ELSEVIER

Atherosclerosis 198 (2008) 264–271

ATHEROSCLEROSIS

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LDL induces parathyroid hormone-related protein expression in vascular smooth muscle cells: Modulation by simvastatin

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Received 11 December 2006; received in revised form 7 February 2008; accepted 7 February 2008
Available online 10 March 2008

Abstract

Background: Parathyroid hormone-related protein (PTHrP) is overexpressed in atherosclerotic plaques by unknown mechanisms. We have examined here the putative mechanism(s) responsible for this overexpression in the atherosclerotic lesion and its potential modulation by simvastatin, both *in vitro* and *in vivo*.

Methods and results: Atherosclerosis was induced in rabbits by femoral endothelial dessication and atherogenic diet. After 2 weeks, animals were randomized to receive either 5 mg/(kg d) simvastatin ($n=7$) or no treatment ($n=6$) during 4 additional weeks. An increase in PTHrP immunostaining was observed in atherosclerotic lesions of hyperlipidemic rabbits, which was significantly reduced by simvastatin. However, PTH/PTHrP type 1 receptor staining was similar in both groups. In cultured vascular smooth muscle cells (VSMCs), atherogenic concentrations of native LDL (0.125–0.5 mg/mL) increased PTHrP expression. This effect was prevented by preincubation with simvastatin (1 μmol/L) and was reversed by mevalonate, geranylgeranylpyrophosphate and, to a lesser extent, by farnesylpyrophosphate. Moreover, in transfection studies, we showed that RhoA appears to participate in the mechanism whereby LDL induces PTHrP in VSMC. Finally, native LDL-induced VSMC growth and this mitogenic effect was blocked by PTHrP silencing.

Conclusions: LDL might be responsible for PTHrP overexpression in atherosclerotic plaques of hyperlipidemic rabbits. The inhibition of this effect by simvastatin provides further insights into the mechanisms of action of statins.

Keywords: Atherosclerosis; Parathyroid hormone-related protein; LDL; Statins

1. Introduction

Atherosclerosis and its complications represent the major cause of death in industralized countries [1]. Atherogenesis is characterized by increased retention of lipoproteins and the accumulation of inflammatory cells in the artery wall. Recruitment of leukocytes to lesion-prone sites is mediated by cell adhesion molecules which are upregulated on endothelial cells in response to high concentrations of low-density lipoproteins (LDL) and other atherogenic stimuli. Infiltrating monocytes/macrophages can subsequently uptake modified LDL-cholesterol in the plaque, leading to a chronic inflammatory response [2].

Parathyroid hormone-related protein (PTHrP) and the common PTH/PTHrP type 1 receptor (PTH1R) are expressed in a wide variety of normal cells and tissues, including vascular smooth muscle cells (VSMCs) [3]. The PTHrP gene has features of early response genes and transcript turnover is rapid, which are common characteristics shared by many proinflammatory cytokines and growth factors [4]. PTHrP is a potent vasodilator and different vasoactive agents, such as angiotensin II (Ang II), endothelin-1 and thrombin stimulate

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doi:10.1016/j.atherosclerosis.2008.02.017

PTHrP expression in rat aortic VSMCs [5]. Moreover, PTHrP is increased during neointimal formation in rat carotid arteries and human restenotic lesions [6], and its expression has been correlated to the severity of coronary atherosclerosis [7]. We previously reported that PTHrP expression was increased in the inflammatory region of human carotid atherosclerotic plaques, potentially contributing to monocyte/macrophage recruitment by inducing monocyte chemoattractant protein-1 (MCP-1) expression [8].

Previous clinical trials have established that lipid lowering with 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) reduces the incidence of acute coronary events [9,10]. This has been generally attributed to a decrease in the plasma levels of atherogenic lipoproteins, mainly LDL. However, some of the beneficial effects of these drugs may involve nonlipid mechanisms [11].

In this study, we have explored the mechanism(s) responsible for PTHrP overexpression in the atherosclerotic lesion and its potential modulation by simvastatin, both *in vitro* and *in vivo*.

