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RESEARCH ARTICLE

Binding of C1q to Galectin-3 Binding Protein on Microvesicles Released by Mononuclear Cells from Patients with Systemic Lupus Erythematosus

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ABSTRACT

Background: Generation of galectin-3 binding protein (G3BP)-expressing microvesicles can be induced in-vitro by Toll-like receptor 9 ligation in mononuclear cells. Microvesicles co-expressing G3BP and double-stranded DNA are associated with active lupus nephritis. However, whether the microvesicular G3BP mainly deposits from circulation or is endogenously derived is unknown. In this study, we aim to delineate the origin of G3BP on in-vitro generated microvesicles by using serum as a source of native G3BP.

Methods: G3BP-expressing microvesicles, generated by stimulation of systemic lupus erythematosus patient-derived mononuclear cells with the Toll-like receptor 9-agonist ODN2395, were incubated with normal human serum, heat-inactivated human serum, recombinant human C1q or human albumin. The expression of G3BP by microvesicles was examined by flow cytometry, and the binding of soluble recombinant human C1q to recombinant human G3BP was investigated by ELISA.

Results: Approximately half of the microvesicles released from mononuclear cells expressed G3BP. Surprisingly, the staining was abrogated by incubation of the microvesicles with normal human serum, while incubation with heat-inactivated human serum did not have a similar effect. Reasoning that C1q might be the heat-labile factor blocking access of our G3BP antibody detection system, we incubated microvesicles with recombinant human C1q, which on average inhibited the detectable proportion of G3BP-bearing microvesicles by 87%. Soluble recombinant human C1q bound to immobilized recombinant human G3BP in a dose-dependent manner.

Conclusion: Our data suggest that soluble C1q binds to G3BP on Toll-like receptor 9-induced microvesicles released from systemic lupus erythematosus patient-derived mononuclear cells. This interaction may exacerbate inflammation in systemic lupus erythematosus but may also serve as a general mechanism for the appropriate clearance of these potentially pathogenic factors.

Keywords: microvesicles, C1q, complement, clearance, Toll-like receptor 9, peripheral blood mononuclear cells, galectin-3 binding protein, systemic lupus erythematosus

Background

Microvesicles (MVs) are extracellular vesicles that range in size from 0.1-1 µm and are shed by most cells, especially during activation and apoptosis [1,2,3,4]. MVs are thought to be important in cellular cross-talk, in general, and in the innate and adaptive immune system both in health and disease [5]. However, the more specific interactions, their clearance and functional roles remain elusive.

Recently, overabundance of the type 1 interferon (IFN)-inducible scavenger receptor galectin-3 binding protein (G3BP) on MVs was discovered as a distinct feature of circulating MVs in autoimmune and thromboembolic diseases [6]. These observations were primarily made in studies of patients with systemic lupus erythematosus (SLE), where MVs are putative major sources of extracellular autoantigens. In SLE patients, the MV-profile deviates markedly from that of normal and disease controls, with increased numbers of circulating MVs bearing G3BP [7], apoptosis-modified chromatin [8], or surface-bound immunoglobulin G (IgG) [9]. Moreover, MVs from SLE patients carry increased amounts of C1q on their surface [9]. These findings are in line with proteome analyses showing an increased content of G3BP, IgG, and C1q in MV-isolates from patient plasma [10,11]. Proteome studies of patients with deep venous thrombosis also identified G3BP as one of few overabundant proteins associated with MVs [12]. This led to the notion that G3BP on MVs could be a general marker of abnormal MVs arising during cell activation or death.

G3BP belongs to the scavenger receptor cysteine rich (SRCR) superfamily; however, its targets and biological functions are poorly explored. G3BP have been shown to interact with galectin-3 and -1, integrins, and proteins such as fibronectin often found in the glomerular basement membrane [13,14]. Accordingly, such binding properties of G3BP combined with accessible autoantigens and C1q on MVs could hold important pathophysiological implications for MVs in SLE and in general [15,16,17,18]. These include complement activation, modulation of MV-cell and MV-matrix interactions e.g., adhesion to the glomerular endothelium and basement membrane, as well as deposition of immune complex (IC)-carrying MVs in the glomeruli. Uncovering these implications is highly warranted and may shed new light on MVs, G3BP, and C1q.

In search for the source of G3BP-expressing MVs, we found that stimulation of mononuclear cells (MNCs) through Toll-like receptor 9 (TLR9) induced the release of MVs bearing G3BP and double-stranded DNA (dsDNA) [19]. Further, we showed that this inducibility was more pronounced in MNCs from patients with active lupus nephritis (LN) compared to healthy donors and patients without active LN [19]. However, whether the majority of the microvesicular G3BP is exogenously or endogenously derived is unknown.

