COMPLEMENT IS ACTIVATED BY COAGULATION AND NOT BY CHITOSAN IN HUMAN WHOLE BLOOD AND PLASMA

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ABSTRACT

Chitosan is a biocompatible and adhesive polysaccharide scaffold composed of glucosamine and N-acetyl glucosamine. We previously showed that liquid chitosan buffered with glycerol phosphate (chitosan-GP) can be homogenously mixed into whole blood to form an in situ-solidifying clot implant that stimulates transient neutrophil chemotaxis during marrow-based regeneration of articular cartilage. Thrombin was recently shown to activate complement, a family of plasma proteases whose activation culminates in the cleavage of a C5 precursor to produce a potent neutrophil chemotactic factor, C5a. Solid chitosan particles were previously reported to activate complement in serum and plasma through the alternative pathway. We therefore tested the hypothesis that liquid chitosan-GP activates complement in human whole blood, plasma, and serum.

INTRODUCTION

Chitosan is a biocompatible and adhesive polysaccharide composed of D-glucosamine and N-acetyl-β-D-glucosamine that has therapeutic effects in clinical cartilage repair applications [1-3]. It was previously reported that solid chitosan particles and cross-linked chitosan membranes activate complement through the alternative pathway [4-6]. Complement activation is propagated through a cascade of plasma serine proteases, and represents the liquid phase innate immune reaction to trauma, biomaterial implants, and infection [7]. To date, four complement activation pathways have been identified: classical, lectin, alternative, and thrombin [7,8]. Activation of complement by the alternative pathway leads to cleavage of C3 followed by C5, culminating in the release the C5a anaphylatoxin peptide fragment. Human complement C5 is a 190 kDa protein that circulates as a disulfide-linked 115 kDa alpha and a 75 kDa beta chain. Proteolytic activation of C5 leads to the cleavage of a cationic 70 amino acid N-terminal peptide fragment C5a which can be further processed to des-Arg-C5a by a carboxypeptidase in the presence of yeast. The release of C5a provides a potent stimulus for neutrophil chemotaxis [9], while C3b and C5b fragments adsorbed onto biomaterial surfaces serve as opsonins or coatings that mediate recognition and clearance by phagocytes.

Chitosan is a polysaccharide polymer derivative of chitin, N-acetyl-β-D-glucosamine (GlcNA), which is also a structural component of yeast cell walls. Hot alkaline deacetylation of chitin under insoluble conditions leads to deacetylation of GlcNA, to produce a polymer with at least 50% glucosamine (Glc, also known as the Degree of Deacetylation, DDA) and variable distribution and retention of GlcNA residues. Chitosans from 70-100% DDA are acid-soluble, although cytocompatible, neutral chitosan solutions can be formed using a disodium β-glycerol phosphate buffer (GP) [10-12]. Chitosan with 80% DDA is biodegradable, retains block GlcNA residues and therefore has structural similarities with zymosan, a crude preparation of yeast cell walls. By comparison, 95% DDA chitosan is mainly composed of glucosamine. Zymosan is frequently used as a positive control for complement activation leading to the production of C5a fragment.

We previously reported that biodegradable chitosan-GP/blood implants stimulate revascularization and repair of surgically treated cartilage lesions [1,13-15]. *In situ* activation of complement by chitosan during acute stages of wound repair could potentially explain the sustained neutrophil chemotaxis we observe towards chitosan-GP/blood implants [16]. Using western blot analysis of C5a and C5 in non-reducing gels, we tested the hypothesis that isotonic solutions of chitosan activate complement in human whole blood, plasma, and serum resulting in the release of C5a chemotactic peptide.

