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Cathepsin G activity lowers plasma LDL and reduces atherosclerosis

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ABSTRACT

Cathepsin G (CatG), a serine protease present in mast cells and neutrophils, can produce angiotensin-II (Ang-II) and degrade elastin. Here we demonstrate increased CatG expression in smooth muscle cells (SMCs), endothelial cells (ECs), macrophages, and T cells from human atherosclerotic lesions. In low-density lipoprotein (LDL) receptor-deficient (*Ldlr*$^{-/-}$) mice, the absence of CatG reduces arterial wall elastin degradation and attenuates early atherosclerosis when mice consume a Western diet for 3 months. When mice consume this diet for 6 months, however, CatG deficiency exacerbates atherosclerosis in aortic arch without affecting lesion inflammatory cell content or extracellular matrix accumulation, but raises plasma total cholesterol and LDL levels without affecting high-density lipoprotein (HDL) or triglyceride levels. Patients with atherosclerosis also have significantly reduced plasma CatG levels that correlate inversely with total cholesterol ($r = -0.535$, $P < 0.0001$) and LDL cholesterol ($r = -0.559$, $P < 0.0001$), but not with HDL cholesterol ($P = 0.901$) or triglycerides ($P = 0.186$). Such inverse correlations with total cholesterol ($r = -0.504$, $P < 0.0001$) and LDL cholesterol ($r = -0.502$, $P < 0.0001$) remain significant after adjusting for lipid lowering treatments among this patient population. Human CatG degrades purified human LDL, but not HDL. This study suggests that CatG promotes early atherogenesis through its elastinolytic activity, but suppresses late progression of atherosclerosis by degrading LDL without affecting HDL or triglycerides.

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1. Introduction

Cathepsin G (CatG), a serine protease, localizes in mast cells and neutrophils [1–3]. The best known function of CatG in a cardiovascular context is its ability to activate the renin–angiotensin system: CatG produces angiotensin II (Ang-II) from angiotensin I (Ang-I) and angiotensinogen [2,4,5]. Considerable data implicate Ang-II in the pathogenesis of atherosclerosis [3,6]. Ang-II affects vascular structure and function. In atherosclerotic lesions, Ang-II induces smooth muscle cell (SMC) growth and migration, activates macrophages, increases platelet aggregation, and causes endothelial dysfunction. It also promotes apoptosis, increases oxidative stress, promotes leukocyte adhesion and migration, stimulates thrombosis, and has proinflammatory effects [7,8]. In addition to producing Ang-II, CatG also activates matrix metalloproteinase (MMP)-1, -2, -3, and -9 zymogens [9–12] or enhances their expression [13]. MMPs participate in atherogenesis [14,15]. CatG is also an elastase [16,17] and collagenase activator [18]. We have recently found that CatG degrades type I collagen [17], the dominant collagen type in human aortic wall. Therefore, CatG may contribute to atherogenesis directly via its elastinolytic and collagenolytic activities or indirectly via its capability of activating collagenases or MMPs, and producing Ang-II.

While the normal human aortas contain negligible CatG, its expression in human atherosclerotic lesions increases by 2-fold as determined by immunoblot analysis, and CatG immunolocalizes to leukocytes and mast cells in the tunica media and adventitia [19]. Increased CatG in aortas may degrade VE-cadherin and fibronectin, thereby enhancing the expression and activation of MMPs [13] and the interaction of blood-borne leukocytes with the luminal endothelium [20]. A recent study using *Apoe*$^{-/-}$ mice demonstrated that CatG insufficiency (*Apoe*$^{-/-}$*Ctsg*$^{+/-}$) significantly reduced lesion collagen and SMC content, and apoptotic cell number [21], supporting a role of CatG in experimental atherosclerosis.

This study used CatG and low-density lipoprotein receptor (LDLr) double-deficient mice (*Ldlr*$^{-/-}$*Ctsg*$^{-/-}$) and examined blood from patients with atherosclerosis to probe further potential roles of CatG in atherosclerosis. The findings implicate CatG in elastin degradation in early atherogenesis, and in LDL catabolism at a later stage. Patients with atherosclerosis have a significant inverse association between plasma LDL and CatG.

