JPP
Journal of
Pharmacy and Pharmacology

JPP 2009, 61: 1681–1688

Received August 6, 2009
Accepted September 16, 2009
DOI 10.1211/jpp/61.12.0014
ISSN 0022-3573

Research Paper

Poloxamer 407 increases soluble adhesion molecules, ICAM-1, VCAM-1 and E-selectin, in C57BL/6 mice

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Abstract

Objectives Soluble shedded forms of cell adhesion molecules (sCAMs) found in plasma are regarded as surrogate markers for the cellular expression of CAMs. The presence of oxidised low-density lipoprotein (ox-LDL) cholesterol and fatty acids in the plasma, hypertriglyceridaemia and reduced plasma concentrations of high-density lipoprotein cholesterol (HDL-C) are all thought to stimulate an increase in the cellular expression of CAMs such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin. Our objectives were to determine how plasma levels of the soluble CAMs were modulated in a mouse model of dyslipidaemia induced chemically with poloxamer 407, and how these changes might be related to changes in the plasma concentrations of total cholesterol, HDL-C, non-HDL-C and triglycerides.

Methods C57BL/6 mice were given a single intraperitoneal dose of poloxamer 407 (0.5 g/kg) and plasma concentrations of lipid fractions and sCAMs were measured at predetermined time points thereafter.

Key findings The plasma concentrations of each sCAM were significantly increased in our mouse model of atherogenic dyslipidaemia compared with control mice administered saline, although the temporal relationship between the plasma sCAM concentration–time profiles and the plasma lipid concentration–time profiles were not coincident.

Conclusions The atherogenic profile in our mouse model was associated with increases in the plasma concentrations of sICAM-1, sVCAM-1 and sE-selectin. These changes precede the formation of atherosclerotic lesions shown in previous work. This suggests the use of these sCAMs as biomarkers of future atheroma formation in this particular animal model.

Keywords atherogenic dyslipidaemia; biomarker; coronary artery disease; Poloxamer 407; soluble cell adhesion molecules

Introduction

Epidemiological studies have clearly established a strong inverse correlation between the concentration of plasma high-density lipoprotein cholesterol (HDL-C) and the incidence of ischaemic heart disease.[1] Accordingly, the HDL-C level was added to the US National Cholesterol Education Program algorithm for the prevention of this disease process,[2] and increasing plasma HDL-C levels, using a variety of innovative approaches, is now considered a major therapeutic target.[3,4]

The role that defects in triglyceride metabolism play in the development of atherosclerosis remains controversial, in contrast to the widely accepted relationship between disturbances in metabolism of low-density lipoprotein (LDL) and the premature development of atherosclerosis. Disturbances in triglyceride metabolism may promote atherogenesis by increasing the expression of vascular cell adhesion molecules (CAMs). Patients with markedly elevated triglyceride levels also have decreased HDL-C levels and abnormalities in fatty acid metabolism. In-vitro studies have demonstrated that low levels of HDL-C and oxidised fatty acids increase the endothelial expression of CAMs in response to cytokines.[5–8]

Adhesion of leucocytes to the vascular endothelium and subsequent transmigration into the intima are key events in atherogenesis.[9] Vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin are CAMs that are expressed on the endothelial cell membrane and mediate the adhesion and transmigration of leucocytes to

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vascular endothelium.[10] The expression of these CAMs is enhanced by a variety of pro-atherogenic stimuli, including inflammatory cytokines and reactive oxygen species.[10] Increased expression of CAMs has been observed in animal models of human atherosclerosis[11,12] and in human atherosclerotic tissues.[13,14] Soluble shedded forms of CAMs (sCAMs) are found in plasma, and their concentration may be regarded as a surrogate marker of cellular expression.[15] The amount of sCAMs released by cultured endothelial cells has been correlated with their cell surface expression,[16] and raised plasma sCAM levels have been found in a variety of pathological conditions in which cell-surface expression is also increased.[15] The concentration of sCAMs has been reported to be elevated in patients with ischaemic heart disease,[17] atherosclerosis,[18,19] hyperlipidaemia[20,21] and diabetes,[22] and high plasma sCAM levels were associated with an increased risk of future coronary events.[19,23–25]