METHODS

Materials

Chitosan (80% DDA, 150 kDa), solutions of filter-sterile 500 mM disodium glycerol-Phosphate/50 mM HCl pH 7.2 (GP), and 2.05% w/w autoclave-sterile solutions chitosan 80% DDA (M_n = 179 kDa) or 95% DDA (M_n =168 kDa) in HCl pH 5.6 were provided by BioSyntech (Laval, QC, Canada). All chitosans were

certified medical-grade (<500 EU/g, <0.2% protein/g, < 5 ppm heavy metals). Isotonic chitosan solutions were formed by combining 400 µL of 2.05% w/w chitosan-HCl with 100 µL GP or 100 µL 750 mM NaCl.

Coagulation and complement activation assays

Fresh peripheral venous blood was obtained from healthy consented non-fasting volunteers under institutional-approved ethics protocols. Unmodified whole blood was homogenously mixed with chitosan-GP at a 3:1 ratio blood:chitosan-GP, deposited in plastic sample cups of a thromboelastograph (TEG, Haemoscope, Niles, IL, USA), and allowed to coagulate for up to 75 minutes at 37ºC. Samples were removed at specific intervals, diluted 10-fold in ice-cold quench buffer (20 mM HEPES, 50 mM EDTA, 10 mM benzamidine, 150 mM NaCl, pH 7.4 with 100 µM PMSF), vortexed, and the serum cleared by centrifugation as previously described [14,17]. Whole unmodified blood was analyzed in parallel as a control. In other experiments, pooled normal human plasma (PrecisionBioLogic, Product CCN-15, Lot No. A1031, Dartmouth, NS, Canada) or pooled human serum (N=4 donors from cleared serum obtained from whole blood coagulated at room temperature for 4 hours) was homogenously mixed at a 3:1 v/v ratio with chitosan-GP. Samples were incubated for 1 hour at 37ºC, diluted 5-fold in quench buffer and analyzed by Western blot for C5 and C5a. Controls consisted in plasma incubated at 37ºC for 1 hour with isotonic GP buffer alone (negative control), Zymosan in isotonic NaCl or GP buffer (positive control), or thrombin at 10 U/mL (Sigma), incubated 1 hr 37 ºC.

Zymosan activated plasma (ZAP) or Zymosan activated serum (ZAS) was used as positive control, by incubating 1 volume of pooled human plasma (or serum) with 1 volume of 8 mg/mL Zymosan particles in 150 mM NaCl for 30 minutes at 37°C, to obtain the same final concentration as chitosan in chitosan-GP/blood mixtures. Zymosan A from Saccharomyces cerevisiea was obtained from Sigma (Oakville, ON).

SDS-PAGE running gels at 5% (C5), 15% or 17.5% (C5a and Platelet Factor 4, PF4) w/v acrylamide (19:1 ratio acrylamide:bisacrylamide) with a 4% w/v acrylamide stacking gel were used with kaleidoscope molecular weight markers from BioRad. All plasma and serum diluted 5- or 10-fold with quench buffer were combined with an equal volume of loading buffer without reducing agent and boiled for 5 minutes prior to loading on the gels. Purified human complement C5 from Sigma (Oakville, ON) was used at a concentration close to normal plasma levels, 80 µg/mL to provide a positive control for C5 precursor. Proteins were transferred to PVDF membranes by semi-dry transfer and immunoblotting was performed using a goat anti-human C5a antibody from R&D Systems (Product No. AF2037, Cedarlane, Hornby, ON), or goat anti-human PF4 clone (Product No. AF796, R&D Systems. Cedarlane, Hornby, ON). Secondary donkey anti-goat horseradish peroxidase antibody was from Jackson Immuno Labs (Montreal, QC). Chemi-luminescent detection was performed with Lumilight (Roche, Montreal, QC) or ECL-plus (BioRad) and Kodak X-ray film. Two molecular weights of C5a were obtained on the western blot results. The higher molecular weight was aligned with the 16.1 kDa molecular weight marker and the more abundant C5a product slightly underneath it. C5 bands were observed slightly beneath the 196.1 kDa molecular weight marker. Urea at 8 M concentration from J.T. Baker was used to dissociate proteins bound to washed pellets of chitosan or zymosan.