In this study, we explore the binding of native G3BP in serum, and other extracellular serum molecules to G3BP-expressing MVs, using our model of TLR9-induced MVs from SLE MNCs [20].

Materials and Methods

CELLS AND SERUM

Nine SLE patients were included from both the inpatient and outpatient rheumatology clinic at Copenhagen University Hospital, Rigshospitalet, fulfilled the American College of Rheumatology (ACR) classification criteria for SLE [21], and were also included in our previous study [20]. The group consisted of eight females and one male, and five of these had active LN at study inclusion. Moreover, the median age was 33 (range 23-72) and the median SLEDAI score was 6 (range 0-26). The study was approved by the Scientific-Ethical Committee of the Capital Region of Denmark (protocol no. H-15004075). MNCs from the patients were isolated by density gradient centrifugation and cryopreserved as previously described [19]. Serum from one healthy donor was included and was heat-inactivated for some of the experiments by heating to 56°C for 30 min.

STIMULATION OF MNCS

The cryopreserved MNCs were thawed at room temperature (RT), washed and resuspended in sterile medium consisting of RPMI-1640 GlutaMAX medium (Lonza, Basel, Schweiz) supplemented with 20% heat-inactivated fetal calf serum (Sigma-Aldrich, St. Louis, USA) and 0.1% gentamicin (BI, Kibbutz Beit Haemek, Israel). The cells were counted, and their viability was confirmed using the NucleoCounter® NC-100™ system (ChemoMetec, Allerød, Denmark) according to the manufacturer's instructions. They were then plated into 48-well plates with *Nunclon*® delta *surface* (Nunc) at approximately 600,000 MNCs per well and rested for 30 min at 37°C and 5% CO_2 before incubation for 24 hours at 37°C and 5% CO_2 with the TLR9-agonist ODN2395 (Invivogen, San Diego, USA) (6 µg/mL), which we previously identified as a stimulus for the release of G3BP-bearing MVs [19].

PREPARATION OF CULTURE SUPERNATANTS

Following incubation, the plates were left at RT for 15 min. Adhered cells were gently loosened, and the cell suspensions were then transferred to FACS tubes (Corning, New York City, USA) and were

centrifuged at 458 x g for 10 min at 24°C to pellet MNCs. The cell-free supernatants were subsequently harvested and aliquoted into cryotubes and were then snap-frozen in liquid nitrogen. Samples were stored at -80°C until analysis.

MV-BINDING EXPERIMENTS

The frozen cell-free supernatants were thawed at RT and transferred to Eppendorf tubes (Corning) (65 µL in each) and the volume was adjusted to 75 µL with PBS (Thermo Fisher Scientific). The samples were then incubated with the following: 25 µL PBS (non-treated control); 25 µL normal human serum (NHS) or 25 µL heat-inactivated human serum (HIS); 25 µL rhC1q (Abcam, Cambridge, UK) (100 µg/mL in PBS) or 25 µL human albumin (100 µg/mL in PBS) (SSI, Copenhagen, Denmark) for 1 hour at RT. For the rhC1q incubations, vehicle (LAL water) was added to the corresponding non-treated controls. Moreover, the serum was MV-depleted before use by ultracentrifugation at 20,000 x g for 30 min at 21°C. The supernatant was aspired down to 50 µL and transferred to new tubes. As a negative control, samples with MV-depleted serum alone (NHS and HIS) were included in parallel.

ISOLATION OF MVS FROM CULTURE SUPERNATANTS

After incubation with serum, rhC1q, or controls, the samples were centrifuged at 20,000 x g for 30 min at 21°C to pellet MVs. The supernatant was subsequently aspired down to 25 µL and discarded, and MVs were resuspended in 75 µL PBS that had been filtered through a 0.2 µm filter (Sartorius, Göttingen, Germany), followed by another ultracentrifugation step. The supernatant was aspirated as before and discarded, and MVs were resuspended in 70 µL filtered PBS to a final volume of 95 µL (MV-isolate).