There still remains a paucity of information regarding the relationship between circulating oxidised LDL (ox-LDL) and CAM expression; specifically, whether circulating ox-LDL stimulates the expression of endothelial cell-membrane-bound CAMs, which are then subsequently shed into the plasma as sCAMs. Several in-vitro studies using human endothelial cells have demonstrated that ox-LDL may induce expression of ICAM-1,[26–30] VCAM-1[28,30] and E-selectin.[26,28] However, conflicting results have also been reported, showing no association between ox-LDL and expression of ICAM-1,[31] VCAM-1[26,29,31] and E-selectin.[30,31] Hence, the relationship between ox-LDL and the expression of CAMs on endothelial cells *in vitro* is unclear. This relationship is even more ambiguous when extrapolated to the in-vivo situation, in which the measurement of sCAMs has been proposed as a potentially useful marker to indicate increased expression of cell-membrane-bound CAMs in response to circulating ox-LDL.

The hypothesis of whether endothelial cells exposed to an atherogenic plasma lipid profile, which includes circulating ox-LDL, results in increased expression of CAMs (as determined by measuring their soluble forms) can be conveniently tested using a mouse model of dyslipidaemia and atherosclerosis developed in our laboratory,[32,33] in which the atherogenic agent poloxamer 407 (P-407) is used to induce dyslipidaemia. P-407 is a nonionic surfactant composed of repeating poly(oxyethylene) and poly(oxypropylene) units. P-407 effectively raises plasma triglyceride levels, as well as significantly lowering plasma HDL-C concentrations, in a dose-dependent fashion.[34] Plasma triglycerides are increased by P-407's ability to inhibit the activity of capillary-bound lipoprotein lipase,[34] whereas the reduction in plasma HDL-C occurs as a result of shifting the cholesterol lipoprotein distribution from primarily HDL-C (normal for a rodent) to LDL and very-low-density lipoprotein (VLDL).[35,36] The formation of aortic atherosclerotic lesions begins approximately 90 days after the repeated administration of P-407 to C57BL/6 mice of either sex.[32] The number and size of the atherosclerotic lesions may be further increased relative to diet-induced models by inclusion of 0.5% cholic acid in the diet,[32] which decreases the activity of cholesterol 7α-hydroxylase and thereby decreases the elimination of cholesterol into bile.[37]

Recently, we have demonstrated that plasma from mice treated with P-407 contains antibodies to malondialdehyde-modified LDL; that is, ox-LDL.[33] Moreover, P-407-treated mice also exhibit increased plasma levels of free fatty acids.[38] In view of the above discussion linking the presence of ox-LDL, hypertriglyceridaemia, low plasma HDL-C levels and oxidized fatty acids to the increased expression of CAMs, we have an ideal animal model with which to evaluate the relationship between these factors and the plasma concentration of the shedded soluble forms of CAMs (sICAM-1, sVCAM-1 and sE-selectin). The purpose of this study was therefore to determine whether the plasma concentrations of sICAM-1, sVCAM-1 and sE-selectin were perturbed in the P-407-induced mouse model of dyslipidaemia and atherosclerosis,[32,36] and to determine any relationship(s) that may exist between a potential rise in plasma sCAM concentrations and the plasma concentrations of total cholesterol, HDL-C, non-HDL-C and triglycerides.

Materials and Methods

Materials

Forty-eight male C57BL/6 mice (~20 g) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). ELISA kits to measure soluble concentrations of VCAM-1, ICAM-1 and E-selectin in mice were purchased from R&D Systems (Minneapolis, MN, USA). Reagents for the determination of total cholesterol, HDL-C and triglycerides in plasma were purchased from Wako Diagnostics, Inc. (Richmond, VA, USA).

In-vivo experiments

These experiments were conducted to determine the temporal relationship between any potential perturbations in the levels of sCAMs and plasma concentrations of total cholesterol, HDL-C, non-HDL-C (calculated) and triglycerides following administration of P-407 to mice. We used two groups of 24 mice. One group were treated with a single intraperitoneal dose of P-407 (0.5 g/kg, 0.5 ml) dissolved in sterile normal saline. The other group were given a single intraperitoneal injection of 0.5 ml sterile normal saline. Blood samples (500 μl) were obtained under isoflurane anaesthesia from each mouse by peri-orbital sampling at predetermined time points post-dosing (three mice per time point). Mice were fasted overnight prior to the experiment, and then for 48 h after the administration of either P-407 or saline. The mice were allowed drinking water *ad libitum* throughout the duration of the experiment and resumed eating after the 48 h blood sample was obtained. Thus, the only time points for which there could potentially have been any postprandial contribution to plasma lipids were the 96- and 120-hour sampling time points.

