DISCUSSION

This flow cytometry study of platelets isolated and concentrated by the Cascade system demonstrate that platelets isolated by this method are intact, express the CD61 antigen and show no appreciable CD62P expression. This profile is consistent with an unactivated/unstimulated phenotype. In contrast, even brief exposure (5 min.) to bovine thrombin, at concentrations commonly employed in other commercial platelet separation systems, results in massive platelet activation (CD62P+), degranulation, and loss of integrity *(Figure 5B; intact platelets colored green)*. These data further support the premise that activation with exogenous thrombin results in rapid irreversible platelet activation and degranulation with subsequent release of growth factors into the aqueous environment. Platelets isolated with the Cascade system *(Figure 5A; intact platelets in green)* without exogenous thrombin, remain intact and retain their growth factor compliment. This allows a more effective, sustained release of growth factors to the wound site following PRFM application. These results, taken together with previous studies, reinforce the premise that the Cascade system, which does not require exogenous thrombin, provides enhanced tissue repair by isolation, concentration and preservation of autologous platelets in a dense fibrin matrix (PRFM). This PRFM is able to deliver a sustained release of concentrated autologous growth factors to the repair site over the space of days and weeks, compared to thrombin-activated PC preparations which release their growth factors within minutes and hours of application.

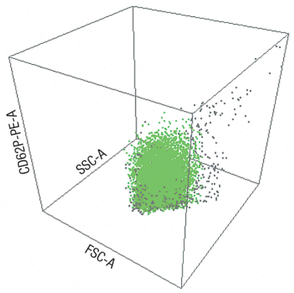

Figure 5A: CD62P-PE vs. Light Scatter Profile of Untreated Platelets

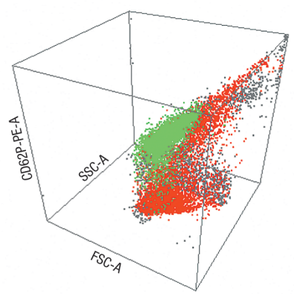

Figure 5B: CD62P-PE vs. Light Scatter Profile of Platelets Treated with Bovine Thrombin (1000U/mL)

REFERENCES

1. Ksander GA, et al. The effect of platelet release on wound healing in animal models. J. Am. Acad. Dermatol. 1990;22:781-791.
2. Carroll RJ, Arnoczky SP, Graham S, O'Connell SM. Characterization of autologous growth factors in Cascade® platelet-rich fibrin matrix (PRFM). Edison, New Jersey: Musculoskeletal Transplant Foundation. 2005.
3. Kevy SV, Jacobson MS. Comparison of methods for point of care preparation of autologous platelet gel. *JECT*. 2004;36:28-35.
4. Swift MJ, Lichtenberger FJ, Marsh C. Characterization of growth factors in platelet rich plasma. *Proc. Ortho. Res. Soc.* 2005.
5. Abrams C, Shattil SJ. Immunological detection of activated platelets in clinical disorders. *Thrombosis and Haemostasis.* 1991;65:467-473.
6. Abrams CS, Ellison N, Budzinski AZ, Shattil SJ. Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood.* 1990;75:128-138.
7. Jackson AL, Warner NL. Preparation, staining, and analysis by flow cytometry of peripheral blood leukocytes. In: Rose NR, Friedman H, Fahey JL, eds. *Manual of Clinical Laboratory Immunology.* 3rd ed. Washington, DC: American Society for Microbiology. 1986;226-235.
8. Modderman PW. Cluster report: CD61. In: Knapp W, Dörken B, Gilks WR, et al, eds. *Leucocyte Typing IV, White Cell Differentiation Antigens.* New York: Oxford University Press. 1989;1025.
9. Fijnheer R, Modderman PW, Veldman H, et al. Detection of platelet activation with monoclonal antibodies and flow cytometry. *Transfusion.* 1990;30:20-25.
10. Shattil SJ, Hoxie JA, Cunningham M, Brass L. Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. *J. Biol. Chem.* 1985;260:11107-11114.
11. Wong DA, Springer TA. CD61 (b3) cluster report. In: Schlossman SF, Boumsell L, Gilks W, et al, eds. *Leucocyte Typing V White Cell Differentiation Antigens.* New York. Oxford University Press. 1995;1664-1665.
12. Larsen E, Celi A, Gilbert GE, et al. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell.* 1989;59:305-312.
13. Ault KA, Rinder HM, Mitchell JG, Rinder CS, Lambrew CT, Hillman RS. Correlated measurement of platelet release and aggregation in whole blood. *Cytometry.* 1989;10:448-455.
14. Metzelaar MJ, Sixma JJ, Nieuwenhuis HK. Detection of platelet activation using activation-specific monoclonal antibodies. *Blood Cells.* 1990;16:85-96.
15. Diacovo T, Springer TA. CD62P (P-selectin) cluster report. In: Schlossman SF, Boumsell L, Gilks W, et al, eds. *Leucocyte Typing V: White Cell Differentiation Antigens.* New York, NY. Oxford University Press. 1995;1500-1501.

