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Modified low density lipoproteins binding requires a lysine cluster region in the murine macrophage scavenger receptor class A type II

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Abstract

Atherosclerosis is a consequence of lipid deposition and foam cell formation in the arterial wall. Macrophage scavenger receptor A II is involved in the uptake of modified low density lipoproteins. It contains an extracellular conserved lysine cluster which has been proposed to form a positively charged groove that interacts with acetylated low density lipoproteins (AcLDL). This study evaluated the role of the murine SRA-II and a lysine mutated SRA-II on AcLDL uptake. Fluorescence labeled AcLDL uptake was quantified using a Laser Scan Cytometer. A significant increase in fluorescence uptake was found in the cells transfected with SRA-II versus those with empty vector. Cells expressing the lysine mutated SRA-II also demonstrated a significant decrease in their uptake of AcLDL. This data supports the concept that the conserved lysine cluster in murine SRA-II is the binding region for AcLDL or contributes to the trimeric structure of SRA-II necessary for AcLDL binding.

Keywords

Low density lipoproteins; Scavenger receptors; Macrophage; Atherosclerosis; Foam cells

Introduction

Cardiovascular disease as a consequence of atherosclerosis is the main causes of death in the United States. The development of atherosclerosis includes lipid deposition and foam cell formation in the arterial walls. Macrophage scavenger receptor A I and II (SRA-I/II) are cell surface receptors involved in the recognition and binding of modified low density lipoproteins (mLDL) such as acetylated LDL (AcLDL) or oxidized LDL. Furthermore, the role of SRA I/II in foam cell formation and atherogenesis has been established in numerous studies [1–5].

Structurally, SRA-I/II is a trimeric transmembrane protein with an N-terminal cytoplasmic region and a glycosylated fibrous coiled extracellular region. SRA-I and SRA-II are splice

variants, containing five domains: cytoplasmic, transmembrane, spacer, α -helical coiled-coiled, and collagenous. A sixth domain called scavenger receptor cysteine rich (SRCR) domain is present only in the SRA-I isoform [6]. A highly conserved lysine cluster is found in the collagenous domain of SRA I/II corresponding to murine, human, rabbit, bovine, rat, and dog when aligned as shown in Fig. 1. It has been proposed that this structure forms a positively charged groove that interacts electrostatically with negatively charged ligands such as mLDL [7]. Doi et al. [8] and Andersson and Freeman [9] independently mutated these lysines in bovine SRA-I and rabbit SRA-II, respectively, and reported in COS transfected cells a remarkable reduction in radiolabeled ^{125}I -AcLDL binding and degradation. Consequent to these results it has been suggested that the conserved lysine cluster is the binding site for mLDL.

Currently there are no studies evaluating the role of the conserved lysine cluster of murine SRA I/II in the uptake of mLDL. The aim of this study was to evaluate the murine version of SRA-II and the role of the conserved lysine cluster when positively charged aminoacids were substituted for negative ones. In addition, this report attempts to validate the use of CHO cells and fluorescence microscopy techniques to obtain qualitative and quantitative information of AcLDL uptake as an alternative to the traditional use of radiolabeled iodide and/or flow cytometry.

Methods

Cell and lipoproteins

Chinese hamster ovarian cells CHO-K1 cells (ATCC:CCL-61) were grown in Ham's F12K medium with 2 mM L-glutamine and 10% fetal bovine serum at 37°C in a humidified 5% CO₂/95% air incubator. Alexa Fluor 488 conjugated AcLDL was obtained from Invitrogen (Carlsbad, CA).

Plasmid constructs and mutagenesis

Mus musculus macrophage scavenger receptor 1 cDNA (SRA-II), was obtained from Invitrogen NIH Mammalian Gene Collection full-length (IRAV), and subcloned into the vector pcDNA3.1/myc-His-A (Invitrogen). The restriction sites KpnI and XbaI were inserted in the SRA-II sequence at the 5' and 3' terminus, respectively in order to introduce it into the pcDNA3.1/myc-His-A vector. Mutations of Lysines 339 and 342 were simultaneously performed in SRA-II construct using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). DH5 α cells (Invitrogen) were transformed with each construct. After verifying the DNA sequence of the constructs, CHO cells were then transfected.

Transfection

Lipofectamine 2000 Transfection Reagent (Invitrogen) was used for CHO cells transfection following manufacturer's instructions. Forty-eight hours after transfection Geneticin antibiotic (Invitrogen) was added at a final concentration of 500 $\mu\text{g/ml}$ for antibiotic selection of transfected cells. Successful clones were isolated and characterized.

