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Impact of serum amyloid A on cellular cholesterol efflux to serum in type 2 diabetes mellitus

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ABSTRACT

Objective: Serum amyloid A (SAA) is an acute phase response protein and has apolipoprotein properties. Since type 2 diabetes is associated with chronic subclinical inflammation, the objective of this study is to investigate the changes in SAA level in type 2 diabetic patients and to evaluate the relationship between SAA and the capacity of serum to induce cellular cholesterol efflux via the two known cholesterol transporters, scavenger receptor class B type I (SR-BI) and ATP-binding cassette transporter G1 (ABCG1).
Methods: 264 patients with type 2 diabetes mellitus (42% with normoalbuminuria, 30% microalbuminuria, and 28% proteinuria) and 275 non-diabetic controls were recruited. SAA was measured by ELISA. SR-BI and ABCG1-mediated cholesterol efflux to serum were determined by measuring the transfer of [^{3}H]cholesterol from Fu5AH rat hepatoma cells expressing SR-BI and from human ABCG1-transfected CHO-K1 cells to the medium containing the tested serum respectively.
Results: SAA was significantly increased in diabetic patients with incipient or overt nephropathy. Both SR-BI and ABCG1-mediated cholesterol efflux to serum were significantly impaired in all three groups of diabetic patients ($p < 0.01$). SAA inversely correlated with SR-BI-mediated cholesterol efflux ($r = -0.36$, $p < 0.01$) but did not correlate with ABCG1-mediated cholesterol efflux. Stepwise linear regression analysis showed that HDL, the presence or absence of diabetes, and log(SAA) were significant independent determinants of SR-BI-mediated cholesterol efflux to serum.
Conclusion: SAA was increased in type 2 diabetic patients with incipient or overt nephropathy, and SAA was associated with impairment of SR-BI-mediated cholesterol efflux to serum.

1. Introduction

Serum amyloid A (SAA) is an acute phase response protein and is a sensitive marker of the acute inflammatory response. It has been suggested that SAA may be involved in the defence mechanisms against pathogens and may function as an effector molecule of the immune system [1]. SAA has apolipoprotein properties and is mainly associated with high density lipoprotein (HDL) in the circulation. Both SAA and apolipoprotein AI (apo AI) share the same amphipathic helical structure, and because of its association with HDL, SAA may also play a role in lipoprotein metabolism [2,3]. Level of SAA increases up to 1000-fold during acute phase inflammation and SAA can displace apo AI from the phospholipid surface of HDL, thus altering the structures and functions of HDL [3,4]. There are conflicting data on the impact of enrichment of HDL with SAA on the property of HDL to induce cholesterol efflux, with some studies reporting a reduction in the ability of HDL to efflux cholesterol from cells *in vitro* [5,6] whilst others have shown the contrary [7–9]. The effect of SAA on cholesterol efflux to HDL seems to be partly dependent on the experimental conditions used and the cholesterol transporters involved. Using a mouse model, Annema et al. has recently reported that SAA impaired reverse cholesterol transport during the acute phase response *in vivo* [10].

The impact of SAA on reverse cholesterol transport has been mainly investigated in acute inflammatory state but the effect of SAA is much less clear in chronic inflammation where the concentration of SAA is much lower. Type 2 diabetes mellitus is associated with chronic subclinical low grade inflammation [11]. We have previously reported that cellular cholesterol efflux to serum in patients with type 2 diabetes is impaired [12]. The capacity of serum to induce cellular cholesterol efflux is influenced not only by the concentrations of lipoproteins, like HDL, that act as cholesterol acceptors but also by other serum components. Hence, we have determined firstly whether there are any significant changes in SAA levels in type 2 diabetic patients with and without nephropathy and secondly, the relationship between SAA and serum capacity to induce cellular cholesterol efflux.

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2. Methods

2.1. Subjects

Type 2 diabetic patients were recruited from the diabetes clinics at Queen Mary Hospital in Hong Kong. Diabetic patients were invited to participate when they attended their annual screening visit for diabetic complications and were subdivided into those with normoalbuminuria (<30 mg/day), microalbuminuria (30–300 mg/day) and proteinuria (>300 mg/day) according to their urinary albumin excretion rate. Patients with history of cardiovascular disease and/or receiving lipid lowering agents were excluded. Recent acute illness was also an exclusion criterion. Healthy non-diabetic controls were recruited from the community. Blood samples were collected after an overnight fast for the measurements of HbA_{1c}, glucose, renal function, lipids, SAA, and cellular cholesterol efflux to serum. The study was approved by the Ethics Committee of the University of Hong Kong, and informed consent was obtained from all subjects.

