



Human Serum CRP – Rich Exosomes Fraction Increases the Activity of Macrophage Paraoxonase 2 (Pon2)

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Abstract

C-Reactive Protein (CRP) is a sensitive systemic marker of inflammation and was identified as a biomarker of cardiovascular diseases. Pathogenesis of atherosclerosis, an inflammatory disease, involves upregulation of oxidative mechanisms and paraoxonases which exhibit antioxidative activities have been shown to inhibit Atherogenesis. Extracellular vesicles such as exosomes and micro particles serve as containers of biological information on various pathophysiological settings. The goal of the present study was to analyze the possible presence of CRP in micro particles or in exosomes fractions isolated from serum samples and the possible effects of the serum CRP-rich, MPs or exosomes fractions on macrophage antioxidative properties such as PON2 activity. Serum exosomes, but not micro particles, exhibited high concentration of the inflammatory marker CRP. Moreover, serum exosome-rich fraction was shown to significantly increase the activity of macrophage PON2, an anti-atherogenic and antioxidative enzyme. In conclusion, the secretion of CRP through exosomes could lead to new directions in understanding the mechanism of action of CRP in inflammatory processes and serum exosomes could have anti-atherogenic and anti-bacterial properties.

Background

C-Reactive Protein (CRP) is released from hepatocytes to the serum during acute-phase response, and is a sensitive systemic marker for inflammation and for tissue damage [1]. CRP also has significant pro-inflammatory effects and is able to trigger complement activation, as a result of infection, inflammation, ischemia, or other pathologies [2]. Moreover, CRP was identified as a biomarker of cardiovascular diseases [3] and could be an active component of the inflammatory cascade during Atherogenesis [4]. Blood serum micro particles (MPs) are a heterogenous population of small vesicles, with a diameter of 100–1000 nm that is shaded from a large number of various cells [5]. Apart from MPs, blood contains also smaller membranous-derived vesicles (40–100 nm) named exosomes. Exosomes contains cell-specific collection of proteins, lipids, and genetic material [6]. Exosomes have pleiotropic biological functions, including immune response, antigen presentation, intracellular communication, and the transfer of RNA and proteins [7]. Arterial inflammation represents a key feature determining the course of Atherogenesis and oxidative stress is a key event in early atherosclerosis. PON2, an hydrolytic enzyme present in macrophages of the arterial wall was shown to protect cells against oxidative damage [8]. The goal of the present study was to analyze the possible presence of CRP in the MPs or in exosomes fractions isolated from serum samples and the possible effects of the serum CRP-rich, MPs or exosomes fractions on macrophage oxidative stress, cellular cholesterol efflux and PON2 activity, processes which are all involved in the pathogenesis of atherosclerosis.

Methods

Microparticles and Exosomes Isolation

Five pools of serum samples with high CRP content (>100mg/L) were used in this study. Sera samples were centrifuged at 19,000×g for 60 min at 4°C and the supernatant represented the MPs-free fractions. The MPs-free fractions were then collected and loaded on top of a 40% sucrose cushion and centrifuged at 100,000×g for 2.5 h. Due to their density, exosomes entered the sucrose cushion. The sucrose solution was harvested, diluted with PBS, and centrifuged for 2.5 h at 100,000×g to pellet the exosomes. Four different fractions were obtained: Microparticles (MPs) and exosomes-free fraction; MPs enriched fraction (contained also part of exosomes); exosomes-free fraction and exosomes-rich fraction. The obtained fractions were analyzed for their CRP content as well

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Table 1: CRP concentration levels and effects on macrophage PON2 activity of MPs and exosomes rich fractions.

Sample Number	CRP Concentration (mg/L)			
	Microparticles- free fraction (2)	Microparticles -enriched fraction (3)	Exosomes free MPs free fraction (4)	Exosomes enriched fraction (5)
1	181	181	6	54 (x9)
2	229	231	9	63 (x7)
3	194	207	8	61 (x8)
4	159	166	14	73 (x5)
5	160	163	7	39 (x6)
Macrophage PON2 Lactonase activity (U/cell protein)				
5	0.086±0.003	0.104±0.006	0.105±0.003	0.128±0.004(*)

Fractions obtained serum samples (Microparticles-free fraction, Microparticles-enriched fraction, Exosomes-free MPs free fraction, Exosomes enriched fraction) were analyzed for their CRP content as well as ability to modulate macrophage PON2 activity. *: <0.05 versus fraction 4.

as macrophage atherogenic properties such as macrophage oxidative status, cholesterol efflux and paraoxonase 2 activity. In addition the exosomes-rich fraction was analyzed by electronic microscopy.

Serum CRP Analysis

Serum CRP was determined using a commercial kit (DF34) on the diagnostic analyzer Dimension RXL (Siemens Germany).

Electron Microscopy

The exosome pellet was resuspended in PBS fixed in 2% paraformaldehyde and loaded onto formvar carbon coated grids (Ted Pella Inc, Redding, USA). Next exosomes were immunostained with mouse anti- CRPs antibody or isotype control (Sigma-Aldrich, St Louis, MO, USA), followed by staining with a 10 nm gold-labelled anti-mouse secondary antibody (Sigma-Aldrich). The exosomes were subsequently fixed in 2.5% glutaraldehyde, washed, contrasted in 2% uranyl acetate and embedded in a mixture of uranyl acetate (0.8%) and methyl cellulose (0.13%) [9]. The preparations were examined in a LEO 912AB Omega electron microscope (Carl Zeiss NTS, Jena, Germany).

