebert N, Süselbeck T, Klüter H, Boggere M, Dempfle CE. Coagulation activation and expression of CD40 ligand on platelets upon in vitro lipopolysaccharide-challenge in patients with unstable angina. Int J Cardiol 2006; 111: 217-223
4. Henn V, Slupszyk JR, Gräfe M, Anagnostopoulos I, Förster R, Müller-Berghaus G, Kroczek RA. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature 1998; 391: 591-594
5. Gawaz M, Brand K, Dickfeld T, Fogatsa-Murray G, Page S, Bogner C, Koch W, Schmitig A, Neumann F. Platelets induce alterations of chemotactic and adhesive properties of endothelial cells mediated through an interleukin-1-dependent mechanism. Implications for atherogenesis. Atherosclerosis 2000; 148: 75-85
6. Galkina E, Ley K. Vascular adhesion molecules in atherosclerosis. Arterioscler Thromb Vasc Biol 2007; 27: 2292-2301
Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. J Biol Chem 2001; 276: 7614-7620

Pepper MS. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arterioscler Thromb Vasc Biol 2001; 21: 1104-1117

Iacovoni A, De Maria R, Gavazzi A. Alcoholic cardiomyopathy. J Cardiovasc Med (Hagerstown) 2010; 11: 884-892

Sand J, Lankisch PG, Nordback I. Alcohol consumption in patients with acute or chronic pancreatitis. Pancreatology 2007; 7: 147-156

Corrao G, Torchio P, Zambon A, D’Amicis A, Lepore AR, di Orio F. Alcohol consumption and micronutrient intake as risk factors for liver cirrhosis: a case-control study. The Provincial Group for the study of Chronic Liver Disease. Ann Epidemiol 1998; 8: 154-159

Thun MJ, Petro R, Lopez AD, Monaco JH, Henley SJ, Heath CW, Doll R. Alcohol consumption and mortality among middle-aged and elderly U.S. adults. N Engl J Med 1997; 337: 1705-1714

Di Castelnuovo A, Rotondo S, Iacoviello L, Donati MB, De Gaetano G. Meta-analysis of wine and beer consumption in relation to vascular risk. Circulation 2002; 105: 2836-2844

Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973; 52: 2745-2756

Gimbrone MA. Culture of vascular endothelium. Prog Hemost Thromb 1976; 3: 1-28

Gawaz M, Neumann FJ, Dickfeld T, Koch W, Laugwitz KL, Adelsberger H, Langenbrink K, Page S, Neumeier D, Schömig A, Brand K. Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. Circulation 1998; 98: 1164-1171

Dickfeld T, Lengyel E, May AE, Massberg S, Brand K, Page S, Thielen C, Langenbrink K, Gawaz M. Transient interaction of activated platelets with endothelial cells induces expression of monocyte-chemotactant protein-1 via a p38 mitogen-activated protein kinase mediated pathway. Implications for atherogenesis. Cardiovasc Res 2001; 49: 189-199

May AE, Kälsch T, Massberg S, Herouy Y, Schmidt R, Gawaz M. Engagement of glycoprotein IIb/IIIa (alpha(IIb)beta3) on platelets upregulates CD40L and triggers CD40L-dependent matrix degradation by endothelial cells. Circulation 2002; 106: 2111-2117

Rimm EB, Giovannucci EL, Willett WC, Colditz GA, Ascherio A, Rosner B, Stampfer MJ. Prospective study of alcohol consumption and risk of coronary disease in men. Lancet 1991; 338: 464-468

Linn S, Carroll M, Johnson C, Fulwood R, Kalsbeek W, Briefel R. High-density lipoprotein cholesterol and alcohol consumption in US white and black adults: data from NHANES II. Am J Public Health 1995; 85: 811-816

Goldberg JJ, Mosca L, Piano MR, Fisher EA. AHA Science Advisory: Wine and your heart: a science advisory for healthcare professionals from the Nutrition Committee, Council on Epidemiology and Prevention, and Council on Cardiovascular Nursing of the American Heart Association. Circulation 2001; 103: 472-475

Gil-Bernabe P, Boveda-Ruiz D, D’Alessandro-Gabazza C, Toda M, Miyake Y, Mifuji-Moroka R, Iwasa M, Morser J, Gabazza EC, Takeda Y. Atherosclerosis amelioration by moderate alcohol consumption is associated with increased circulating levels of stromal cell-derived factor-1. Circ J 2011; 75: 2269-2279

S-Editor Cheng JX  L-Editor Cant MR  E-Editor Zheng XM

Stach K et al. Ethanol: Friend or foe in atherosclerosis?