RESULTS

Complement is activated in coagulating whole blood and mixtures of chitosan-GP/blood

Whole blood and chitosan-GP/blood were allowed to coagulate during thromboelastography, a rheological-type setup used to evaluate clotting time and clot tensile strength, and placed in quench buffer to arrest proteolysis at different time intervals. In these unmodified blood samples, thrombin generation and coagulation occurred spontaneously after 15-30 minutes. Platelets (PF4) and complement (C5a) were co-activated in both chitosan-GP/blood and whole blood (Figure 1, representative results of donor 3, out of N=4 donors).

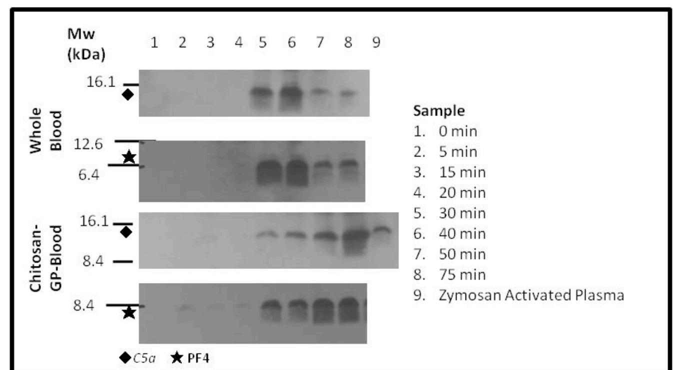

Figure 1. Analysis of complement and platelet activation in human whole blood and chitosan-GP/blood.

Complement is not activated in plasma homogenously mixed with liquid chitosan-GP

In human plasma alone, zymosan stimulated C5a production as expected (Fig. 2A, lanes 1 & 2).

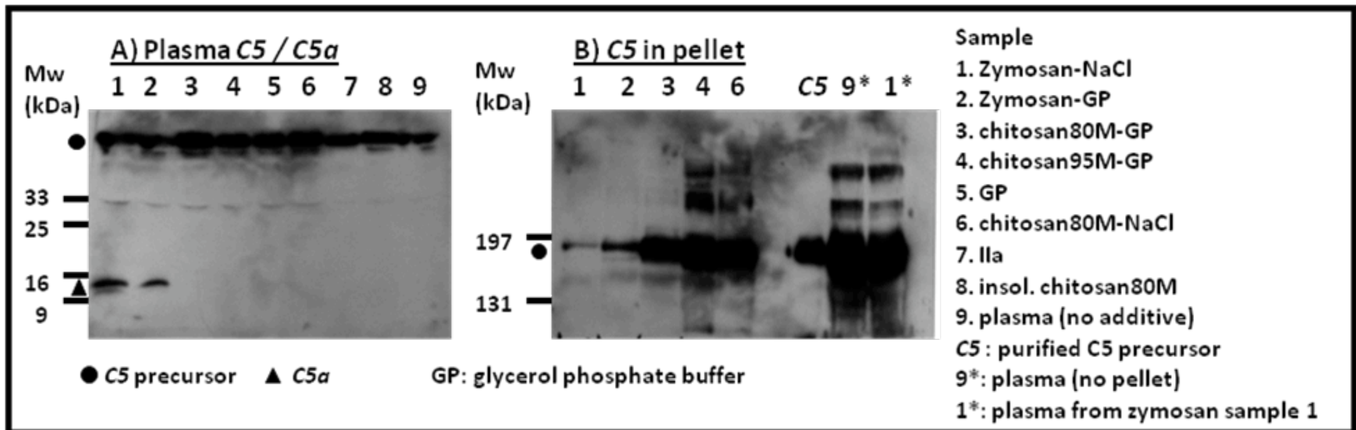

Figure 2. Complement activation in human plasma

However, chitosan-GP at the same mass/volume as Zymosan (4 mg/mL) failed to activate complement in human plasma (Fig. 2A, lanes 3, 4 & 6). The failure to activate complement was not due to GP buffer, as incubation of Zymosan-GP in plasma still activated complement, and incubation of chitosan-NaCl still failed to activate complement.