All blood samples were collected into heparinised tubes, the plasma obtained, and the samples stored at −80°C until the time of lipid and sCAM analysis. All procedures for P-407 administration and subsequent blood collection were in accordance with the institution's guide for the care and use of laboratory animals; the treatment protocol was approved

by the Institutional Animal Care and Use Committee (IACUC) at the University of Missouri-Kansas City.

Determination of plasma lipid and sCAM concentrations

Plasma samples were analysed for total cholesterol and triglycerides using standard enzymatic colorimetric assay kits. Triglycerides were assayed by the method of Bucolo and David,[39] and total cholesterol by the technique described by Allain *et al*.[40] The concentration of HDL-C was determined by first precipitating the VLDL and LDL fractions with a phosphotungstic acid/magnesium solution[41] and then analysing the supernatant obtained after centrifugation at 10 000*g* for total cholesterol. Non-HDL-C was calculated as total cholesterol minus HDL-C.

The plasma concentrations of sVCAM-1, sICAM-1 and sE-selectin were determined using monoclonal antibody-based ELISAs according to the manufacturers' protocols. Assays were performed in duplicate for each sample. Intra-assay and inter-assay coefficients of variation for all measured sCAMs were less than 4.1% and less than 6.7%, respectively.

To determine whether P-407 interfered with the sCAM assays, we included increasing concentrations of P-407 with a known concentration of each sCAM standard prepared in mouse plasma. Using sICAM-1 as an example, we added a known volume of the mouse sICAM-1 standard contained in the ELISA kit to blank mouse plasma to obtain a sICAM-1 concentration of 10 ng/ml. To six separate tubes prepared in this fashion, P-407 was then added to obtain final P-407 concentrations of 0.1, 1.0, 10, 100, 500 and 1000 μmol/l. These six samples were assayed in duplicate. Similar P-407 assay interference experiments were also conducted for sVCAM-1 and sE-selectin. These concentrations of P-407 represent the range of plasma concentrations previously observed when animals were injected with 0.5 g/kg P-407.[42] The plasma concentration–time profile of P-407 (the hyperlipidaemic agent) and the plasma concentration–time profiles for the lipids (the pharmacodynamic response elicited by P-407 treatment) are not superimposable in this animal model of dyslipidaemia and atherosclerosis: the maximum plasma concentration of P-407 occurs at 12 h[42] whereas maximum plasma concentrations of plasma lipids occur 24 h after injection of P-407.[32,36]

Statistical analysis

The mean plasma concentration of total cholesterol, HDL-C, non-HDL-C (calculated), triglycerides, sVCAM-1, sICAM-1 and sE-selectin at each time point for P-407-treated mice were individually compared with the corresponding mean values in the control mice using the Student's *t*-test.[43] A P value less than 0.05 was deemed significant.

Results

Plasma lipid concentrations

As shown in Figure 1a, plasma total cholesterol increased from a baseline of approximately 75 mg/dl to 701 mg/dl by 24 h after administration of P-407. Beginning at 6 h, plasma total cholesterol concentrations were significantly higher in P-407-treated mice than saline controls ($P < 0.05$).

The plasma concentration–time profiles for non-HDL-C (calculated), HDL-C and triglycerides are shown in Figures 1b, 1c and 1d, respectively. As with the total cholesterol concentrations, plasma non-HDL-C and triglyceride levels were significantly increased in P-407-treated mice compared with controls ($P < 0.05$), beginning at 6 h, and lasting as long as 48 h post-dosing (Figures 1b and 1d). In each case, the plasma lipid concentration reached a maximum at 24 h after P-407 administration (Figures 1b and 1d). However, in contrast to total cholesterol, non-HDL-C and triglycerides, there was a significant decrease in the plasma concentration of HDL-C beginning 6 h after P-407 administration ($P < 0.05$), with the maximum reduction occurring 24 h after P-407 administration (Figure 1c). Finally, each plasma lipid returned to baseline concentrations by 96 h after P-407 administration (Figures 1a–1d).