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Flow Cytometric Characterization of CASCADE® Platelet-Rich Fibrin Matrix (PRFM): The Impact of Exogenous Thrombin on Platelet Concentrates (PC)

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INTRODUCTION

Platelet-derived growth factors (GF) have been shown to be critically involved in the early (bFGF, PDGF, IGF) and later stages (EGF, VEGF, TGF-b, IGF) of the healing process in bone, cartilage and soft tissue repair. A variety of methods have been employed to harness autologous platelet growth factors as an aid to optimize healing in a number of therapeutic arenas, including orthopaedics. Most of these methods produce platelet-rich plasma (PRP) by first isolating and concentrating the platelets from whole blood. However, the platelet concentrate (PC) is unstable and difficult to administer in a number of clinical applications. To produce a stable clot, calcium chloride together with an exogenous activator, such as thrombin, is used to activate the platelets and residual fibrin in the PC. Excess thrombin treatment is thought to result in irreversible platelet degranulation with loss of platelet integrity and immediate growth factor release.[1]

The CASCADE® Autologous Platelet System was developed with the goal of preserving platelets and their associated GF to facilitate optimal tissue repair. The system achieves this goal by employing a novel strategy of concentrating both platelets and fibrin in a dense cross-linked platelet-rich fibrin matrix (PRFM) *without the use of excess exogenous activators such as thrombin.* The lack of excess thrombin would tend to ensure platelet integrity during PRFM processing and allow a gradual release of GFs versus premature total release. Indeed, previous studies by our group have demonstrated a gradual release of the platelet growth factors over a seven day time course[2] indicating that the platelets remain functionally viable PRFM production in the Cascade system. In contrast, addition of exogenous thrombin (bovine, 1000U/mL) (BoThr) resulted in a rapid release of the total measured GF from the platelets.[3,4] The current study was designed to assess the effect of the Cascade system on the platelets with and without the addition of exogenous thrombin.

Flow cytometry, together with fluorescence immuno-cytochemistry, was used to determine the extent of platelet activation, degranulation and loss of physical integrity. Autologous platelets were isolated with the Cascade system, with and without the addition of exogenous BoThr. BoThr was chosen since it is the most common activator used to produce a PC. For flow cytometry, the platelets were suspended in solution and stained with fluorescently tagged monoclonal antibodies (MAbs) that recognize specific cell surface markers which distinguish between unactivated and activated platelets. The stained platelets were then analyzed on the laser-based flow cytometer. The flow cytometer is able to perform a variety of simultaneous measurements on each platelet as it passes through the machine. In this manner, the activation profile, size and density (granularity) of each platelet in the suspension can be rapidly measured and the effect of exogenous activators determined.

MATERIALS AND METHODS: TWO-COLOR FLOW CYTOMETRY OF PLATELET ACTIVATION

Design: The platelets isolated from the Cascade system are stained, fixed, and then analyzed on a BD™ LSR II brand flow cytometer (Becton Dickinson, San Jose, CA). With two-color analysis, one fluorescent color (MAb) can be used to detect platelets that bind an activation-independent, platelet-specific antibody.[5-7] The second fluorescent color/MAb can be used to simultaneously detect the binding of platelet-associated, activation-dependent antibodies. Thus, the combination of CD61-PerCP (red fluorescence, all platelets), and CD62P-PE (orange, activated platelets) MAbs represents a two-color assay that differentiates activated platelets from intact, unactivated platelets.[7]

CD61 recognizes a Mr 110-kdalton protein, also known as gpIIIa, the common b subunit (integrin b3-chain) of the gpIIb/IIIa complex and the vitronectin receptor (VNR). *The CD61 antigen is found on all normal resting and activated platelets*.[8-11] The CD62P antigen, *also known as platelet activation-dependent granule-external membrane* (PADGEM) *protein or granule membrane protein* (GMP-140), is a 140-kilodalton (kd) single chain polypeptide[12-15] *and is found on the surface of all activated platelets.*

Venous blood typically demonstrates three subpopulations of platelets by routine light scatter *(Figure 1A)*. The majority of the particles consist of single intact platelets. A second population, typically representing <5% of all particles, exhibits greater light scatter than single platelets and represents platelets associated with large white blood cells (WBCs).[5-7] A third population, representing 5% to 15% of the particles whose light scatter is lower than single intact platelets, includes platelet-derived micro particles with an average diameter of 0.1µm.[6] Figure 1B shows the side-angle scatter (SSC) vs. CD61-PerCP Fl. profile. The intact platelets are seen as a distinct single population (region R1).