It was previously reported that purified thrombin at 0.5 to 15 units has C5 convertase activity in murine plasma [8]. However incubation of human plasma with purified human thrombin at 10 U/mL for 60 minutes at 37ºC did not yield a 10 – 15 kDa C5a fragment (Fig. 2A, lane 7). Analysis of C5 bound to the insoluble particulate fraction showed that the C5 precursor bound to zymosan (samples 1 & 2), 80% DDA chitosan (samples 3 & 6) and 95% DDA chitosan (sample 4). Comparatively less C5 was eluted from Zymosan than chitosan particles (Fig. 2B, compare lanes 1 & 2 with 3, 4 & 6). C5 eluted from insoluble chitosan particles co-migrated with purified C5, and C5 in several plasma samples (Fig. 2B, lanes 9* and 1*). However it is important to note that a quantitative comparison cannot be made between the C5 in plasma samples 9* and 1*, and C5 eluted from pellets as they were prepared differently.

Complement is not activated in serum homogenously mixed with liquid chitosan-GP

Chitosan-dependent complement activation was previously reported using either plasma, or serum [4,5,6]. We considered the possibility that our failure to detect chitosan-dependent C5a fragment generation was due to the use of plasma instead of serum. Moreover, given that C5a was activated by coagulation alone (Fig. 1, bottom panels), we considered that serum under various conditions traditionally used in research could produce variable amounts of C5a in the starting sample. We therefore prepared serum by 6 different methods. Sterile whole blood was clotted for 30 minutes in plastic vials, borosilicate glass vials, or Tiger vacutainer tubes at 37ºC or room temperature (RT), then held at 4ºC or RT for 4 hours.

Zymosan activated complement C5a in serum (Fig. 3A & B, lane 1), but GP buffer alone and chitosan-GP failed to generate C5a (Fig. 3A, lanes 2 & 3). Low levels of C5a were detected in all serum samples, migrating slightly above 16.1 kDa, variably accompanied by a ~15 kDa C5a fragment (Fig. 3B, lanes 4-9).

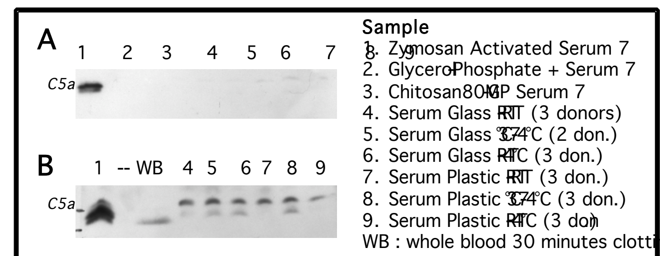

Figure 3. Analysis of C5a in serum generated in zymosan-serum, chitosan-GP/serum, and normal serum by different methods. (A) low-intensity chemiluminescence exposure and (B) ECL-plus high intensity exposure Western blot.

CONCLUSIONS

The first assay showed burst activation of platelets and C5a around 30-40 minutes in coagulating chitosan-GP/blood or whole blood. Our second assay showed that purified thrombin failed to generate abundant levels of C5a in citrated plasma after 1 hour of incubation at 37ºC. Our third assay showed that C5a generated during whole blood coagulation was still present but at low levels in serum samples 4 hours post-coagulation. These data suggest that C5a fragments are labile proteins that can be degraded in serum. Our collective data support previous data

indicating that C5a is activated through the thrombin pathway [8], and extend these results to demonstrate that C5a is co-activated with platelets.