Using average values from Figures 1B and 1C, the ratio of plasma HDL-C to non-HDL-C concentrations in P-407-treated mice ranged from 3.4 at time 0 h (prior to injection) to 0.03 at 24 h. This represents more than a 100-fold difference in the plasma concentrations of HDL-C and non-HDL-C following P-407 administration. P-407 treatment therefore causes most of the cholesterol to reside in the LDL and VLDL lipoprotein fractions, rather than in the HDL fraction.[36]

Circulating soluble cell adhesion molecules

The plasma concentrations of all three sCAMs were significantly increased relative to corresponding concentrations in saline-treated mice ($P < 0.05$). Only sVCAM-1 showed a slight delay (6 h) in attaining a significantly increased plasma concentration and took 12 h after P-407 dosing to show a significant rise in concentration relative to controls ($P < 0.05$; Figure 2a). In contrast, plasma levels of sICAM-1 and sE-selectin both showed significant increases compared with controls as little as 6 h after the administration of P-407 ($P < 0.05$; Figures 2b and 2c). All the sCAM plasma concentration–time profiles showed a maximum concentration at 48 h after a single injection of P-407. All profiles returned to baseline by 120 h.

Discussion

The present study demonstrated that treatment of C57BL/6 mice with P-407 resulted in an atherogenic plasma lipid profile, with a phenotypic expression of reduced HDL-C but elevated concentrations of total cholesterol, non-HDL-C and triglycerides. We have previously shown that P-407-treated mice display a 31% decrease in circulating apolipoprotein A-1,[44] an increase in oxidized lipids in the plasma, especially ox-LDL (since we detected autoantibodies to ox-LDL[33]), and more than a 50% increase in non-esterified (free) fatty acids for 24 h following a single dose of P-407,[38] all of which exacerbate the atherogenic plasma lipid profile. In the present study we have shown that the atherogenic plasma lipid phenotype manifested in P-407-treated mice is accompanied by an increase in the plasma concentrations of sVCAM-1, sICAM-1 and sE-selectin.

