CHARACTERISATION OF SELECTIN-MEDIATED ADHESION USING SYNTHETIC GLYCOCONJUGATE LIGANDS AND SCINTILLATION PROXIMITY ASSAY (SPA)

Stephen M. Game[1*], P. Kalpana Rajapurohit[1], Merwyn Clifford[1], Michael I. Bird[2], Nicolai V. Bovin[3] , Nikolay E. Nifant'ev[3] and Gerard O'Beirne[1].

[1]Amersham International plc, Forest Farm, Whitchurch, Cardiff CF4 7YT, U.K. [Telephone: 44 1222 526000, Fax: 44 1222 526230], [2]GlaxoWellcome Research Ltd, Gunnells Wood Road, Stevenage SG1 2NY, U.K. and [3]Syntesome GmbH., Heimdall Str. 4, D-81739 Munchen, Germany.

Introduction

The selectins (E-, P- and L-selectin) are a family of cell surface glycoproteins that mediate the initial interaction between leukocytes and endothelial cells at sites of inflammation[(1)]. The selectins are known to bind to carbohydrate ligands on a number of glycoprotein counterstructures[(2)], but the exact nature of these carbohydrate ligands remains to be determined. The tetrasaccharide, sialyl Lewis^x appears to be a minimal carbohydrate ligand, with all three selectins capable of binding sialyl Lewis^x under appropriate conditions.

Recent studies have shown that selectin antagonists may be useful therapeutic agents for the treatment of inflammatory disorders[(3)]. As such, a demand exists for robust assays suitable for screening of potential selectin inhibitors. Scintillation proximity assay (SPA)™ technology has been used to develop homogeneous screening assays for many targets including cell adhesion molecules[(4)]. We have utilised SPA, along with synthetic radiolabelled glycoconjugate ligands, to develop a simple, robust, cell-free selectin adhesion assay, without the need for separation steps (Figure 1). This assay is ideally suited for high throughput screening purposes. We have also used the assay to evaluate different carbohydrate structures as high affinity selectin ligands, by directly measuring adhesion. These studies demonstrate the versatility and utility of the assay format.

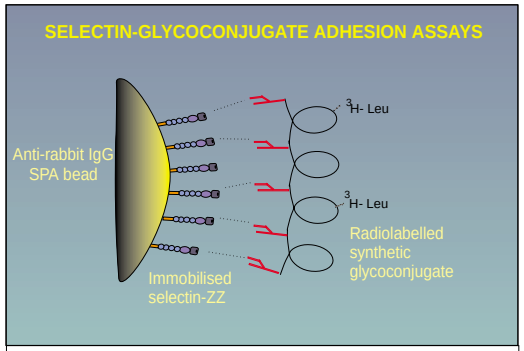

Figure 1. *Diagramatic representation of the selectin SPA adhesion assay concept.*

Experimental

Synthetic glycoconjugate ligands were in the form of a poly-(2-hydroxyethyl) acrylamide backbone[(5)] containing varying amounts of sialyl Lewis$^{A/X}$. Precursors of these glycoconjugates were labelled with L-[^{3}H]leucine. For cell-based SPA adhesion assays, HL60 human promyelocytic cells were metabolically labelled with L-[^{3}H]leucine. Adhesion assays were set up in microplates in a final volume of 100µl. E-, P- and L-selectin fusion proteins, containing the ZZ domain of protein A, were immobilised on anti-rabbit IgG SPA beads using rabbit IgG as a bridging antibody. Labelled glycoconjugate ligand was added to the wells and the plate incubated at room temperature on a shaker for 90 minutes. Adhesion was determined at equilibrium by counting the plate on a flatbed scintillation counter (Wallac MicroBeta™).

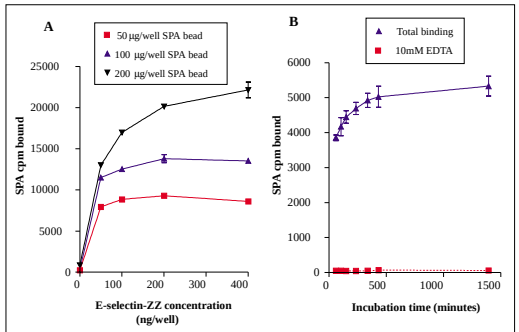

Figure 2. *(A) Effect of varying SPA bead and E-selectin fusion protein concentration on assay signal. The assay used 1 x 10^6 cpm sialyl Lewis^x (20%mol) glycoconjugate ligand. (B) Time course for E-selectin SPA adhesion assay. The assay contained 0.1mg/well bead, 100ng/well E-selectin-ZZ and 2 x 10^5 cpm sialyl Lewis^x (20%Mol) glycoconjugate ligand. Assays were carried out in 25mM Hepes buffered saline containing 0.1%(w/v) BSA at pH 7.4*

Optimisation of the SPA format was carried out using the E-selectin assay. The assay proved to be simple to set up, reproducible and economical with reagents (Figure 2A). Under optimal conditions, high signal to noise ratios (>100:1) were achieved (Figure 2B). The figure also shows the time course for equilibration of the assay.

