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Urokinase receptor surface expression regulates monocyte migration and is associated with accelerated atherosclerosis

Wei Chen [a], Wei-Quan Jin [a], Lian-Feng Chen [a], Taiya Williams [b], Wen-ling Zhu [a], Quan Fang [a,*]

[a] Department of Cardiology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China, 100730
[b] Section of Cardiovascular Sciences, Baylor College of Medicine, Houston, TX, 77030, United States

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ABSTRACT

Background: The urokinase receptor (uPAR) is a key regulator of pericellular proteolysis, cell adhesion and migration, all of which are fundamental processes in atherogenesis. We hypothesized that increased monocytic uPAR expression in circulation is associated with the formation and development of atherosclerosis.
Methods: A total of 42 male apoE−/− mice were ramdonly divided into high-fat (HF) diet and normal diet (n = 21 per group). The percentage of uPAR expressing monocytes (PUEM) and the expression of uPAR within different types of atherosclerotic plaques were measured at an interval of 3 weeks from week 10 to week 16. In vitro, uPAR expression upon ox-LDL stimulation and the migration of monocytes were examined.
Results: PUEM in circulation was significantly higher in animals with HF diet compared with those having normal diet ($p<0.03$). The augmented levels of PUEM were associated with body weight, visceral fat weight and numbers of uPAR + macrophages within atherosclerotic lesions. Accumulation of uPAR + macrophages increased with the progression of atherosclerosis. Monocytes upon ox-LDL stimulation exhibited an increased uPAR expression and uPAR antibody markedly suppressed monocyte migration induced by monocyte chemotactic protein-1. uPAR modulated monocyte migration was accelerated by uPA and was suppressed by amino terminal fragment of uPA dependent.
Conclusions: Over-expression of uPAR both in circulating monocytes and in atherosclerotic lesions is associated with the development of atherosclerotic plaques. uPAR and its interaction with uPA may contribute to enhanced monocyte migration. Thus, uPAR may be a novel target for prevention of uncontrolled monocyte recruitment in inflammatory atherogenic process.

1. Introduction

The infiltration and congregation of monocytes/macrophages within the intima of arterial wall is one of the key characters of atherogenesis [1]. The motility of monocytes/macrophages as well as smooth muscle cells (SMCs) needs not only the breaking-down of cell-cell, cell-extracellular matrix connections but also the new establishment of connections among infiltrating cells and the infiltrated neointima [2,3]. The local proteolytic processes, modulating cell migration, tissue remodeling and plaque destabilization, are thought to be regulated mostly by urokinase-type plasminogen activator (uPA), its membrane receptor (uPAR, also named as CD87) and matrix metalloproteinases (MMPs) [4,5]. uPAR, a 55–60 kD membrane protein, is expressed mainly on invasive cells, such as monocytes/macrophages, SMCs and metastatic tumor cells [6]. The high affinity binding of uPA to uPAR localizes the pericellular proteolysis to the leading edge of migrating cells [7]. uPAR itself can be endocytosed rapidly and recycled to the cell surfaces [2,8]. The redistribution of uPAR on specific regions of cell membranes guarantees the accuracy of proteolysis and is strongly associated with cell motility [4]. In addition to uPA, uPAR has other ligands such as integrins [4,9] and high molecular weight kininogen (HK) [10,11]. The interactions of uPAR with both uPA and other ligands suggest that uPAR may critically involve in a broad spectrum of pathophysiological processes including cardiovascular diseases and vascular remodeling.

Experimental evidence from immunohistolocalization analyses of human coronary arteries has demonstrated that uPAR is highly expressed in atherosclerotic plaques [12,13]. Consistent co-localization of uPAR and macrophages has been observed in most of these investigations. Moreover, in symptomatic carotid atherosclerosis, macrophage dependent uPAR over-expression is associated with ruptured plaque segments [14]. High levels of uPAR were also expressed on circulating monocytes in patients with acute myocardial infarction [15]. Isolated monocytes from these patients showed enhanced adhesiveness to endothelial cells compared with those in patients with chronic stable angina [15].

Although an enhanced uPAR expression has been described in atherosclerotic lesions and on circulating monocytes, the effects of monocytic uPAR expression on atherogenesis and the underlying mechanisms are

* Corresponding author. Tel.: +86 10 65295062; fax: +86 10 65295068.
E-mail address: quanfang2002@yahoo.com.cn (Q. Fang).