Immunoprecipitation and western blot

Ten million cells were incubated on ice for 30 min in 200 μl of lysis buffer (150 mM NaCl, 10 mM EDTA, 10 mM NaN₃, 10 mM Tris [pH 8.0], 1 mM PMSF, 5 mM iodoacetamide and 1% Nonidet P-40 [NP-40]). Thereafter, immunoprecipitation was performed with rat anti-mouse CD204 antibody from Serotec (Oxford, UK) and western blot with a monoclonal anti-6X His tag antibody from Abcam (Cambridge, MA) as described in the detailed online protocol. Images were taken using a Versadoc Imaging System 3000 from Bio-Rad (Hercules, CA).

AcLDL uptake

Cells were plated in a Lab-Tek II eight-well chamber slide from Nalge Nunc International (Rochester, NY) at 5×10^4 cells per well 24 h before exposure. Cells were pretreated with the inhibitors Polyinosinic acid (PolyI) from Sigma–Aldrich (Saint Louis, MO) at 100 µg/ml or anti-CD204 blocking antibody (Serotec) at 5 µg/ml for 30 min. Alexa Fluor 488 conjugated AcLDL was then added at a final concentration of 10 µg/ml and incubated for 90 min at 37° C. After the incubation cells were washed, fixed and stained with propidium iodide (PI) as described in the detailed online protocol. Images were taken at 400× magnification with a Zeiss Axioskop Fluorescence Microscope and fluorescence quantified using a laser scan cytometer (LSC) with a 20× objective (see detailed online protocol).

Statistical analysis

Experiments were repeated at least three times. Results were presented as the mean $\pm$ SEM. Data was analyzed by pairing related means using the paired *t*-test with the software GB-Stat version 6.5.6. Alpha error was set at $P < 0.05$.

Results

SRA-II expression in CHO cells

The protein expression of SRA-II in transfected CHO cells was confirmed by immunoprecipitation (IP) using the anti-CD204 antibody followed by WB with the anti-6X His Tag antibody as described in “Methods” (Fig. 2a). The SRA-II monomer has a molecular weight of approximately 75 kD, which is seen in the WB. Also a band of approximately 60 kD could be seen in each of the different groups. This band could be the result of some unspecific binding by the heavy chain of the anti-CD204 IgG used for IP. These presence of the 75 kD band not only confirm the expression of SRA-II, but also that the full length of the SRA-II including the 6X His tag was expressed.

AcLDL uptake

After verifying SRA-II protein expression in transfected CHO cells by WB, the uptake of AcLDL was determined to evaluate functionality of the SRA-II construct in CHO cells. Transfected cells were incubated with Alexa Fluor 488 labeled AcLDL as described in “Methods”. Cells transfected with SRA-II showed uptake of fluorescence AcLDL while cells transfected with empty vector (EV) did not (Fig. 2b). AcLDL uptake was quantified using the LSC and the increase in fluorescence was analyzed as shown in Fig. 3. After normalizing each group with its respective control, an increase in fluorescence of more than two-fold was found in the cells transfected with SRA-II compare to EV. This increase was statistically significant.

Effect of SR inhibitors in AcLDL uptake

To verify that SRA-II expression was responsible for the increase of AcLDL uptake, cells were pretreated with SRA-II inhibitors. The specific inhibitor anti-CD204 anti-body and the nonspecific inhibitor PolyI were used for this experiment. The use of anti-CD204 antibody and PolyI produced a decrease in AcLDL uptake of almost one-half (Fig. 4). As expected, a greater decrease was found with PolyI than with the anti-CD204 antibody due to its cross reactivity, since PolyI would block AcLDL uptake by endogenous receptors.

Expression of lysine mutated SRA-II

All previous experiments were directed to confirm expression and functionality of the murine SRA-II in CHO transfected cells and to establish this as a model for AcLDL uptake. After verifying the validity of this model, the role of the conserved lysine cluster was evaluated using a lysine mutated SRA-II (K-E SRA-II) construct. CHO cells were transfected with the K-E

SRA-II construct and protein expression was verified by IP using anti CD204 antibody followed by WB with the anti-6X His Tag antibody as previously described in “Methods” (Fig. 5). The WB showed a band of a molecular weight of approximately 75 kD, confirming the protein expression of SRA-II and K-E SRA-II.

AcLDL uptake of lysine mutated SRA-II

Transfected cells were incubated with Alexa Fluor 488 labeled AcLDL as previously described. Cells transfected with K-E SRA-II and cells transfected with empty vector showed no uptake of fluorescence AcLDL in comparison with SRA-II transfected cells (data not shown). Quantification of fluorescence using the LSC showed that the uptake of AcLDL in the cells transfected with K-E SRA-II was similar to EV transfected cells, confirming that mutation of the conserved lysine cluster prevented the uptake of AcLDL (Fig. 6).