2.2. Ex vivo cellular cholesterol efflux to serum

Scavenger receptor class B type I (SR-BI) and ATP-binding cassette transporters G1 (ABCG1) are the main cholesterol transporters that mediate cholesterol efflux to HDL. To measure the SR-BI-mediated cellular cholesterol efflux to serum and ABCG1-mediated cellular cholesterol efflux to serum, Fu5AH rat hepatoma cells which express only SR-BI but no functional ABCs [13], and human ABCG1-transfected Chinese Hamster Ovary-K1 (CHO-K1) cells (a generous gift from Prof. W. Jessup) were used respectively [14]. Cells were cultured in minimum essential medium (MEM) and Ham's F-12K medium respectively (Gibco, Grand Island, NY, USA), which were also supplemented with 5% FBS. Eighty per cent confluent cells were labelled with [^{3}H]-cholesterol for 18 h (37kBq/well; Amersham Biosciences, Pittsburgh, PA, USA). After that, the medium was removed and cells were incubated with fresh medium containing 0.5% BSA for a further 4 h to allow the incorporated [^{3}H]-cholesterol to equilibrate among the cellular cholesterol pools. Cells were then washed with PBS and incubated with serum samples from either diabetic patients or healthy controls (diluted 1:20 with medium) for 6 h at 37 °C. Supernatants were collected and cells were lysed with 0.1% Triton X-100 in PBS (500 μl). The amount of [^{3}H]-cholesterol in each fraction was assessed by scintillation counting using a Packard scintillation counter (Perkin Elmer, Waltham, MA, USA). Cholesterol efflux was calculated as the percentage of [^{3}H]-cholesterol recovered in the supernatant compared to the total cellular [^{3}H]-label for each sample. All samples were assayed in duplicate, and inter-assay coefficients of variation for SR-BI-mediated cholesterol efflux assay and ABCG1-mediated cholesterol efflux assay was 8.0% and 9.7% respectively.

2.3. Ex vivo cellular cholesterol efflux to HDL

Protocol for cholesterol efflux to HDL was similar to that of serum, but HDL (25 μg/ml HDL-protein) was used as the cholesterol acceptor for each sample. To prepare HDL from plasma, a one-step ultracentrifugation protocol was used. Briefly, plasma was first salted with KBr to establish a density at 1.24 g/ml and was overlaid on top with a single gradient of 1.076 g/ml NaBr solution. Mixed plasma was then ultracentrifuged (Beckman Coulter, Brea, CA, USA) for 3.5 h at 100,000 rpm at 10 °C and purified HDL (1.063–1.21 g/ml) was extracted from the tube. Lastly, HDL was desalted with Econo-Pac® 10DG desalting column (Bio-Rad, Hercules, CA, USA). Protein concentration was measured by the method of Lowry et al. [15].

2.4. Biochemical assays

Serum amyloid A was measured by commercial sandwich ELISA kit (abcam, San Francisco, CA, USA). Plasma total cholesterol and triglyceride were determined enzymatically on a Hitachi 912 analyser (Roche Diagnostics GmbH, Mannheim, Germany). HDL-cholesterol was measured using a homogenous method with polyethylene glycol-modified enzymes and alpha-cyclodextrin. Plasma apo AI and apo B were measured by rate nephelometry using the Beckman Array System (Beckman Coulter, Brea, CA, USA). HbA_{1c} was measured in whole blood samples using ion-exchange high performance liquid chromatography with the Bio-Rad Variant Haemoglobin Testing System (Bio-Rad, Hercules, CA, USA). Plasma creatinine was measured by the Jaffe method. Urinary albumin excretion rate was determined from the mean of two consecutive 12-h overnight urine collections. Urine albumin was measured by rate nephelometry using the Beckman Array 360 Analyser (Beckman Coulter, Brea, CA, USA). Estimated glomerular filtration rate (eGFR) was calculated by Modification of Diet in Renal Disease (MDRD) Study equation.

2.5. HDL binding assay

Fu5AH rat hepatoma cells were seeded in 24-well plate until confluent in MEM with 5% FBS. Cells were then fasted with plain medium for 16 h and incubated with ice-cold MEM with various concentrations of lipid-free SAA (Peprotech, Rocky Hill, NJ) for 2 h in 4 °C. The medium was removed and 5 μg/ml DiI-HDL (BTI, Stoughton, MA) in MEM was added and incubated for 2 h in 4 °C. Cells were then washed once with ice-cold PBS and lysed with 0.1% Triton X-100 in PBS (200 μl). The fluorescence intensity of the cell lysate reflecting the amount of bound HDL was measured on black 96-well microtiter plate (Costar, Palo Alto, CA), with excitation and emission wavelengths set at 520 and 578 nm respectively by the Tecan plate reader (Männedorf, Schweiz). Results were expressed as percentage of the control value obtained in the absence of SAA. Each of the binding experiment shown is representative of the duplicate results obtained in three individual experiments.

2.6. Statistics

Numerical data were expressed as mean ± standard deviation of the mean (SD) or median (inter-quartile range). Data that were not normally distributed were logarithmically transformed before analyses were made. Analysis of variance (ANOVA) was used to compare continuous variables for multiple groups followed by *post-hoc* multiple comparisons using Dunnett *t*-tests with the non-diabetic control as the reference group. Pearson's correlations were used to test the relationship between variables, and multiple stepwise linear regression analysis was used to assess the relationships between cholesterol efflux to serum and various variables simultaneously.

3. Results

The clinical characteristics of control and type 2 diabetic patients are shown in Table 1. Control subjects were younger than the diabetic patients. All three groups of diabetic patients had larger waist circumference and higher systolic blood pressure. Glycaemic control was comparable in the three groups of diabetic patients (Table 2) and none of the patients was on pioglitazone which could affect HDL efflux capacity. Diabetic patients had hypertriglyceridaemia and low plasma HDL-cholesterol (HDL-C) and apo AI compared to healthy controls. Serum amyloid A was significantly higher in diabetic patients with microalbuminuria or proteinuria