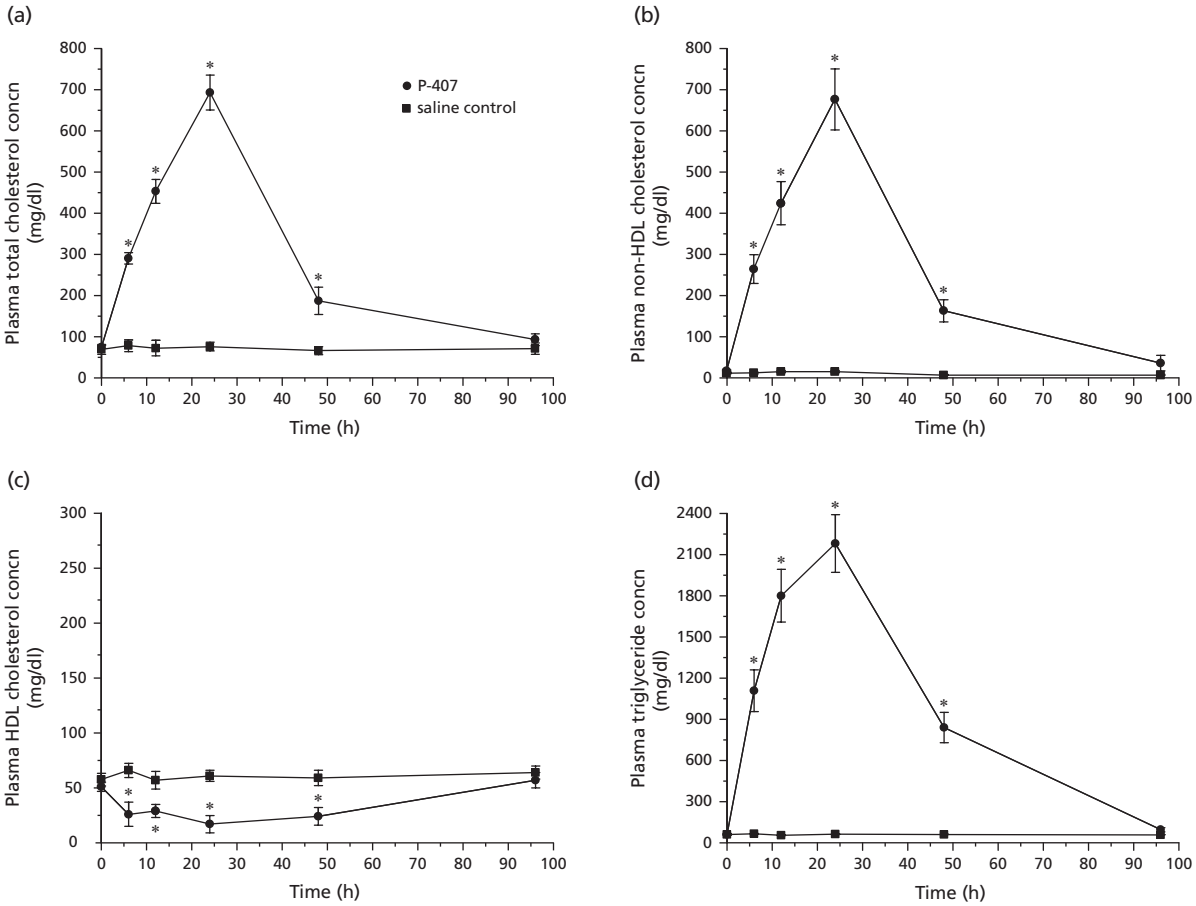

Figure 1 Effect of P-407 on plasma concentrations of lipids. Plasma concentrations of (a) total cholesterol, (b) non-high-density-lipoprotein (HDL)-cholesterol, (c) HDL-cholesterol and (d) triglycerides are shown following a single intraperitoneal injection of P-407 (0.5 g/kg) or saline to C57BL/6 mice. Graphs show means ± SD (n = 3 per time point). $^{*}P < 0.05$ vs controls.

The present investigation is significant because of the continuing questions surrounding the premise of whether the soluble forms of CAMs can function as surrogate markers for predicting future atherosclerotic coronary artery disease. The present work demonstrates that the sCAMs we evaluated do increase in the context of an atherogenic plasma lipid profile. Whilst we did not treat mice with P-407 for an extended period of time in the present study, we have reported previously that mice treated chronically with P-407 begin to exhibit fibrofatty lesions in their aortas approximately 90 days after starting P-407 treatment.[32,36,45,46] This suggests that the elevation in the plasma concentrations of the sCAMs we evaluated (sVCAM-1, sICAM-1 and sE-selectin) following a single dose of P-407 may be related to the subsequent formation of atherosclerotic lesions in our model. Thus, we suggest that the elevation in plasma concentration of sCAMs is a prelude to the formation of atherosclerotic lesions and may therefore serve as a biomarker for the genesis of future aortic atherosclerotic lesions in our model.

With regard to sCAMs and atheroclerosis, it has been suggested that sICAM-1 is more predictive of future coronary artery disease,[23,47] whereas sVCAM-1 is more predictive of existing atherosclerosis.[16,48,49] However, in this acute study (a single injection of P-407), all three sCAMs were elevated in the presence of an atherogenic plasma lipid profile but in the absence of atheroma formation. Several groups have provided evidence to support the fact that levels of the sCAMs we evaluated were elevated as a result of the atherogenic plasma lipid profile produced in P-407-treated mice. Lupattelli *et al.*[50] reported that low HDL-C resulted in increased levels of sICAM-1 and sVCAM-1, whereas elevated ox-LDL resulted in increased plasma concentrations of sVCAM-1 in patients with hyperlipidaemia. Recently, Verna *et al.*[51] demonstrated that sustained atherogenic LDL blood levels, produced by injecting native LDL into LDL receptor-deficient (LDLR(−/−)) mice, induced increased expression of ICAM-1 and VCAM-1 in aortic endothelial cells after only 24 h. Bro *et al.*[52] claim it is well established that inhibition of the expression of adhesion molecules retards the development of classic atherosclerosis in animal models, probably by slowing down the recruitment of inflammatory cells from the blood.[53–55] Thus, many investigators are now beginning to show that sCAMs may be convenient biomarkers that predict the likelihood of future coronary artery disease. Whether the results of the present study, together with work from other animal models that has