Discussion

Scavenger receptors are members of a family of membrane receptors, initially described as a binding site on macrophages that mediate uptake and degradation of AcLDL [10]. Today it is well recognized that scavenger receptors are a growing family of glycoproteins implicated as pattern recognition receptors, that not only have a broad ligand binding ability to polyanions, but also are expressed in different cell types [11]. The scavenger receptors class A (SRA) are expressed in macrophages and have the ability to bind ligands such as mLDL, negatively charged particles, asbestos [12], and LPS [13]. SRAs also have an important role mediating cell adhesion [14]. There are six members of the SRA family: I, II, III, MARCO (macrophage receptor with collagenous structure) [15], SRCL (SR with C-type lectin) [16], and SCARA5 [17]. SRA-I, SRA-II, and SRA-III are three alternative splice variants of the same gene. SRA-III is not functional and remains in the endoplasmic reticulum [18].

As previously described, SRA-I has an SRCR domain at the C-terminus, which is truncated in SRA-II. Despite this structural difference, binding assays performed with both receptors showed the same ability to bind mLDL [19]. Due to this observation, it was established that the SRCR was not required for mLDL binding. As previously pointed out, Doi et al. [8] and Andersson and Freeman [9] proposed the conserved lysine cluster to be the mLDL binding region due to a decrease in mLDL binding at 37°C when this region is mutated. However, when the same experiments were performed at 4°C binding of AcLDL was rescued. Acton et al. [20] reported that the collagenous domain of the Complement component C1q, which lacks a lysine cluster, has the ability to bind polyanions such as PolyI, fucodain, and maleylated-BSA with similar specificity than SRA-I/II when experiments are performed at 4°C. All these findings suggest that the conserved lysine cluster and the collagenous domain conformation of SRA-I/II are both important for ligand binding, with the conserved lysine cluster more important than the collagenous domain at 37°C, and the opposite at 4°C. In addition, it has been reported that synthesized peptides containing the SRA-I/II conserved lysine cluster adopt a triple-helical conformation [21], which is pH and temperature sensitive. It is possible, therefore, that the conserved lysine cluster is required to stabilize the trimeric conformation of the collagenous domain required for ligand binding at 37°C and not at 4°C.

Doi et al. [8] replaced lysines with alanines, Andersson and Freeman [9] replaced lysines with asparagines or methionine. All these substitutions are different, however, considering that alanine and methionine are non-polar non-charged aminoacids and asparagine is a polar non-charged aminoacid. In the current report lysines were substituted with glutamic acids, which are polar negatively charged aminoacids having similar molecular weight. This substitution changed the positive charge groove of the conserved lysine cluster to a negative one in order to prevent any electrostatic interaction with negatively charged ligands such as AcLDL. As results were similar to other previous substitutions, it is possible to conclude that the decrease

in AcLDL binding is independent of the type of amino acid substitution. Although it would be interesting to determine if substitution of lysine for another polar positive charged amino acid such as arginine or histidine has any effect on AcLDL binding. This last experiment would help to determine if it is the charge or the conformation of the conserved lysine cluster that is more important in AcLDL binding. In conclusion, our experiments have demonstrated using imaging fluorescence analysis that the integrity of the conserved lysine cluster in the murine SRA-II is required for AcLDL uptake supporting the idea that the lysine cluster domain is the binding region of AcLDL and/or is necessary for maintaining the trimeric structure of SRA-II. Because SRA-II binds different type of ligands, further studies will be necessary to determine if the conserved lysine cluster is the binding region for these other ligands.

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Fig. 1.

The SRA-II lysine cluster is conserved among different species. Alignment of SRA I/II protein sequences corresponding to murine (GI: 13277876), human (GI: 4505259), rabbit (GI: 346605), bovine (GI: 27806535), rat (GI: 62662711), and dog (GI: 73979397) showing the conserved lysine cluster

Fig. 2. Expression and functionality of transfected SRA-II. **a** Protein expression of SRA-II was confirmed in CHO transfected cells (arrowhead at 75 kD). **b** Uptake of AcLDL by transfected CHO cells. Cells were exposed to fluorescence labeled AcLDL. Pictures were taken at 400× magnification

Fig. 3. Uptake of AcLDL by transfected CHO cells. Cells were exposed to fluorescence labeled AcLDL for 90 min. Quantification was performed using a Laser Scan Cytometer. Data shown are presented as Mean $\pm$ SEM (* $P < 0.01$; $n = 6$)

Fig. 4. Inhibition of AcLDL uptake. Uptake of AcLDL by transfected CHO cells when exposed to specific (aCD204) and non-specific (PolyI) SRA II inhibitors. Quantification was performed using a Laser Scan Cytometer. Results shown are representative of two experiments and presented as Mean $\pm$ SEM

Fig. 5. Expression of K-E SRA-II. Protein expression of K-E SRA-II was confirmed in CHO transfected cells (*arrowhead* at 75 kD). Untransfected and empty vector transfected cells showed *no band*

Fig. 6. Uptake of AcLDL by transfected CHO cells. Cells transfected with K-E SRA-II showed a significant decrease in uptake. Quantification was performed using a Laser Scan Cytometer. Results shown are pooled from two separate experiments representative of three experiments and presented as Mean $\pm$ SEM (* $P < 0.01$)