STAINING OF MVS

Calcein was used as a fluorescent marker of MVs as previously described [19,22]. Fcγ-receptors on isolated MVs were blocked by adding 5 µL Fc-blocker (BD) to MV-isolates for 15 min at RT. After this incubation, 10 µL MV-isolate was pipetted into 27.5 µL filtered PBS in FACS tubes followed by addition of 1 µg/mL mouse anti-human G3BP antibody of IgG2b isotype (Proteintech, Manchester, UK), 0.5 µg/mL APC-conjugated goat anti-mouse IgG2b antibody (Southern Biotech, Birmingham, USA), and 2.5 µg/mL calcein acetoxymethyl ester (calcein-AM) (Sigma-Aldrich). Some samples were stained for both G3BP and dsDNA. In these cases, 10 µL MV-isolate was pipetted into 17.5 µL filtered PBS in FACS tubes, and 0.5 µg/mL mouse anti-dsDNA antibody of IgG2a isotype (SSI) was then added together with the anti-human G3BP antibody (Proteintech) (1 µg/mL), followed by the addition of 0.5 µg/mL APC-conjugated goat anti-mouse IgG2b antibody (Southern Biotech), 0.5 µg/mL BV510-conjugated rat anti-mouse IgG2a antibody (BD), and 2.5 µg/mL calcein-AM (Sigma-Aldrich), all in filtered PBS and yielding a final volume of 50 µL. Unstained controls and isotype controls were included for some of the samples. The tubes were incubated for 1 hour at RT in the dark. After incubation, ~9,870 TruCount beads (BD) were added to each tube, and the volume was adjusted to 300 µL with filtered PBS before acquisition on a FACSCanto II flow cytometer (BD) in logarithmic mode, at low flowrate, and with lowest SSC threshold (=200).

INDIRECT ELISA FOR C1Q BOUND TO G3BP

The rhG3BP (R&D systems, Minneapolis, USA) was diluted at 1 µg/mL in bicarbonate buffer (pH 9.6) and coated onto 96-well immobilizer amino plates in duplicates (Nunc, Roskilde, Denmark). As a negative control, wells were coated with bicarbonate buffer alone to measure background binding. The plates were left overnight at 4°C. The liquid was discarded, and the wells were washed three times in phosphate-buffered saline supplemented with 0.05% Tween (PBS-T) followed by blocking for 30 min with 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, USA) dissolved in PBS-T. The liquid was discarded, and the wells were then incubated for 1 hour with 100 µL serial dilutions of rhC1q (Abcam) (5-1.25 µg/mL in PBS-T) or with PBS-T containing vehicle only. The wells were washed as before followed by incubation with mouse anti-C1q antibody (Bio-Rad, Hercules, USA) (1 µg/mL in PBS-T) for 1 hour. Following another three washes, the wells were incubated for 1 hour with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) (1:1000 in PBS-T). The wells were washed again, and the plates were developed by incubating the wells with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution (Thermo Fisher Scientific, Waltham, USA) for 15 min in the dark, followed by the addition of stop solution (H_2SO^4) (0.5 M final) to each well. The plates were subsequently read on a microplate reader at 450 nm with a reference wavelength of 650 nm.

STATISTICAL ANALYSIS

The Wilcoxon signed-rank test (two-tailed) was used for two-group comparisons of the paired continuous data and were carried out using GraphPad Prism software 8 (GraphPad Software Inc, San Diego, USA). P *values* less than 0.05 were considered statistically significant.

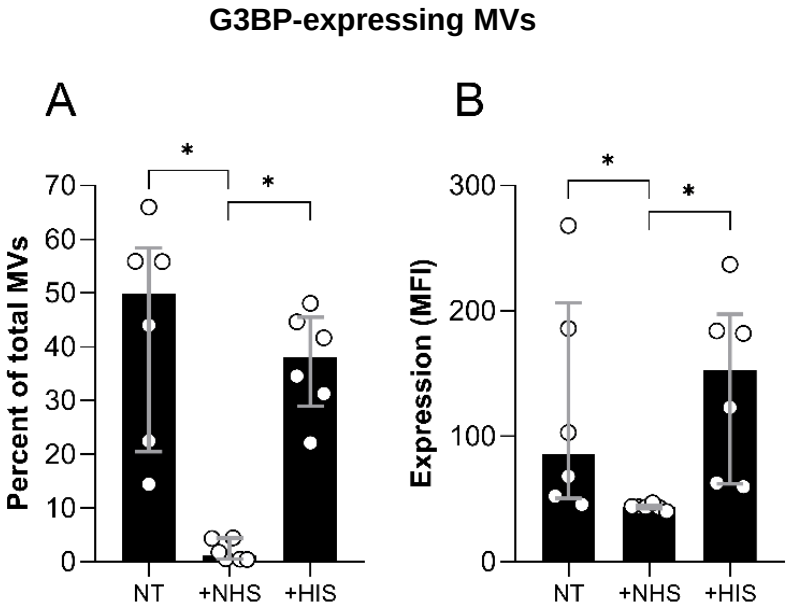

Figure 1. Inhibition of G3BP staining by serum. MNCs from six SLE patients were stimulated with the TLR9-agonist ODN2395 for 24 hours. (A) The proportion of detectable G3BP-expressing MVs in the culture supernatants after incubation with normal human serum (+NHS), heat-inactivated human serum (+HIS) or without serum (NT). (B) The corresponding G3BP-derived median fluorescence intensity (MFI) values. *P <0.05. Columns and error bars represent median values and interquartile range.