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still to be determined. We hypothesize that increased monocytic uPAR expression in circulation is associated with the formation and development of atherosclerosis. In this study, we prospectively studied the surface expression of uPAR on circulating monocytes in a murine model of accelerated atherosclerosis, and analyzed in vitro the possible role of uPAR in modulating monocyte migration.

2. Materials and methods

2.1. Animals

A total of 42 male apoE−/− mice were obtained from the Peking University Health Science Center. Mice were housed in specific pathogen-free rooms and fed a normal laboratory diet prior to the commencement of studies in the animal lab of Peking Union Medical College Hospital. All studies were approved by the Animal Care and Use Committee of Chinese Academy of Medical Sciences. All mice received diets ad lib and had free access to water. Mice were weighed at baseline and randomly divided into controls with normal diet and experimental groups (n = 21 per group) with diets containing 21% lard and 0.15% cholesterol (wt/wt) (Institute of Laboratory Animal Sciences, CAMS&PUMC) since 8-week age [16]. Mice were weighed and sacrificed at an interval of 3 weeks from week 10 to week 16 (n = 7 per group). Blood, visceral fat (including caul and perirenal fat) and aortic arteries were collected at each time point. After sacrifice, the chests were opened, PBS were perfused into blood vessels through left ventricle using a syringe, then 10% paraformaldehyde was applied to perfuse for 5 min. Aortic arteries were dissected from the roots to segments before renal arteries. All samples were fixed with 10% paraformaldehyde for 3 days, then stored in 70% ethanol till further essay.

2.2. Analysis of uPAR on circulating monocytes in mice

Blood specimens for uPAR analysis were collected in standard tubes containing citrate (Vacutainer, BD Biosciences, Vienna, Austria). 90 μl whole blood was taken immediately after blood sample collection and stained with rat anti-mouse CD87 monoclonal antibody (R&D, MAB531), kept at room temperature for 30 min and washed twice with PBS. Then cells were incubated with goat anti rat FITC-IgG at 4 °C in darkness for 30 min. After lysis of erythrocytes, cells were fixed with paraformaldehyde (1%) and analyzed by flow cytometry (FACSCalibur, BD, Mountain View, CA) immediately. Isotype control and blank control was prepared for each sample. Monocytes were defined by the featured FSC and SSC data of blood cells. PUEM = (CD87 positive cell count-isotype control cell count)/monocyte cell count × 100%. The intra- and interassay precision of the FACScalibur flow cytometer were <1.0%.

2.3. Quantitative histology and immunohistochemistry

Classification of atherosclerosis was performed as described previously [13]. Briefly, macroscopically normal areas (MNAs) were defined as segments without atherosclerosis. Early lesions (ELs) were segments with yellowish mild intimal thickening. Fatty streaks were characterized by accumulating foam cells under endothelium with moderate proteoglycan and synthesized smooth muscle cells. Fibrous plaques (FPs) were classified as an accumulation of lipid-laden cells underneath the intima of the arteries with a fibrous "cap" covering the atheromatous "core" of the plaque. Calcified plaques (CPs) were defined if calcifications were macroscopically detectable.

Serial slicing was from the root of aorta (tip of aortic valve) to the beginning of renal arteries, including brachiocephalic artery and part of carotid artery. The thickness of sclicing was 4 μm, and 4 to 6 slices were obtained at an interval of 100 μm. Every mouse had 80 slices on average. Adjacent sections were immunostained using rat anti mouse antibodies against uPAR (R&D systems, MAB531) and CD68 (Santa Cruz Biotechnology, Santa Cruz, CA). Fluorescence studies were performed by using uPAR antibody (R&D systems, MAB531), secondary FITC-conjugated goat anti rat IgG (ZhongShan GoldenBridge Biotechnology, ZF-0315) and embedded in Aqua-Poly-Mount mounting media (Polysciences, Inc.). Images were captured by a digital camera (DM3000, DM5000, Leica Microsystems), imported into IPwin32 imaging software (Media Cybernectics, Inc.), and analyzed on at least 4–6 areas per slice and 4 different slices.