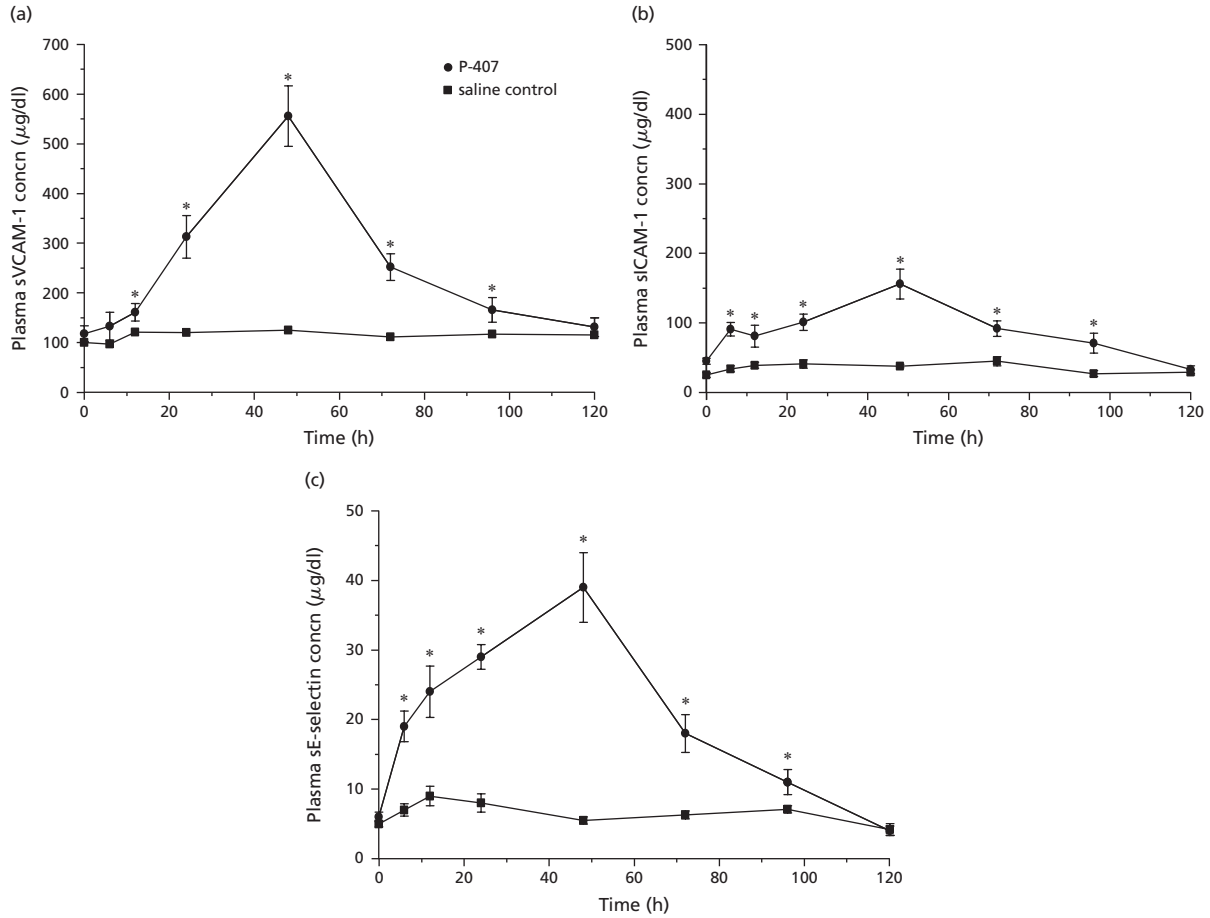

Figure 2 Effect of P-407 on plasma concentrations of soluble cell adhesion molecules. Plasma concentrations of (a) soluble vascular cell adhesion molecule-1 (sVCAM-1), (b) soluble intercellular adhesion molecule-1 (sICAM-1) and (c) sE-selectin are shown following a single intraperitoneal injection of P-407 (0.5 g/kg) or saline to C57BL/6 mice. Graphs show means ± SD (n = 3 per time point). $^{*}P < 0.05$ vs controls.

evaluated the expression of soluble adhesion molecules as possible biomarkers for the prognosis of atherosclerosis, can be extrapolated to the human condition remains to be determined.

An interesting finding in the present investigation is the temporal relationship between the rise in plasma lipids and the rise in plasma sCAMs following P-407 treatment. Figures 1a, 1b, 1d, 2a, 2b and 2c show that plasma total cholesterol, non-HDL-C and triglycerides reach a maximum plasma concentration at 24 h after P-407 administration, whereas the plasma concentration of each sCAM reaches a maximum at 48 h. The plasma concentration–time profiles for the lipids therefore cannot be superimposed on the plasma concentration–time profiles for the sCAMs. Verna *et al.*[51] demonstrated that maximal adhesion molecule response in LDLR(−/−) mice was established at 24 h after injection of LDL. At this time point, expression of ICAM-1 and VCAM-1 in aortic endothelial cells was strongly increased and this was related to elevated blood cholesterol levels.[51] In all of the aortas in which it was evaluated, ICAM-1 expression was much more pervasive, whereas VCAM-1 expression appeared more focal and was somewhat less elevated. These expression patterns of ICAM-1 and VCAM-1 were similar to those observed by Iiyama *et al.*[56] in the aortas of LDLR(−/−) mice fed a high-fat diet. Verna *et al.*[51] measured many blood parameters (total cholesterol, HDL, LDL, VLDL and triglycerides) and found that expression of adhesion molecules was most closely related to the value for total blood cholesterol. The drastic increase in plasma total cholesterol seen in the present study may explain why we observed a greater increase in the plasma concentration of sVCAM-1 than Verna *et al.*[51] and Iiyama *et al.*[56]