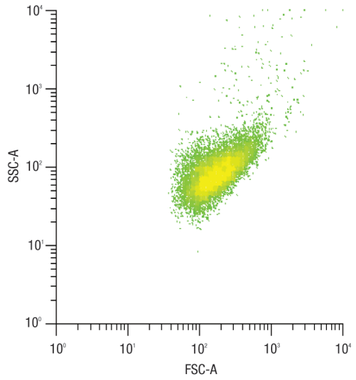

Figure 1A: Platelet Light Scatter Profile of Untreated Platelets

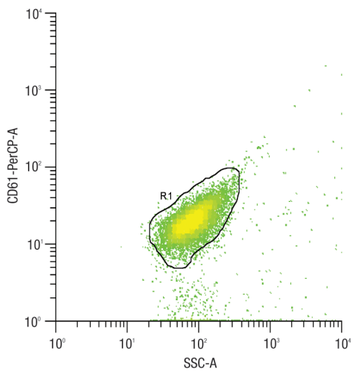

Figure 1B: SSC vs. CD61-PerCP-A Profile of Untreated Platelets

This sample was used to determine any degree of activation (baseline) prior to the addition of BoThr, as well as to identify intact platelets.

Processing of the PRP: The Cascade system (Cascade Medical Enterprises, Wayne, NJ) was used to produce 4.5mL of PRP from a 9cc blood draw. Replicate samples were drawn from two donors. The process involves a six-minute, 1100g spin in a separator tube.

MAb Staining: Immediately following centrifugation, 0.45mL of PRP was incubated with either an isotype control MAb (IgG1-PerCP or IgG1-PE) or CD61-PerCP and CD62P-PE following washing in Basal Medium Eagle (Invitrogen Corp., Grand Island, NY) supplemented with 0.35% bovine serum albumin (Sigma, St. Louis, MO) to inhibit non-specific background MAb binding. Each sample was then mixed and incubated at room temperature (20° to 25°C) for 5 minutes, washed and fixed in 1% para-formaldehyde and stored at 4°C in the dark for later analysis.

Exogenous Activation of Platelets in PRP: For the BoThr-treated groups, 0.45 mL of PRP was treated with 1000U/mL bovine thrombin (Sigma, St. Louis, MO), mixed, and incubated at room temperature (20° to 25°C) for 5 minutes prior to MAb staining as described above.

Flow Cytometry: Logarithmic signals were collected for all parameters measured. SSC vs. CD61-PerCP events were used to trigger acquisition and analysis gating *(R1, Figure 1B)*. Platelets stained with isotype controls were used to determine non-specific background fluorescence levels for both PerCP and PE signals *(Figure 2A, B; open curve histograms outlined in grey)*. Orange *(shown in red, Figure 2B)* versus red *(shown in green, Figure 2A)* fluorescence compensation was set using calibrated bead standards (B-D Calibrite™) and CD61-PerCP/IgG1-PE isotype stained, unstimulated platelets. For each run, 20,000 to 30,000 particles were collected. Data was collected as listmode files and analyzed using the WinList™ (Verity Software House) software package.

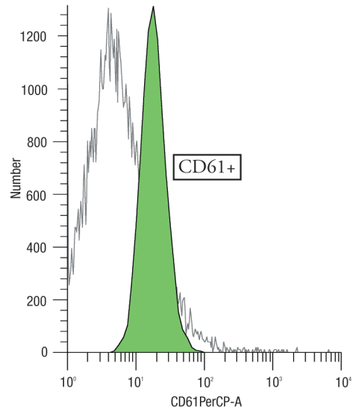

Figure 2A: CD61-PerCP Expression of Untreated Platelets

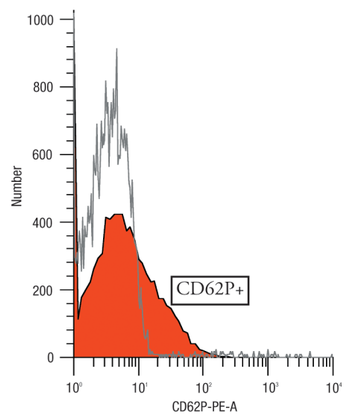

Figure 2B: CD62P-PE-A Expression of Untreated Platelets

RESULTS

Unactivated Platelets: Physical Integrity

Flow cytometry of platelets isolated from the Cascade system demonstrate that these platelets represent a single homogenous, physically intact population as measured by light scatter. Figure 1A shows the forward light scatter (FSC-A) versus side scatter (SSC-A) profile of unstained platelets isolated and concentrated using the Cascade system. FSC-A is the log of the forward light scatter and is a measure of the platelets size (cross-sectional area), SSC-A is the log of the 90° or side light scatter and is associated with platelet density or granularity. The platelets display a single cell population with uniform size (FSC-A) and density (SSC-A).