Macrophage Atherogenic Properties

For these experiments, J774-A1 macrophages cell line was used. Macrophage intracellular oxidative stress analysis in cells incubated with the different obtained fractions was performed by the DCFH assay [10]. The effect of the four obtained fractions on Cholesterol efflux from macrophages was measured following cell incubation with [3H]-labeled cholesterol (2 mCi/ml) for 1 h at 37°C [11].

Macrophage PON2 Lactonase Activity

The cells were grown in a 12 wells plate. After cell wash they were incubated with 1 ml of 1mM DHC in 50mM Tris HCl, pH 8.0 +1mM CaCl₂. Initial rates of hydrolysis were determined spectrophotometrically at 270nm after 10 minutes incubation. Nonenzymatic hydrolysis of DHC was subtracted from the total rate of hydrolysis. One unit of lactonase activity equals 1µmol of DHC hydrolyzed/min/mL [12]. The cells' protein content was measured by the Lowry assay.

Results

Four fractions were isolated from serum samples with high CRP levels: Microparticles (MPs) and exosomes- free fraction; MPs enriched fraction (contained also part of exosomes); exosomes-free fraction and exosomes-rich fraction. The exosomes rich fraction was first analyzed by electron microscopy to assess the CRP content of the fraction. Specific staining for CRP revealed that the exosomes particles

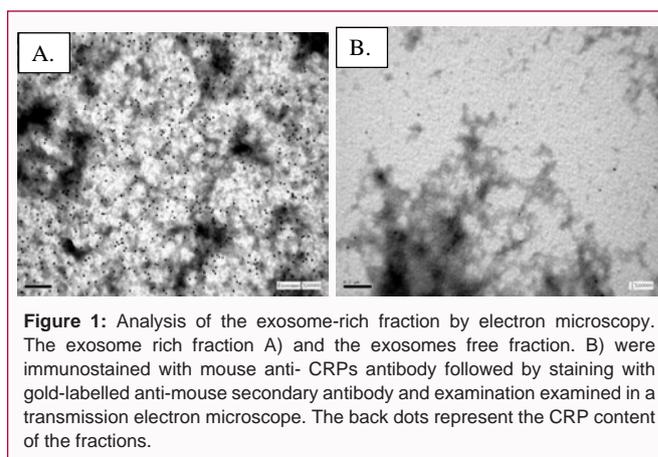


Figure 1: Analysis of the exosome-rich fraction by electron microscopy. The exosome rich fraction A) and the exosomes free fraction. B) were immunostained with mouse anti- CRPs antibody followed by staining with gold-labelled anti-mouse secondary antibody and examination examined in a transmission electron microscope. The back dots represent the CRP content of the fractions.

contained a substantial amount of CRP (Figure 1A) whereas the exosomes free fraction did not contain CRP (Figure 1B). These results were further assessed in quantitative analyses of five serum different samples with high CRP levels (over 100 mg/l). When measuring CRP in the different fractions obtained from the serum samples, there were no significant differences in CRP concentration between the MPs free fraction and the MPs-enriched fraction. However the exosomes-rich fraction revealed up to 9 fold in CRP concentration in comparison to MPs and exosomes free fraction (Table 1). The effects of CRP - rich serum MPs or exosomes on serum lipids peroxidation and on serum PON1 activity, as well as on, macrophage oxidative stress, PON2 activity, and cellular cholesterol efflux, were then analyzed. There were no significant effects of serum MPs or exosomes on serum oxidation or on PON1 activity, as well as on macrophage oxidation or on macrophage cholesterol efflux (data not shown), but macrophage PON2 activity was significantly increased following macrophage exposure to exosomes isolated from high CRP serum samples, in comparison to exposure to exosomes-free fraction (Table 1).

Discussion

Serum exosomes, but not MPs, exhibited high concentration of the inflammatory marker CRP. Moreover, serum exosome-rich fraction was shown to significantly increase the activity of macrophage PON2, an anti-atherogenic and antioxidative enzyme. CRP is a member of the class of acute-phase reactants, as its levels rise dramatically in acute inflammation and inflammatory processes [2]. Its major role resides in assisting the complement system by binding to foreign and damaged cells and enhances phagocytosis by macrophages (opsonin-mediated phagocytosis), which express

a receptor for CRP [4]. CRP is mainly secreted from hepatocytes to the circulation following infection. However there is no data on the mechanisms of secretion of CRP and whether vesicles (microparticles or exosomes) are involved in this mechanism of excretion. In this study, we have shown that a substantial fraction of CRP in the serum was measured in the exosomes-rich fraction, whereas no differences could be found in CRP concentration between the MPs rich fraction and the MPs free fraction, thus illustrating that CRP is secreted to the serum through exosomes but not through microparticles. Exosomes have been previously implicated in the packaging transport and protection of biomarkers involved in atherosclerosis [13]. Moreover Microvesicles (exosomes and microparticles) were recently identified as cell-cell messengers in cardiovascular diseases [14]. When evaluating the possible effect of the different fractions obtained from serum samples on processes involved in the pathogenesis of atherosclerosis, an inflammatory disease, serum CRP-rich exosomes was shown to significantly increase the activity of macrophage PON2, a most potent cellular anti oxidative, anti atherosclerotic lactonase enzyme. Hydrolytic activity against acyl homoserine lactones, important bacterial quorum-sensing mediators, suggests that PON2 may also play a role in defense responses to pathogenic bacteria [8]. We hypothesize that in this way CRP-rich exosomes may play important role in protecting cells from oxidative stress and in early defense system against infections. In conclusion, since exosomes exhibit pleiotropic biologic activities within the immune system, the secretion of CRP through exosomes could lead to new directions in understanding the mechanism of action of CRP in inflammatory processes. Moreover, these results imply that serum exosomes could have anti-atherogenic and anti-bacterial properties.

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