2.4. Monocyte isolation and stimulation

To characterize the expression of uPAR in monocytes and its effect on cell migration, mononuclear cells (MNCs) were isolated from peripheral blood by Ficoll gradient centrifugation (20 minutes at 4 °C at 800 g). MNCs were cultured in 96-well plates at an initial density of 1×10^4 cells/well or in 60 × 15 mm dishes at a density of 1×10^5 cells/dish at 37 °C in humidified 5% CO_2 for 24 h, then unattached cells were washed off by changing medium. The medium was DMEM (Gibcobrl) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FBS) (Invitrogen) , 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, Grand Island, NY) and glutamine (Sigma). Monocytes in plates were incubated with 25 μg/mL or 50 μg/mL of ox-LDL (Lab of Biochemistry and Molecular Biology, Institute of Basic Medical Sciences, CAMS) for 6 to 48 h with or without atorvastatin (5.0 μmol/L, Honghui Medicine CO., LTD). Monocytes in dishes were detached by 0.4% EDTA for 5 to 10 min and stained by CD14-FITC and CD87-PE (both 20ul/10^6 cells, BD) for 30 min. Following two times of PBS wash and trituration through 40 μm nylon mesh, uPAR + monocytes were isolated (FACS Vantage SE Diva) and collected for migration experiments.

2.5. ELISA

At the end of the experiment, cells were extracted with 0.1 M phosphate-buffered saline (pH7.4) containing 1% Triton X-100 and centrifuged at 14000 *g* for 20 min. Supernatants were collected and protein concentrations were measured. AssayMax urokinase receptor ELISA kit (Assaypro) was used to quantitate the uPAR expression levels following monocyte stimulation.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells by using an RNeasy mini-kit (Qiagen, Germany). PCR was performed using the Qiagen Onestep RT-PCR kit following the manufacturer's guidelines. Annealing temperature for uPAR and β-actin (internal standard) was 58 °C. Real-time PCR analysis was done with a SYBR Green PCR reagents kit and an ABI Prism 7700 sequence detection system (Applied Biosystems, FosterCity, CA). The following sets of primers were used in the study: uPAR (forward) CGGGCTCCAATGGTTTCC, (reverse) TTAGCAGGGTGATGGTGAGG; β-actin (forward) ATGGATGATGATATCGCCGCGC, (reverse) CTAGAAGCATTTGCGGTGGACG. Each sample was measured in triplicate.

2.7. Migration assay

1×10^4 monocytes and isolated uPAR + monocytes were seeded in the upper chamber of 8 micron Transwells (Costar Inc). 600 μl of DMEM medium supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, glutamine and 25 ng/mL monocyte chemotactic protein-1 (MCP-1) was placed in the lower chambers. First, cells in the upper chamber were incubate for 12 h with 10 ng/mL to 160 ng/mL anti-uPAR antibody (R&D systems, MAB531), 1 μg/mL uPA (US Biological), 10 ng/mL PAI-1 (Chemicon International), 4 μg/mL amino-terminal fragment of uPA (ATF, Chemicon International) or medium alone as control, respectively. Then cells were allowed to migrate for 72 hours at 37 °C. At the end of the migration assays, the filter side of the upper chamber was cleansed with a cotton swab and the filter was stained for 5 min with 1:2000 DAPI (Roche) in 2% ethanol and then rinsed in water. The filter was cut from the chamber and images were captured by an immunofluorescent microscope (Nikon ECLIPSE 80i). Five to eight fields from each filter were used for quantitative analysis and an average was calculated and expressed as cells/well. For each migration condition, three identical replicates were performed.

3. Statistical analyses

Data were analyzed by Student's *t*-test or ANOVA with repeated measures, as appropriate. Nonparametric data was analyzed by Mann-Whitney Rank sum test. Correlation analysis between variables was carried out by Pearson's (r) or Spearman's correlation coefficient (r_s), as appropriate. All data analyses were performed using SPSS 14.0 (SPSS, Chicago, IL). $p<0.05$ values were considered to be statistically significant.

4. Results

4.1. Body weights and visceral fat weights

Body weight started to differ following 3 weeks of feeding with HF compared to normal diet (Fig. 1A), which was persistent throughout the whole study. The quantification of retroperitoneal adipose tissue showed that mice with HF diet have a marked increase in the ratio of retroperitoneal adipose tissue/total body weight (from 0.76% of normal diet vs.6.61% of HF diet at 10-week to 1.54% vs.8.16% at 16-week, $p<0.01$) (Fig. 1A and B).

4.2. Atherosclerotic plaques in apoE knock-out mice

Different stages of atherosclerotic plaques were found in HF diet mice (Fig. 3, A, C, and E), but none were found in control groups in the whole study period. FP and CP were detected more frequently in the brachiocephalic and coronary arteries when compared with the common carotid artery. The distribution of atherosclerotic plaques showed that early lesions, such as fatty streaks were more easily observed in HF mice on week 10. While progressive or advanced