Determinants of Scavenger Receptor Expression Levels in Human Macrophages

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Abstract

Background

Atherosclerosis involves the accumulation of oxidized low-density lipoprotein (OxLDL)-laden foam cells (FC) within the blood vessel intima. Macrophages are transformed to FCs by the uptake of OxLDL, a process mediated by scavenger receptors (SRs), which include MSR1, CD36 and CD68. Atherosclerosis differs in prevalence across ethnic groups, being less common in African than Indian or European populations. Therefore, our aim was to measure SRs gene expression from macrophages isolated from these three ethnic groups.

Methods

Ten participants were recruited from each ethnic group (African, European and Indian). Anthropometry and fasting serum lipid and glucose levels were measured. Monocytes were isolated from whole blood and converted to macrophages using standard cell culture procedures. Macrophage RNA was isolated, and reverse transcribed to cDNA. Relative gene expression was calculated using the $\Delta\Delta C_t$ relative quantification method with β -actin used as the normalization control. Multivariable regression analysis was performed to identify the determinants of macrophage SRs expression.

Results

Expression of the *CD36* gene correlated with age ($\beta = 0.33$, $p = 0.02$), LDL ($\beta = 0.29$, $p = 0.03$) and *CD68* expression ($\beta = 0.57$, $p < 0.001$). Expression of *CD68* correlated with triglycerides ($\beta = 0.45$, $p = 0.005$) and European ethnicity ($\beta = 0.56$, $p < 0.001$). Expression levels of *MSR1* correlated positively with LDL ($\beta = 0.27$, $p = 0.01$) and negatively with HDL ($\beta = -0.33$, $p = 0.003$) and African ethnicity ($\beta = -0.73$, $p < 0.001$).

Conclusions

These data suggest that serum lipids may modulate foam cell formation via effects on macrophage SRs gene expression. Furthermore, ethnic differences in atherosclerotic plaque formation may be mediated through differential *CD68* and *MSR1* expression levels within macrophages.

Background

The major cause of heart disease and myocardial infarction in western society is atherosclerosis (1). Atherosclerosis involves the accumulation of oxidized low-density lipoprotein (OxLDL)-laden foam cells (FC) within the blood vessel intima (1). Foam cell formation is the principal event in the development of atherosclerotic plaques. Foams cells form from macrophages following macrophage uptake of oxidised-LDL (oxLDL) (2). The uptake of oxLDL is accomplished by specific receptors on the macrophage cell

surface, the scavenger receptors (SRs). Scavenger receptors are a 'superfamily' of membrane-bound receptors that bind and internalize modified LDL. Identification of these SRs in macrophages was first described in the 1970s by Brown and colleagues (3).

There are 3 main sub-types of SRs: macrophage scavenger receptor 1 (MSR1), CD36 and CD68 (2). The CD36 SR is an 88-kDa transmembrane glycoprotein receptor that belongs to the class B SR family (4). It is encoded by the *CD36* gene, located on chromosome 7 (5, 6). This gene is expressed on various cell types including monocytes and macrophages and its expression levels are increased by a fat-rich diet, inflammation and oxidative stress (5, 6). The MSR1 protein is a 77-kDa cell surface glycoprotein and is a member of the class A SR family (7). It is highly expressed in macrophages but is also present on vascular smooth muscle and endothelial tissues and it mediates the uptake of oxLDL into macrophages (8). The CD68 molecule is a 110-kDa glycoprotein that is a member of the class D SR family (9). The *CD68* gene is located on human chromosome 17 and it is highly expressed by monocytes and tissue macrophages (10, 11).

Macrophages develop from monocytes in response to specific immunological stimuli, including macrophage-colony stimulating factor (M-CSF) (12). This molecule is the main facilitator of the differentiation of monocytes into macrophages *in vitro* and its presence has been demonstrated in atherosclerotic lesions (13).

The risk of atherosclerosis is modified by a number of factors and disease states. Thus, increasing age, high low density lipoprotein- cholesterol (LDL-C), low high density lipoprotein- cholesterol (HDL-C) and high glucose levels, increased body mass index (BMI) and low socioeconomic status are all associated with an increased risk of atherosclerotic disease (14). Atherosclerosis differs in prevalence across ethnic groups, being less common in African than Indian or European populations (15). Furthermore, in South Africa Norman and colleagues found that cholesterol-attributable mortality is highest in Indian subjects (22.2%), followed by European (20.5%) and mixed-ancestry (8.8%) population groups. Cholesterol attributable mortality was lowest in the black African group, at only 1.8% (16).

