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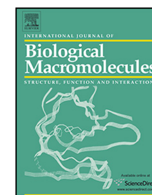

Erythrocytes in the combined milieu of high glucose and high cholesterol shows glycosaminoglycan-dependent cytoadherence to extracellular matrix components

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ABSTRACT

Pathological conditions are bound to affect the molecules on erythrocytes, and accordingly affect their functions. Chondroitin sulphate/dermatan sulphate (CS/DS), one of the classes of molecules found to be expressed in erythrocytes was previously observed by us to be either overexpressed in diabetic condition or undergo structural changes in hypercholesterolemic condition. Both of them had implications on their binding to extracellular matrix components (ECM). In the present work, we have explored the quantitative changes in erythrocyte glycosaminoglycans (GAGs) and their role in erythrocyte binding towards ECM components in the combined milieu of both diabetes and hypercholesterolemia (SFHD). Membrane cholesterol was significantly higher in SFHD group compared to control (SFC) and diabetic groups (SFD). Interestingly, there were no quantitative changes in CS/DS compared to SFC erythrocytes, but showed significantly increased cytoadherence to selected ECM components to various extents. Binding was partly dependent on CS/DS as digesting the chains resulted in relatively decreased cytoadherence. It also showed significantly increased binding to chondroitin sulphate and heparan sulphate. Thus, combined milieu of high glucose and high cholesterol can have more deleterious consequences than either of them independently.

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1. Introduction

Pathological disorders, such as diabetes mellitus and hypercholesterolemia are characterized by increased blood glucose and cholesterol levels, respectively. These can have different consequences on erythrocytes. Membrane lipids in particular, are major active players in the fluidity, aggregability, adherence and deformability of erythrocytes. Fluidity of erythrocytes decreases with increasing membrane cholesterol [1]. Interestingly, erythrocytes have been localized in coronary atherosclerotic plaques [2]. Increased proportion of membrane cholesterol has been predicted to contribute to instability of atherosclerotic plaque [3,4] and progression of atherosclerosis [5,6]. Total erythrocyte membrane cholesterol is now known to be a new marker of acute coronary disease [3]. Thus, hypercholesterolemia is a risk factor for the development of atherosclerotic vascular disease [7].

Diabetes mellitus, on the other hand, causes increased erythrocyte aggregation, increased adherence of erythrocytes to endothelial cells and decreased deformability [8]. Erythrocyte ultrastructure has been reported to get altered in type II diabetes, the causative factor being iron overload and the subsequent non-enzymatic fibrinogen polymerization [9]. Disturbance in the structure and function of the erythrocyte membrane has been associated with diabetes [10]. During diabetes, decrease in erythrocyte membrane fluidity has been correlated with an increase in cholesterol and phospholipid contents [11].

Glycosaminoglycans (GAGs) are polymeric macromolecules which are present in virtually all the cells of the body and play important roles in myriad functions, such as growth factor binding, cell adhesion, cell signalling, proliferation and migration, coagulation, tissue repair and immune responses [12,13]. Among various classes of GAGs, chondroitin sulphate/dermatan sulphate (CS/DS) and heparan sulphate (HS) are the major classes, which play a vital

Abbreviations: BSA, bovine serum albumin; CS/DS, chondroitin sulphate/ermatan sulphate; DMMB, 1,9-dimethylmethylene blue; ECM, extra cellular matrix; GAGs, glycosaminoglycans; HS, heparan sulphate; HDL, high density lipoprotein; LDL, low density lipoprotein; PG(s), proteoglycan(s); P-orn, Poly-L-Ornithine; STZ, streptozotocin; SFC, control; SFD, diabetic; SFHD, starch fed hypercholesterolemia and diabetic.

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role in cell adhesion and proliferation [14], migration, cell signalling [15], and development [12]. CS-A has been recognized to be a specific receptor for *Plasmodium falciparum*-infected erythrocytes, and they bound to Chinese hamster ovary cells and C32 melanoma cells in a CS-A dependent manner [16]. Furthermore, adhesion of *P. falciparum*-infected erythrocytes to endothelial cells is known to be mediated by HS via the DBL1α domain of PfEMP1 [17]. Findings from our group revealed the presence of CS/DS class of GAGs in erythrocytes for the first time. In conditions of diabetes, they were overexpressed, which resulted in increased cytoadherence to ECM components [18]. On the other hand, in diet-induced hypercholesterolemic rats, fine structural alterations were observed, which also led to increased cytoadherence [manuscript, under revision]. Both of the above studies showed that there is a propensity of change occurring in erythrocyte depending on the milieu in which they were subjected to. The next question which arises is what would happen to erythrocyte GAGs and its role in cytoadherence in conditions of both hyperglycemia and hypercholesterolemia.

Hence, the current study investigates the effect of high cholesterol diet fed to streptozotocin-induced diabetic rats on erythrocyte GAGs and their role in adhesion of erythrocytes to various ECM components.