2. Methods

2.1. Studies in a rabbit model of atherosclerosis

2.1.1. Induction of atherosclerosis

Thirteen New Zealand male rabbits weighing 3.5–4 kg were housed in individual cages, quarantined for 7 days before use, and treated according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. An atherogenic diet (2% cholesterol and 6% peanut oil, Letica) was started on day 0. One week later, vascular injury was induced in the femoral arteries using desiccated N_2, according to a previously described technique [12]. One week after surgery, the animals were randomized to receive (5 mg/(kg day)) simvastatin (SV, $n=7$) or no treatment (NT, $n=6$) while kept on atherogenic diet during 4 weeks. The dose of simvastatin was chosen on the basis of previous experiments designed to rule out toxicity and any effect of the drug on chow consumption. It is important to note that rabbits need higher doses of statins than patients to obtain a similar reduction in blood lipids. Animal weight was controlled weekly to adjust the dose of drug.

2.1.2. Harvesting of damaged vessels

At the time of sacrifice, rabbits were anesthetized and femoral arteries were sequentially exposed and cannulated, fixed *in situ* with 4% buffered formaldehyde, at 100 mmHg pressure, and removed. The arteries were kept for 24 h in 4% buffered formaldehyde, and then in 70% ethanol until paraffin embedding.

2.1.3. Blood lipid measurement

Rabbits were bled from a marginal ear vein after 24-h fasting on day 0 and at the end of weeks 1, 2 (randomization), and 6 (sacrifice). Total cholesterol, LDL, high-density lipoproteins (HDL), very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), and triglycerides were measured by enzymatic assays (Sigma Diagnostics).

2.1.4. Immunohistochemistry

Paraffin-embedded femoral arteries were cross-sectioned into 4-μm thick pieces at 5-mm intervals, dewaxed and rehydrated. Primary antibodies: a monoclonal anti-rabbit macrophage antibody (RAM11, Dako), monoclonal antiα-actin (HHF-35, Sigma), rabbit polyclonal anti-PTHrP antiserum C6 recognizing a C-terminal epitope in the intact PTHrP molecule, and an affinity-purified anti-PTH1R antibody (Ab-VII, Covance, Berkeley, CA) were used as previously described [8,12]. For PTHrP immunodetection, tissue samples were first incubated with 0.02 U/mL neuraminidase (Sigma) for 1 h. Then, they were incubated with 6% swine serum/4% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h to block nonspecific staining. Antibodies were diluted in 1% swine serum/4% BSA in PBS. As secondary antibodies, a biotinylated swine anti-rabbit IgG (Amersham) (for PTHrP, PTH1R) or a biotinylated goat anti-mouse IgG (Dako) (for monocytes/macrophages and VSMCs) were used. Tissue sections were subsequently incubated with the avidin–biotin-peroxidase complex (Dako) and 3,3′-diaminobenzidine (Dako) as chromogen. Then, sections were counterstained with hematoxylin and mounted in Pertex (Medite). For colocalization studies, immunohistochemistry for macrophages, VSMCs and PTHrP was carried out on serial tissue sections. In each experiment, negative controls either without the primary antibody or using the corresponding IgG were included to check for nonspecific staining.

2.1.5. Image analysis

The morphometric analysis was performed on hematoxylin-stained arterial sections with an Olympus microscope (BH-2) linked to a microcomputer using Olympus software by a pathologist who was blind to the groups the sections belongs to, as previously described [12]. Sections with maximal lesion were chosen for quantification. For morphometric analysis, the intimal area was measured. For immunohistochemisty, intimal areas with positive staining for PTHrP, PTH1R and macrophages from arterial cross-sections were segmented and quantified by area unit (mm^2). In this way, the results were expressed as percentage of immunostained area.

2.2. In vitro studies

2.2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, trypsin–EDTA and fetal bovine serum (FBS) were supplied by BioWhittaker. Native LDL (nLDL, density 1.019–1.063 g/mL) was isolated from plasma of normocholesterolemic subjects by differential ultracentrifu-