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2. Materials and methods

2.1. Human atherosclerotic lesion immunohistology and immunoblot analysis

Human atherosclerotic plaques were obtained at endarterectomy (n = 12), and non-atherosclerotic carotid arteries from heart transplant donors (n = 10), according to protocols pre-approved by the Human Investigative Review Committee of Brigham and Women's Hospital. Human atherosclerosis serial cryostat sections (6 μm) were stained for CatG (1:200, Calbiochem, San Diego, CA), CD68 (macrophages, 1:700, Dako, Carpinteria, CA). For localization of CatG to cell types rabbit anti-CatG (1:50) antibody mixed with mouse anti-CD68 (1:50, Dako), or anti-CD31 (endothelial cells [ECs], 1:30, Dako), or -α-actin (SMCs, 1:30, Enzo Diagnostics Inc., Farmingdale, NY), or -CD4 (T cells, 1:20, BD Biosciences, San Jose, CA) antibodies. Consequently, sections incubated with mixture of anti-rabbit Alexa 533 (red, 1:500) and anti-mouse Alexa 488 (green, 1:300, both from Invitrogen, Grand Island, NY). Nuclei stained with Dapi (NucBlue® Fixed Cell ReadyProbes® Reagent, Molecular Probes, Eugene, OR) and slides were coverslip with fluorescent mounting medium (Dako). Human carotid atherosclerotic lesions (n = 7) and non-atherosclerotic carotid arteries (n = 6) were also lysed in a protein lysis buffer containing 10 mM Tris.HCl, (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton, 0.1% Sodium deoxycholate, 0.1% SDS, and 140 mM NaCl. Equal protein from each sample was separated on a 12% SDS-PAGE for immunoblot analysis with rabbit anti-human CatG polyclonal antibody (1:1000, Calbiochem). Mouse anti-actin monoclonal antibody (1:2000, Santa Cruz Biotechnology, Inc., Dallas, TX) was used to ensure equal protein loading.

2.2. Mouse experimental atherosclerosis

We crossbred $Ctsg^{-/-}$ mice (C57BL/6/129/SvJ) [22] with atherosclerosis-prone $Ldlr^{-/-}$ mice (C57BL/6, N11, The Jackson Laboratory, Bar Harbor, ME) to generate $Ldlr^{+/-}Ctsg^{+/-}$ breeding pairs to produce $Ldlr^{-/-}Ctsg^{-/-}$ mice and their littermate $Ldlr^{-/-}Ctsg^{+/+}$ control mice. All mice used in this study were male. To induce atherosclerosis, 6-week-old mice from each group consumed a Western diet (C12108, Research Diets Inc.) for 3 months or 6 months. At each time point, blood pressures were measured and plasma collected. Lesion characterizations for mouse atherosclerosis, including thoracic and abdominal aorta oil-red O staining, aortic arch lesion intima and media areas, lesion macrophages (Mac-3, 1:900, BD Biosciences), T cells (CD4, 1:90, BD Biosciences), MHC class II–positive cells (MHC class-II, 1:250, BD Biosciences), SMCs (α-actin, 1:750, Sigma), collagen (picrosirius red birefringence), elastin (Verhoeff–van Gieson), arch lipid deposition (0.5% oil-red O), and apoptosis (TUNEL, EMD Millipore, Billerica, MA) were performed as described previously [23]. The 3-mm long aortic arch from the brachiocephalic artery to the ascending artery perpendicular line toward the descending artery was used for atherosclerotic lesion analysis, as previously described [24]. We captured images digitally; and measured the stained area using computer-assisted image quantification (Image-Pro Plus software, Media Cybernetics), and immunopositive cells were counted manually. All mouse experiments were performed, and data were analyzed in a blinded fashion, by at least three observers. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health, and were approved by the Harvard Medical School Standing Committee on Animals (protocol # 03759).