The study by Verna *et al.*[51] showed that expression of adhesion molecules does not appear immediately after LDL injection but reached a maximum 24 h after treatment with LDL. This delay was also observed in endothelial cell culture, and indicates a similarity between the in-vivo and in-vitro activation pathways. These authors proposed two explanations for their findings: a sufficient amount of LDL-C must enter and accumulate in the cells before activation of the adhesion-molecule pathways can begin, and a certain amount of signalling intermediate, such as c-Jun, must be formed before sufficient adhesion-molecule protein can be produced. The authors suggested that both of these possibilities could be true. In the present investigation, there was a 24-h time lag between the maximum elevation

in plasma lipids (24 h) and maximum plasma sCAM concentrations (48 h).

An important aspect of the present investigation is the rapid decrease in the concentration of sCAMs in response to decreasing plasma concentrations of total cholesterol, non-HDL-C and triglycerides and increasing plasma concentrations of HDL-C. It has been demonstrated in humans that when LDL levels are precipitously reduced, rapid correction of vascular change occurs,[57,58] indicating that certain LDL- or cholesterol-induced effects are likely to be reversible. The work by Verna *et al*. appears to corroborate the reversibility effect because there was almost no ICAM-1 or VCAM-1 after 48 h, when cholesterol levels were approaching baseline in the LDLR−/− mice treated with LDL. The reversibility of adhesion-molecule expression corroborates recent evidence that atherosclerosis can be reversed as cholesterol is removed from the circulation.[59–63] Verna *et al*. also showed that even a 24-h exposure to LDL may be sufficient to begin the atherogenic process, indicating that any exposure of the aortic endothelial cells to LDL may be problematic. In the present work, non-HDL-C (intermediate-density lipoprotein, LDL and VLDL) reached a maximum at 24 h after treatment with P-407. While we did not measure circulating levels of ox-LDL, if the time course for ox-LDL even somewhat resembled that for non-HDL-C, the aortic endothelial cells would have been exposed to maximum concentrations of LDL, and the more injurious ox-LDL, for 24 h before initiation of the atherogenic process (i.e. the increased expression of CAMs).

One explanation for the increase in plasma sCAMs following the administration of P-407 might be inflammation caused by the polymeric surfactant (P-407), although this seems unlikely as we have previously shown that incubation of P-407 with cultured human umbilical-vein endothelial cells did not influence cell proliferation or production of interleukin-6 or -8.[64] We therefore concluded that the formation of atherosclerotic lesions in this mouse model of atherosclerosis does not result from either direct stimulation of endothelial cells or macrophage activation by P-407.[64]

Conclusions

We have shown that sCAMs are detected in the plasma of P-407-treated mice and reach a maximum approximately 24 h after the atherogenic lipids (total cholesterol, non-HDL-C and triglycerides) reach a maximum and anti-atherogenic HDL-C reaches a minimum. The decline in plasma sCAMs from 48 h onwards parallels the decline in atherogenic lipids from 24 h onwards, demonstrating that slow removal of atherogenic lipids from the circulation is followed by a corresponding slow decline in the sCAMs. Finally, there was a positive relationship between the elevation in total cholesterol, non-HDL-C and triglycerides and the increase in plasma concentration of all sCAMs, but an inverse relationship between the elevation in plasma sCAMs and the reduction in plasma HDL-C. The elevation in the plasma concentrations of sCAMs precedes the formation of atherosclerotic lesions in this mouse model of dyslipidaemia, and suggests the use of these sCAMs as a biomarker of future atheroma formation in this particular animal model. Future work will examine whether long-term administration of P-407 sustains the elevation of sCAMs until the time at which aortic atherosclerotic lesions are first observed.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

The School of Pharmacy, University of Missouri-Kansas City, provided financial support for this work.

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