Figure 1B shows the SSC-A versus CD61-PerCP profile of platelets stained with CD61-PerCP and CD62P-PE. This two-parameter histogram shows that all the platelets within the single population (R1) stain positive for CD61 (>93%). However, looking at the single parameter histograms for CD61 (PerCP-A) and CD62P (PE-A) *(Figures 2A, B; CD61 positive populations in green, CD62P positive activated populations in red, isotype controls outlined in grey)* we see that these same platelets are negative for CD62P when compared to the isotype controls (95.7% CD61+; 4.6% CD62P+). These results are consistent with a single intact, unactivated population of platelets.

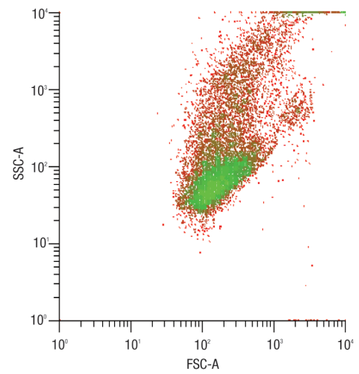

Figure 3A: Platelet Light Scatter Profile of Platelets Treated with Bovine Thrombin (1000U/mL, 5 min.)

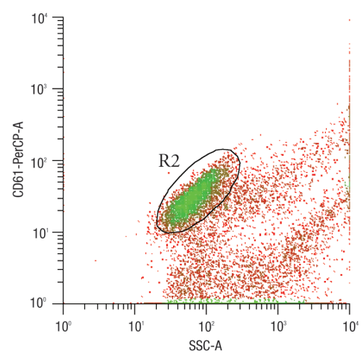

Figure 3B: SSC vs. CD61-PerCP-A Profile of Platelets Treated with Bovine Thrombin (1000U/mL, 5 min.)

Effect of Bovine Thrombin: Activation and Degranulation

Figure 3B shows the SSC-A versus CD61-PerCP profile of platelets exposed to 1000U/mL BoThr for 5 minutes (intact platelets are shown in green -R2, degranulated platelets are colored red). Marked changes in the SSC-A are seen for many of the platelets (>60% of the platelets are out of the singlet R2 gate). This effect can also be seen on the FSC-A versus SSC-A scattergram *(Figure 3A; intact platelets colored green, degranulated platelets colored red)* which shows many platelets with increased forward scatter as well as side light scatter. Major shifts are also noted in both the CD61 and CD62P profiles *(Figures 4A, B; CD61 and CD62P positive intact platelets shown in green, CD62P positive intact activated platelets in red, total fluorescence shown in grey and isotype controls outlined in grey)*. The CD61 positive count has dropped from 96% to 60% while the CD62P has increased from 5% to >60% for platelets within the singlet gate (compare Figure 2 with Figure 4). These results are consistent with significant platelet degranulation, loss of platelet integrity and the appearance of substantial numbers of platelet-associated micro-particles.

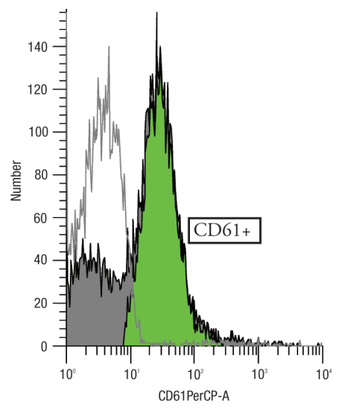

Figure 4A: CD61-PerCP Expression of Platelets Treated with Bovine Thrombin (1000U/mL)

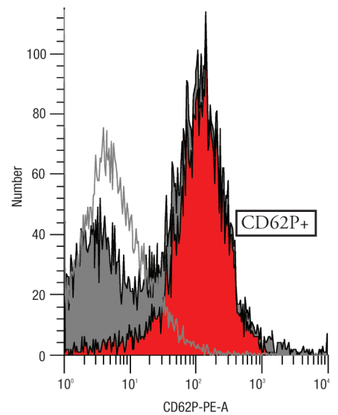

Figure 4B: CD62P-PE Expression of Platelets Treated with Bovine Thrombin (1000U/mL)