The reasons for these ethnic variations in the prevalence of atherosclerosis and the contribution of cholesterol to mortality are not fully understood. Given that factors associated with a higher risk for atherosclerosis can modulate SR gene expression, we hypothesised that ethnic differences in the level of atherosclerotic disease may be related to differential macrophage expression of the SRs across ethnic groups. The aims of the current study were therefore to measure SR gene expression from macrophages isolated from African, European and Indian populations to determine if SR expression levels mirror the reported ethnic differences in the prevalence of atherosclerosis. We also sort to determine whether anthropometric, demographic and cardiometabolic factors influence macrophage SR gene expression.

Materials And Methods

Participant recruitment

Ten healthy participants per ethnic group (African, European, Indian) aged ≥ 40 years were recruited to participate in the study from the greater Johannesburg area by convenience sampling. The three groups were matched for gender. Anthropometric measurements (weight, height, and hip and waist circumferences) were collected for all participants and blood drawn through venous sampling. Body mass index (BMI) was calculated using weight and height ($\text{BMI} = \text{weight (kg)} / \text{height}^2 \text{ (m)}$). The participant's ethnicity was self-reported. Participants receiving therapy for high lipid levels, participants with diabetes or those with a history of cardiovascular problems were excluded from the study. Informed consent was obtained from all participants and ethical approval for the study was obtained from University of Witwatersrand Human Research Ethics Committee (Medical).

Measurement of cardiometabolic variables

Fasting lipograms and glucose were measured on the Cobas® 8100 (Roche, Basel, Switzerland) auto-analyzer in the routine Chemical Pathology Laboratory of the National Health Laboratory Service (NHLS) based at Charlotte Maxeke Johannesburg Academic Hospital. The coefficients of variation (CV) for cholesterol were 1.6 % and 1.4 %, for triglycerides were 2.7 % and 1.1 %, for HDL-C were 0.9 % and 0.9 % and for LDL-C were 0.9 % and 0.9 % for low and high control samples, respectively. The detection limit for cholesterol, triglycerides and LDL-C was 0.1 mmol/L, while for HDL-C it was 0.08 mmol/L.

Cell culture

Monocyte isolation

Human monocytes were isolated using a modified method based on that described by de Almeida and colleagues (17). Buffy coat was retrieved from 45 ml of blood in ethylenediaminetetraacetic acid (EDTA) tubes (Beckman Dickenson, Franklin Lakes, NJ, USA), overlaid on Histopaque®-1077 (density 1.077 g/ml, [Sigma, St Louis, MO, USA]) and centrifuged at $2360 \times g$ for 10 min. Following centrifugation, the peripheral blood mononuclear cells (PBMCs) were isolated and plated into a 6 well cell culture plate (Nest Biotechnology, Wuxi, China) at a concentration of 2×10^6 cells per well in RPMI growth medium (Lonza Bioscience, Basel, Switzerland) supplemented with 10% fetal bovine serum, 25mM HEPES, 2mM L-glutamine, and 100U/ml Penicillin/Streptomycin. The cells were maintained under standard conditions (in an incubator at 37°C and 5% CO_2 in humidified air). After 24 hours of culture the monocytes adhered to the culture plate and non-adherent lymphocytes were removed by rinsing the culture plate with 2 ml growth medium and the isolated monocytes were subsequently converted to macrophages. One well of a 6-well culture plate (containing a coverslip) was used for determining the monocyte purity by incubating the cells with a primary antibody that binds to calprotectin, an intracytoplasmic protein expressed by monocytes and macrophages (18). Random sections of the cover slip were chosen, and 100 cells were counted. Both stained and unstained cells were counted, and the percentage of stained cells was calculated.

Macrophage differentiation

After 24 hours of culture (see above), monocytes were differentiated into macrophages by incubation with 1ml growth media supplemented with macrophage colony-stimulating factor (M-CSF (100 ng/ml) [Biolegend, San Diego, CA, USA]) for 5 days under standard conditions. Macrophage differentiation was confirmed through microscopic detection of morphological changes (elongated or stellate morphology) (19).