Results

BINDING OF SERUM FACTORS TO G3BP ON IN-VITRO-GENERATED MVS

Stimulation of MNCs from nine SLE patients with the TLR9-agonist ODN2395 resulted in release of MVs, of which on average 45% expressed G3BP, as measured by flow cytometry (Figure 1).

To study the deposition of G3BP on MVs under near-physiological conditions, we incubated the MVs with NHS. Surprisingly, however, this on average reduced the proportion of MVs with detectable G3BP to 5.5% (Figure 1A, B), suggesting that factors contained in the serum blocked the access of the primary antibody to G3BP or of the secondary fluorescence-conjugated antibody to the former. In contrast, incubation of the MV-preparation with NHS did not affect the proportion of MVs bearing DNA, as detected with an anti-DNA antibody (data not shown).

The binding of the anti-G3BP antibody to MVs was only inhibited to a small extent by heat-inactivated serum (+HIS in Figure 1), suggesting that the serum factor(s) blocking the access of the anti-G3BP antibody was heat-labile.

BINDING OF SOLUBLE C1Q TO IN-VITRO-GENERATED MVS EXPRESSING G3BP

Since C1q is known to be “sticky” and heat-labile [23], we suspected that C1q was the factor that blocked access of the anti-G3BP antibody and incubated the MV-containing culture supernatants with rhC1q, resulting in a blockade of G3BP similar to that observed in the presence of NHS (Figure 2). This effect was not due to unspecific protein-protein interaction in general, since exposure of the MVs to human albumin, at a similar concentration to that of C1q, had no significant effect (+HA in Figure 2).

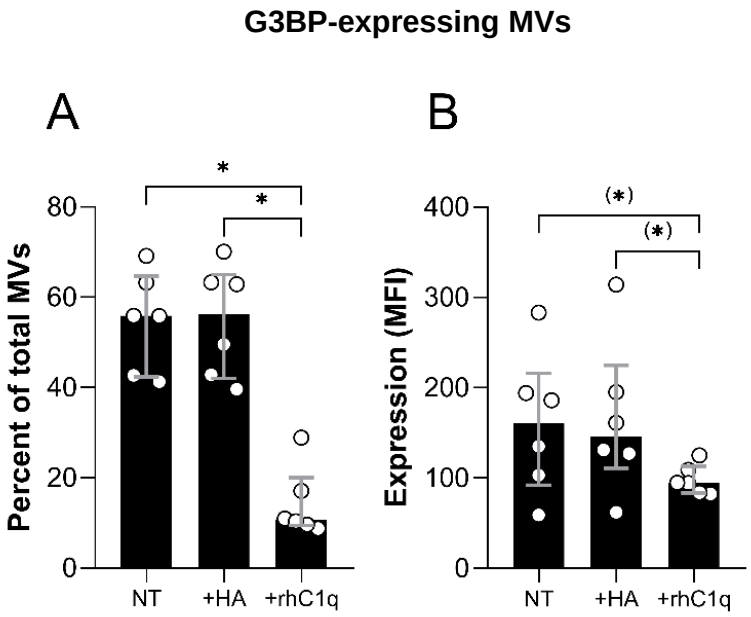

Figure 2. Inhibition of G3BP staining by soluble C1q. MNCs from six SLE patients were stimulated with the TLR9-agonist ODN2395 for 24 hours. (A) The proportion of detectable G3BP-expressing MVs in the culture supernatants after incubation without (NT) or with rhC1q (100 µg/mL) or human albumin (+HA) (100 µg/mL). (B) The corresponding G3BP-derived median fluorescence intensity (MFI) values. (*) P <0.1. *P <0.05. Columns and error bars represent median values and interquartile range.

BINDING OF SOLUBLE C1Q TO IMMOBILIZED G3BP

To confirm the ability of C1q to bind to G3BP, soluble rhC1q was added to immobilized rhG3BP and assessed for binding by ELISA and proved to bind to rhG3BP in a dose-dependent manner (Figure 3).