The second assay showed that C5a was not activated in plasma by chitosan-GP. Our data furthermore showed C5 precursor strongly adsorbed to chitosan, and lesser amounts to zymosan. These data indicate that C5 can opsonize carbohydrate structures containing N-acetyl-β-D-glucosamine (zymosan and 80% DDA chitosan) or glucosamine (95% DDA chitosan), but that additional factors present in zymosan but not chitosan are required to trigger C5 convertase activity. Our data indicate that chitosan alone is unable to activate complement C5 through the alternative pathway, contrary to the conclusions drawn from previous studies [4,5,6]. These previous conclusions [4,5,6] were based on depletion of C5 and C3 and not actual appearance of the proteolytically activated forms of these two complement proteins. Our data indicated that chitosan can deplete C5 from plasma without generating the C5a fragment. The failure to observe C5a generation in mixtures of chitosan-GP/plasma or serum is not explained by irreversible tethering of C5a to chitosan, since this C5a peptide is cationic like chitosan, and was freely liberated to serum in mixtures of chitosan-GP/whole blood (Fig. 1).

This last conclusion brings a set of new questions. First, it is important to clarify the role of chitosan on C5a generation during clotting. Future experiments will determine whether chitosan can enhance the generation of C5a, protect C5a from degradation or influence burst of activation that is observed in whole blood clots. Secondly, the fate of C3 in hybrid chitosan-blood mixtures needs to be investigated to clarify the pathway used in complement activation during clotting of chitosan-GP/blood.

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REFERENCES

[1] Hoemann CD, Hurtig M, Rossomacha E, Sun J, Chevrier A, Shive MS, and Buschmann MD J Bone Joint Surg AM 2005;87A(12),2671-2686

[2] Shive MS, Hoemann CD, Restrepo A, Hurtig MB, Duval N, Ranger P, Stanish W, and Buschmann MD Operative Tech in Orthopaedics 2006;16(4):271-278

[3] Buschmann MD, Hoemann CD, Hurtig MB, and Shive MS Strategies in Cartilage Repair. Ed Riley J Williams 2006, Humana Press

[4] Minami S, Suzuki H, Okamoto Y, Fujinaga T, and Shigemasa Y. Carb Polymers 1998;36, 151-155

[5] Usami Y, Minami S, Okamoto Y, Matsuhashi A, and Shigemasa Y Carb Polymers 1997;32(2), 115-122

[6] Benesch J, and Tengvall P. Biomaterials 2002;23(12), 2561-2568

[7] Nilsson B, Ekdahl KN, Mollnes TE, and Lambris JD. Molecular Immunology 2007;44(1-3), 82-94

[8] Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, Lambris JD, Warner RL, Flierl MA, et al. Nature Medicine 2006;12(6), 682-687

[9] Frevert CW, Wong VA, Goodman RB, Goodwin R, and Martin TR J Immunol Methods 1998;213(1), 41-52

[10] Chenite A, Chaput C, Wang D, Combes C, Buschmann MD, Hoemann CD, et al Biomaterials 2000;21(21), 2155-2161

[11] Filion D, Lavertu M, and Buschmann MD Biomacromolecules 2007;8(10), 3224-3234

[12] Lavertu M, Filion D, and Buschmann MD. Biomacromolecules 2008;9(2), 640-650

[13] Hoemann CD, Chenite A, Sun J, Hurtig M, Serreqi A, Lu Z, et al J Biomed Mat Res-A 2007;83A(2), 521-529

[14] Marchand C, Rivard G, Sun J, and Hoemann CD. Osteoarthritis and Cartilage 15(Suppl 2), 2007;B147-189

[15] Iliescu M, Hoemann CD, Shive MS, Chenite A, and Buschmann MD Microscopy Research Technique 2007;71(3), 236-247

[16] Chevrier A, Hoemann CD, Sun J, and Buschmann MD. Osteoarthritis Cartilage 2007;15(3), 316-327

[17] Rivard GE, Brummel-Ziedins KE, Mann KG, Fan L, Hofer A, and Cohen E. J Thrombosis Haemostasis 2005;3(9), 2039-2043.