Quantification of scavenger receptor expression by quantitative real time PCR

Total RNA was extracted from macrophages using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) and DNase I (Sigma) was used to eliminate DNA from RNA template according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MS, USA). Real-Time PCR was performed using Maxima SYBR® Green master mix (Thermo Scientific, Waltham, MS, USA). The quantification of the levels of expression of the scavenger receptor genes was carried out on the StepOnePlus real time PCR system (Thermo Scientific, Waltham, MS, USA). Relative gene expression of SRs *CD36*, *CD68* and *MSR1* was calculated using the $\Delta\Delta C_t$ relative quantification method with beta (β)-actin used as the normalization control. The cDNA amplification of *CD36*, *CD68* and *MSR1* was performed using the following conditions over 45 cycles: initial denaturation at 95°C for 2 minutes; denaturation at 95°C for 30 seconds; annealing at 55°C for 30 seconds; extension at 72°C for 1 minute. The primer sequences used for quantification were as follows: *CD36* forward primer 5'-AAGCCAGGTATTGCAGTTCTTT-3', reverse primer 5'-GCATTTGCTGATGTCTAG CACA-3'; *CD68* forward primer 5'- GGAAATGCCACGGTTCATCCA-3', reverse primer 5'-TGGGGTTTCAGTACAGAGATGC-3'; *MSR1* forward primer 5'- GCAGTGGGATCACTTT CACAA-3', reverse primer 5'-AGCTGTCATTGAGCGAGCATC- 3'; beta actin forward primer 5'-CATGTACGTTGCTATCCAGGC-3', reverse primer 5'- CTCCTTAATGTAC GCACGAT-3'.

Statistical analysis

Continuous variables with a normal or skewed distribution were expressed as means $\pm$ SD or median (interquartile range), respectively. Categorical variables were expressed as percentages. Continuous data were compared across the three ethnic groups using ANOVA, with skewed data log normalised before being analysed and paired comparisons performed using a Tukey *post hoc* test. Categorical variables were compared across the groups using a χ^2 test. Univariate Spearman correlations of SR levels with gender, ethnicity, age, anthropometry, blood lipid and glucose levels were performed and variables with $p < 0.20$ were included as independent variables in multivariable linear regression models. Multivariable linear regression analysis was performed using a backward stepwise model to find the main determinants of SR expression levels. A $p < 0.05$ was considered significant. Statistical analyses were performed using Statistica software version 13.0 (Statsoft, Tulsa, OK, USA).

Sample sizes were not calculated due to lack of data on ethnic differences in macrophage SR expression. The sample size chosen (10 subjects per group) was based on study infrastructural constraints, but with allowance for expansion of the sample size if significant differences were not observed with $n = 10$.

Results

Monocyte purity was assessed for all 30 isolates and was found to be 96.0 ± 6.0 %. The purity was compared across the 3 ethnic groups and no statistical differences were observed ($p = 0.28$).

Table 1 shows the data comparison across ethnicity. Africans had a significantly higher BMI than Europeans ($p = 0.03$). Hip circumference was significantly higher in Africans as compared to Europeans ($p = 0.01$) and Indians ($p = 0.004$). No differences were observed in terms of fasting lipograms (total cholesterol, HDL, LDL, and triglycerides) and glucose between the ethnic groups.

Table 1
Data comparison across ethnic groups

Variables	Africans (n = 10)	Europeans (n = 10)	Indians (n = 10)
Males (%)	50.0	50.0	50.0
Age (years)	46.9 ± 5.1	53.8 ± 10.6	54.4 ± 9.8
BMI (kg/m ²)	30.2 (27.8, 40.4)	24.1 (20.8, 25.9) *	26.9 (21.1, 29.0)
Waist (cm)	96.6 ± 9.1	88.7 ± 10.6	85.2 ± 15.7
Hip (cm)	115.5 ± 14.9	$100.6 \pm 6.6^*$	$98.7 \pm 9.0^{**}$
Cholesterol (mmol/L)	4.6 ± 0.7	5.1 ± 0.6	5.2 ± 0.8
HDL-C (mmol/L)	1.4 (1.0, 1.7)	1.5 (1.3, 1.9)	1.5 (1.1, 1.6)
LDL-C (mmol/L)	3.0 ± 0.6	3.5 ± 0.5	3.5 ± 0.9
Triglycerides (mmol/L)	0.9 (0.7, 1.1)	0.7 (0.6, 1.2)	0.9 (0.7, 1.1)
Glucose (mmol/L)	4.7 ± 0.8	5.1 ± 0.6	5.3 ± 1.0
Data given as mean $\pm$ SD or median (interquartile range); * $p < 0.05$, ** $p < 0.005$ vs African.			