Sialyl Lewis^x-glycoconjugate was validated as a counterligand for E-selectin by comparing the inhibition profiles of a range of carbohydrates in both HL60 cell and glycoconjugate based SPA adhesion assays. The data in Figure 3 shows that the inhibition profiles, when using the glycoconjugate ligand, are very similar to that obtained with the labelled HL60 cells. The glycoconjugate assay is also slightly more sensitive to inhibition by monomeric carbohydrates than the cell-based assay. The data also shows that a blocking monoclonal antibody completely inhibits specific binding in both assay formats.

It is now well established that multivalency enhances the avidity of selectin-carbohydrate interactions. Figure 4 demonstrates the effect of changing the density of the tetrasaccharides, sialyl Lewis^x and sialyl Lewis^A, on the adhesion signal generated. The data indicates that both tetrasaccharides are capable of adhering to

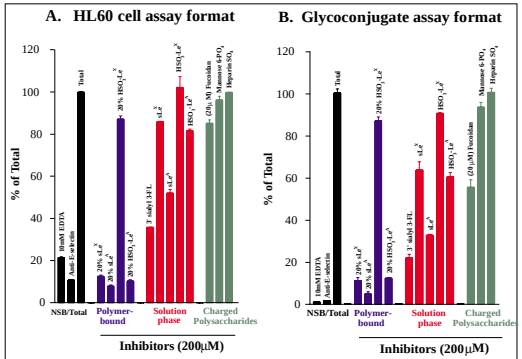

Figure 3. *Inhibition profile for the (A) HL60 cell-based and (B) sialyl Lewis^x-glycoconjugate assay formats. Assays contained 2 x 10^5 cpm glycoconjugate ligand or 50,000 HL60 cells (7 x 10^5 cpm). Anti-E-selectin antibody concentration: 5µg/ml (MAB2150, Chemicon Intl. Inc.).*

all three selectins in this assay format. E-selectin is more sensitive to carbohydrate density than P- or L-selectin, with glycoconjugate containing 20% (molar ratio) sialyl Lewis$^{A/X}$ giving a significantly higher assay signal than those with lower carbohydrate densities.

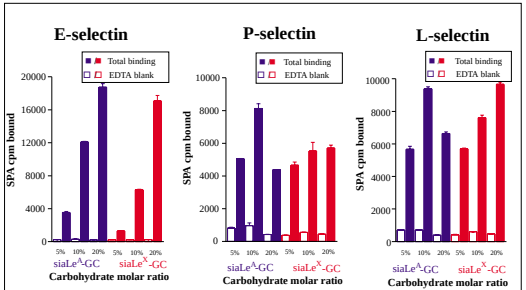

Figure 4. *Adhesion of sialyl Lewis^A- and sialyl Lewis^x-containing labelled glycoconjugates to E-, P- and L-selectin. The glycoconjugates contained 5%, 10% and 20% (molar ratio) of tetrasaccharide. The assay contained 0.1mg SPA bead, 100ng selectin-ZZ and 1 x 10^6 cpm glycoconjugate.*

Recent research into the natural carbohydrate ligands for P- and L-selectins has highlighted the role of sulphate groups in mediating high avidity adhesion[(6,7)]. A derivative of the labelled glycoconjugate ligand containing sialyl Lewis^x and additional sulphate groups was evaluated as a ligand in all three selectin assays. The results, shown in Figure 5, indicate that this ligand shows greatly enhanced adhesion to P- and L-selectin compared to sialyl Lewis^x-glycoconjugate alone. No significant difference in adhesion to E-selectin was evident. The high levels of cation-independent adhesion in the P-selectin assay when using the sulphated ligand suggests that the sulphate-mediated binding makes a significant contribution to adhesion in this assay.

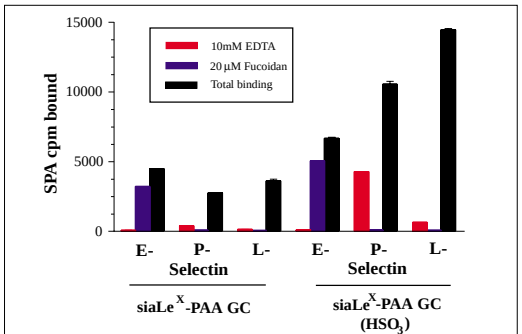

Figure 5. *Effect of incorporation of sulphate into the glycoconjugate ligand structure on the adhesion to E-, P- and L-selectin. Assay contained 2 x 10^5 cpm/well glycoconjugate, 0.1mg SPA bead, 100ng selectin-ZZ.*

Conclusions

The scintillation proximity assay format, in conjunction with soluble synthetic glycoconjugate ligands, offers a versatile and convenient assay for measuring selectin-mediated adhesion. This configuration preserves the capacity for multivalent interactions whilst retaining good sensitivity to inhibition. The assay is thus well suited to high throughput screening applications. The polyacrylamide-based glycoconjugates have also proved to be versatile tools in characterising the selectin-carbohydrate interaction. This data demonstrates that the selectins show differential sensitivity to carbohydrate density. Furthermore, sulphate-containing sialyl Lewis^x-glycoconjugates show markedly enhanced levels of binding to P- and L-selectin.

References

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