2. Materials and methods

2.1. Materials

Anti-chondroitin sulphate (CS-56), FITC-conjugated secondary antibody (anti-IgM), streptozotocin (STZ), type IV collagen, laminin, fibronectin, dinitro-salicylic acid, 1,9-dimethylmethylene blue (DMMB), heparin, papain from *Carica papaya* were obtained from Sigma, USA. Bovine serum albumin (BSA) was from Genei, Bangalore. Chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4), Standard chondroitin sulphate unsaturated disaccharides, Chondroitin sulphate B were from Associates of Cape Cod, USA. Glutaraldehyde was from Himedia, India. Pre-packed disposable PD-10 columns containing Sephadex G-25 (medium) was from GE Healthcare, USA. Glucose estimation kit (Enzymatic GOD–POD method), Cholesterol, triglycerides, HDL and LDL kits were procured from Agappe Pvt. Ltd., India. All other chemicals and reagents used were of analytical grade.

2.2. Animals, diet and induction of diabetes

Male albino Wistar rats [OUTB-Wistar-IND CFTRI (2c)] weighing around 110–130 g were housed in individual cages under a 12-h light/dark cycle. The rats were acclimatized to the basal diet (AIN-93) for at least one week. After a week's adaptation, the animals were divided into two groups based on body weight – control (SFC) and diabetic. Animals in diabetic groups were injected with streptozotocin (STZ) at the dosage of 45 mg/kg body weight as reported earlier [19]. After a week, the animals in the diabetic group were further subdivided into diabetic (SFD), and diabetic + hypercholesterolemic (SFHD) based on blood glucose levels. While, animals in SFC and SFD groups were fed with AIN-93 diet, SFHD animals were fed with AIN-93 diet supplemented with 0.5% cholesterol [20]. Rats had free access to water and diet. All animal work was performed in accordance to relevant national and international guidelines. The work was approved by Institute Animal Ethical Committee. The experimental duration after diabetes induction was for a period of 2 months.

2.3. Blood sampling and plasma biochemistry

Plasma biochemical analyses were carried out on blood samples collected after a fasting period of 12 h. Rats were anesthetized, and blood was collected from the orbital sinus into tubes containing heparin as an anticoagulant [21]. Blood was centrifuged at 180 × *g* and plasma was taken for analysis of various biochemical parameters. Blood glucose, total cholesterol, triglycerides, HDL and LDL were determined in plasma by using respective kits procured from Agappe Pvt. Ltd., India.

2.4. Estimation of RBC membrane cholesterol

Lipids were extracted from the erythrocyte membranes as described earlier [22]. Briefly, blood was collected in heparinized tubes and centrifuged at 180 × *g* for 10 min. Pellet was washed thrice with 5 volumes of 0.9% NaCl and buffy coat was removed. 1 mL distilled water was added to 1 mL of packed erythrocytes and allowed to stand for 5 min. To this 11 mL of isopropanol was added slowly with mixing for 1 h. After 1 h, 7 mL of chloroform was added and mixed, then incubated for 1 h. The tubes were then centrifuged at 500 × *g* for 30 min. Supernatant was made to convenient volume with isopropanol:chloroform mixture (3:2) [23] and taken for estimation.

2.5. Isolation of glycosaminoglycans

It was carried out as described previously [18]. At the end of the experimental period, rats were sacrificed, and blood was collected into individual tubes containing 3.8% sodium citrate by heart puncture. An aliquot of the collected blood sample was taken for RBC counting. Collected blood samples were centrifuged at 180 × *g* for about 15 min, and buffy coat was removed [24]. Packed erythrocytes were then washed thrice with 5 mM PBS. Following three washes, erythrocytes were hemolyzed by adding chilled 5 mM hypotonic phosphate buffer, pH 8.0. The membrane pellet obtained after centrifugation was washed sequentially with 2.5 mM and 1.25 mM phosphate buffer, pH 8. Wet pellet weight was recorded, and membrane pellets were taken for further analysis. Finally, membrane pellet was subjected to papain digestion by reconstituting in 0.1 M phosphate buffer, pH 6.5. Digestion was carried out for 48 h by adding freshly prepared enzyme aliquots at regular intervals. Digested samples were centrifuged at 300 × *g* at 4 °C for 15 min and to the supernatant one-third volume of 40% TCA was added to precipitate proteins. Precipitated proteins were removed by centrifugation and GAGs were precipitated by adding 4 volumes of ethanol containing 1.2% potassium acetate to the supernatant. The precipitate was collected and dried under nitrogen gas, reconstituted in a minimal volume of water. They were then desalted on a PD10 column using pyridine–acetate buffer and collected fractions were dried under speed vac, reconstituted in the known volume of water and stored at −20 °C until use.

2.6. Cytoadherence of erythrocytes to extracellular matrix proteins

Adhesion assays were performed as described previously [18]. Briefly, 96-welled flat-bottomed polystyrene microtiter plates (Nunc-immunoplate, USA) were coated overnight at 4 °C with 0.1 mL of coating buffer containing varying amounts of type IV collagen, laminin, fibronectin (0–200 ng/well) individually. In experiments involving cytoadherence to CS-C and HS, wells were priorly coated with poly-L-lysine (400 ng/well) followed by coating with CS-C and HS (0–200 ng) overnight [19]. The wells were then washed thrice with PBS to remove unbound ECM components. Then the wells were blocked with 2% BSA in PBS for 2 h to prevent the non-specific binding. Wells were washed thrice with PBS and 1×10^8 erythrocytes in 0.1 mL were added and incubated overnight at 4 °C. After the incubation, wells were subjected to gentle washing with saline containing $CaCl_2$ to remove unbound erythrocytes.

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