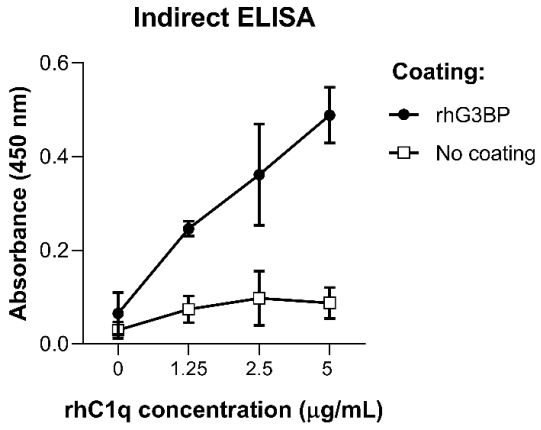

Figure 3. Binding of soluble C1q to G3BP. The binding of soluble C1q to immobilized G3BP was evaluated with an in-house ELISA (n=3), using 96-well microplates coated (closed circles) or not coated (open squares) with rhG3BP and incubated with various concentrations of soluble rhC1q. The symbols and whiskers represent the mean absorbance and standard deviation, respectively.

Discussion

Tagging with C1q plays an important role in clearance of apoptotic material and soluble immune complexes from the circulation [24,25,26]. This study suggests for the first time that C1q also binds to microvesicular G3BP.

We observed that incubation of the MVs with NHS or rhC1q, but not with HIS, significantly inhibited the binding of our G3BP antibody detection system, which strongly suggested that C1q was responsible for the inhibition. This notion was confirmed by the finding of a direct interaction between rhC1q and rhG3BP and may further imply that flow cytometry severely underestimates the plasma concentration of G3BP-expressing MVs.

A number of cell surface proteins expressed by various cell types, including macrophages, have been shown to bind C1q [27], and may directly be involved in clearance of C1q-bearing MVs from the bloodstream. Moreover, activation of the complement system via the classical pathway, of which binding of C1q is the initial event, may lead to deposition of C3 fragments that further enhance clearance by phagocytic cells. Thus, MVs expressing significant amounts of G3BP and, in turn, capturing C1q are marked for accelerated removal.

The present study focuses on MVs released from SLE patient-derived MNCs, but the G3BP-C1q interaction likely serves as a general opsonizing strategy for maintaining homeostasis rather than being confined to an SLE setting. The discoveries made here could provide new clues to the role of MVs in IC-deposition, activation of complement, and to clearance of MVs in SLE and in general.

How ICs arise and deposit in the glomeruli is a key unanswered question in LN pathogenesis. We suspect G3BP-bearing MVs of having a pathogenic role as carriers of autoantigens and ICs in SLE, and to deposit in the glomeruli and induce inflammation through aberrant local complement activation [17]. This concept is particularly substantiated based on the higher concentration of these vesicles in the blood of SLE patients, and their glomerulophilic properties and extracellular accessible DNA content [7,14,19]. Moreover, their ability to fix complement as demonstrated in this study may render G3BP-bearing MVs potent glomerular C1q-fixatives. In support of this, both G3BP [7] and C1q [28] colocalize with deposited ICs in electron dense deposits in kidney biopsies from LN patients.

In light of our observations, the reduced serum C1q levels in SLE patients and particularly in patients with LN [29] may predispose to diminished clearance, more available microvesicular G3BP, and consequently, peripheral abundancy and increased glomerular deposition of G3BP-bearing MVs, not least in a TLR9-ligand charged environment. Furthermore, the SLE-related deficiencies of complement receptor type 1 (CR1) [30] may add to this picture. CR1 recognizes the opsonins C3b and C4b, which are likely deposited on MVs as a consequence of C1q-binding, and is vital for the removal of ICs and presumably also MVs from the circulation [30,31,32,33]. Corroborating this notion, Gasser et al. demonstrated that MVs released by neutrophils adhere to erythrocytes in the presence of complement [31]. Moreover, Winberg et al. found that erythrocytes restrict MV-induced activation of polymorphonuclear leukocytes in a complement-dependent manner [32].

Our study has limitations with respect to the small sample sizes, usage of MNCs from SLE patients only, and the indirect quality of our findings. Moreover, our flow cytometric MV-assay has an estimated lower size-limit of approximately 0.5 µm, and thus, a large part of the vesicular spectrum is neglected.

Conclusion

In conclusion, our results suggest that soluble C1q binds to G3BP on TLR9-induced MVs released from SLE patient-derived MNCs. This interaction may cause aberrant immune activation via tissue-deposited MVs and thereby exacerbate inflammation in SLE. In healthy individuals, it may serve as a general mechanism for the appropriate clearance of these potentially pathogenic factors.

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Author contributions

SJ, CTN, CHN created the research concept and supervised the research and the preparation of the manuscript. NSR, CTN, SJ, CHN designed the research. NSR conducted the experiments, analyzed data and wrote the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

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