2.3. Mouse plasma ELISA

Blood samples were collected from $Ldlr^{-/-}Ctsg^{-/-}$ mice and their littermate $Ldlr^{-/-}Ctsg^{+/+}$ control mice at harvest by retro-orbital venous plexus puncture. Plasma total cholesterol, triglyceride, and HDL were determined using ELISA kits according to the manufacturer's instructions (Pointe Scientific, Inc., Canton, MI). LDL cholesterol was calculated as follows: serum LDL cholesterol concentration (mg/dl) = total cholesterol − HDL cholesterol − (triglycerides/5). Mouse plasma Ang-II and angiotensin-converting enzyme (ACE) levels were determined using the Ang-II (USCN Life Science Inc., Houston, TX) and ACE (R&D Systems, Minneapolis, MN) ELISA kits, respectively, according to the manufacturers' instructions. Both albumin and alanine aminotransferase (ALT) levels were determined from plasma samples from mice that consumed a Western diet for 3 or 6 months to assess whether CatG deficiency affected mouse liver functions (Mouse Metabolic Phenotyping Center, Yale University School of Medicine, New Haven, CT).

2.4. Blood pressure measurement

Mouse blood pressures were measured at different time points while consuming a Western diet. To measure blood pressures from live mice, we used the CODA standard non-invasive blood pressure system — the tail–cuff method, according to the manufacturer's instructions (Kent Scientific Corporation, Torrington, CT). Briefly, mice were trained for 3–5 times before the experiments to confirm that they became accustomed to the tail–cuff procedure. A single investigator recorded blood pressures in a quiet environment without disturbance. At least 30 measurements were obtained from each mouse to determine the mean values of systolic and diastolic blood pressures and heart rate.

2.5. Human patient population and plasma CatG ELISA

A total of 232 patients from Wuhan Union Hospital, Wuhan, China, were enrolled in the study due to symptoms of chest pain or electrocardiogram abnormalities including ST-T abnormalities, and were scheduled to undergo coronary angiography. The local Hospital Review Committee approved the human study protocol, and all patients gave informed consent. 171 of the 232 subjects were diagnosed with coronary heart disease (CHD) with one or more major coronary arteries having ≥50% stenosis; 61 subjects had <50% or no luminal narrowing of the coronary artery, and were selected as non-CHD controls. Among the 171 patients with CHD, 59 were diagnosed with acute myocardial infarction (AMI) due to increased levels of creatinine kinase-MB (twofold higher than the upper reference limit) or troponin-I (fivefold higher than the upper reference limit), ischemic symptoms, or ST-T abnormalities by electrocardiography indicative of ischemia and/or infarction; 67 were diagnosed with unstable angina pectoris (UAP) due to the progression of ischemic symptoms less than 3 months before admission to the hospital; and 45 were diagnosed with stable angina pectoris (SAP) based on predictable exertional chest discomfort more than 3 months before enrollment. Patients who were treated with anti-inflammatory drugs; who had connective tissue disease, thromboembolism, disseminated intravascular coagulation, advanced liver disease, renal failure, malignant disease, or other inflammatory diseases (such as septicemia or pneumonia); who had other heart diseases such as rheumatic heart disease, valvular heart disease, congenital heart disease; who had atrial fibrillation or had a pacemaker, were excluded. Among all (232) selected patients, 6/61 (9.8%) non-CHD, 22/45 (49%) SAP, 37/67 (55%) UAP, and 37/59 (63%) AMI patients received lipid-lowering treatment with statins. Human plasma CatG levels were determined using commercial ELISA kits according to manufacturer's recommendation (Alpco, Salem, NH).

2.6. Isolation and modification of LDL and HDL_3

Human LDL (d = 1.019–1.050 g/ml) and human HDL_3 (d = 1.125–1.210 g/mL) were isolated from plasma of healthy volunteers (from the Finnish Red Cross Blood Service, Helsinki, Finland) by sequential ultracentrifugation in the presence of 3 mM EDTA [25,26]. Lipoprotein amounts are expressed in terms of their protein concentrations.

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