Scavenger receptor gene expression from macrophages isolated from African, European and Indian populations

Scavenger receptor gene expression were compared across ethnic groups (Fig. 1). Expression of *CD36* was significantly lower in African ($p = 0.0005$) and Indian ($p = 0.04$) compared to European participants. The expression levels of *CD68* in Indian ($p = 0.007$) participants were significantly lower compared to

European participants whilst *MSR1* expression was higher in European ($p = 0.0002$) and Indian ($p = 0.0001$) than African participants.

Determinants of macrophage SR gene expression

Note that Table 2 and 3 the $n = 28$ instead of $n = 30$, this is because when we performed normal distribution of scavenger receptors, we found outliers and we removed them from our data. Table 2 outlines the factors that are associated with the scavenger receptor expression in macrophages. Univariate correlation analysis of *CD36* gene expression demonstrated positive associations with age ($p = 0.01$), *CD68* expression ($p < 0.001$) and *MSR1* expression ($p = 0.02$). whilst *CD68* gene expression associated positively with serum triglycerides ($p = 0.04$). Gene expression of *MSR1* was associated positively with serum LDL-C ($p = 0.001$) and plasma glucose ($p = 0.005$).

Table 2
Univariate correlations for macrophage scavenger receptor expression

Variables	R value (p-value)		
	CD36 (n = 28)	CD68 (n = 28)	MSR1 (n = 28)
Age (Years)	0.455 (0.013)	0.130 (0.500)	0.256 (0.189)
BMI (kg/m ²)	-0.260 (0.173)	-0.084 (0.666)	-0.266 (0.172)
Waist (cm)	0.027 (0.891)	0.119 (0.537)	-0.026 (0.896)
Hip (cm)	-0.239 (0.212)	0.030 (0.878)	-0.334 (0.082)
Cholesterol (mmol/L)	0.291 (0.226)	-0.039 (0.766)	0.345 (0.072)
HDL-C (mmol/L)	-0.010 (0.895)	-0.117 (0.517)	-0.273 (0.159)
LDL-C (mmol/L)	0.333 (0.114)	-0.023 (0.915)	0.575 (0.001)
Triglycerides (mmol/L)	0.112 (0.721)	0.377 (0.031)	-0.022 (0.910)
Glucose (mmol/L)	0.105 (0.498)	-0.117 (0.680)	0.516 (0.005)
CD36 expression		0.607 (0.000)	0.424 (0.015)
CD68 expression	0.607 (0.000)		0.072 (0.717)
MSR1 expression	0.424 (0.015)	0.072 (0.717)	

Table 3

Multivariate regression models for macrophage scavenger receptor expression

Dependent variable	Independent variables with β -coefficient (p-value)	Adjusted R ² (p-value)
CD36 (n=28)	Age; 0.33 (0.02) LDL-C; 0.29 (0.03) CD68; 0.57 (<0.001)	0.55 (<0.001)
CD68 (n=28)	Europeans; 0.56 (<0.001) Triglycerides; 0.45 (0.005) Africans; -0.73 (<0.001)	0.41 (<0.001)
MSR1 (n=28)	HDL-C; -0.33 (0.003) LDL-C; 0.27 (0.015)	0.77 (<0.001)

Using the data from Fig. 1 and Table 2, backward, stepwise linear multivariate regression analysis of gene expression was performed, and the results are shown in Table 3. The *CD36* scavenger receptor expression correlated with age ($p = 0.02$), LDL ($p = 0.03$) and *CD68* expression ($p < 0.001$) whilst *CD68* expression correlated positively with triglycerides ($p = 0.005$) and European ethnicity ($p < 0.001$). Gene expression of *MSR1* correlated positively with LDL ($p = 0.015$) and negatively with HDL ($p = 0.003$) and African ethnicity ($p < 0.001$).

Discussion

The current study shows clear associations of serum lipid levels and ethnicity with macrophage SR gene expression. Thus, *CD36* and *MSR1* expression were both positively associated with serum LDL levels, and *MSR1* was also negatively associated with HDL. Gene expression of *CD68* was positively associated with serum triglyceride levels. In terms of the association of ethnicity with SR expression, *CD68* was expressed at higher levels in subjects with European ancestry, whilst *MSR1* was expressed at lower levels in African subjects.

When comparing data between the 3 ethnic groups, the African participants had significantly higher levels of both BMI and hip circumference compared to the other groups. It is known that obesity is more prevalent in African than other ethnic groups in South Africa, particularly in females (20). In addition, LDL and total cholesterol, although not statistically significantly so, were lower in the African compared to the

other groups. Similar ethnic differences in lipid profiles have been reported in other South African studies (21, 22).

Scavenger receptors *MSR1* and *CD36* have been shown to be the primary receptors responsible for the uptake of modified LDL into macrophages leading to foam cell formation (23) (2). The interaction of *CD68* with modified lipoproteins is still controversial and their relevance is still unclear (2). However, a study by Tsukamoto and colleagues (2002) reported that *CD68* may play a crucial role in foam cell formation through uptake of oxLDL by macrophages (24). In our study *CD36* expression was lower in African and Indian compared to European participants, whereas the expression of *CD68* was lower in Indian compared to European participants. The expression of the *MSR1* gene was higher in European and Indian than African participants. Therefore, lower levels of expression of *CD36* and *MSR1* in African participants mirror a lower level of atherosclerotic disease observed in this population (15).

Scavenger receptor expression levels correlated with baseline fasting lipid profiles, ethnicity and age. Thus, *CD36* expression levels correlated positively with LDL-C, *CD68* expression and age, whilst *CD68* expression correlated positively with triglycerides and European ethnicity. The expression of the *MSR1* gene correlated positively with LDL-C, and negatively with both HDL-C and African ethnicity. The positive association of serum LDL levels with macrophage SR gene expression is a novel finding and is supported by a study showing that LDL upregulates the expression of macrophage SRs in a murine macrophage cell line (25). Lipoproteins, particularly minimally modified LDL and oxidized LDL can modulate signal transduction and gene expression in macrophages and other cells. Thus, LDL stimulates phosphoinositide turnover and elevates cytosolic calcium levels in vascular smooth muscle cells. Minimally modified LDL induces the expression of monocyte adhesion molecules, tissue factor, macrophage chemotactic protein-1 (MCP-1) and M-CSF in endothelial cells (25). Furthermore, high levels of plasma LDL has the ability to activate signal transduction and gene expression in macrophages (26), through stimulation of phosphoinositide turnover.

The association of serum lipid levels with macrophage SR expression that is described in the present study mirrors the effect of these lipid molecules on the risk of atherosclerosis. These data suggest that serum lipids may modulate atherosclerotic plaque formation by modifying the expression levels of scavenger receptors in circulating macrophages even before exposure to oxLDL.

Our findings were limited by the relatively small sample size however, we were still able to observe differences in SR levels across ethnicities and significant associations within the regression models. The other limitation is the analysis of associations between *in vitro* data i.e. SR expression, and *in vivo* data i.e. serum lipid levels. In performing these analyses we are assuming that lipid levels have an effect on macrophage SR expression that is not modified by monocyte isolation and cell culture conditions. Despite this limitation, it is interesting to note that the associations observed between serum lipid levels and *in vitro* macrophage SR expression are supported by other studies from the literature and are scientifically plausible.

Conclusion

Our data suggests that serum lipids may modulate atherosclerotic plaque formation by modifying the expression levels of scavenger receptors in macrophages even before exposure to oxLDL in the intima. Furthermore, ethnic differences in atherosclerotic plaque formation may be mediated through differential *CD68* and *MSR1* SRs expression levels within macrophages.

Abbreviations

FC: foam cells; OxLDL: oxidized low-density lipoprotein; SRs: scavenger receptors; MSR1: macrophage scavenger receptor 1; M-CSF: macrophage-colony stimulating factor; LDL-C: low density lipoprotein-cholesterol; HDL-C: high density lipoprotein-cholesterol; BMI: body mass index; NHLS: National Health Laboratory Service; CV: coefficients of variation; EDTA: ethylenediaminetetraacetic acid; PBMCs: peripheral blood mononuclear cells; MCP-1: macrophage chemotactic protein-1

Declarations

Acknowledgments

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Author Contributions

Maile G Ralefatane: Writing - original draft, analysis and interpretation of the data, Drafting of the manuscript, acquisition of the data. **Eleanor Cave:** Writing - review & editing, supervision. **Nigel J Crowther:** Writing - review & editing, supervision, funding acquisition, gave final approval of the version of the manuscript submitted for publication.

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Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The study was approved by University of Witwatersrand Human Research Ethics Committee (Medical). Informed consent was obtained from all participants.

Consent for publication

Each participant has a signed consent form.

Competing interests

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Figures

Figure 1

Macrophage SR expression levels across ethnic groups. Data given as mean $\pm$ SD; + $p < 0.05$ vs Europeans, *** $p < 0.0005$ vs Africans.