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Perspectives Series: Cell Adhesion in Vascular Biology

Therapeutic Inhibition of Carbohydrate–Protein Interactions In Vivo

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Leukocytes and the trafficking processes that direct these cells to their tissue sites of function, represent mainstays in the mammalian armamentarium against microbial pathogens. Trafficking processes are equally relevant to recruitment of immune cells which engage in functions that include localized immune response and tissue remodeling activities. The remarkable vigor with which leukocytes seek out and destroy pathogens, pathogen-infected cells, and dead cells and their products has come at a price; evolution has been incompletely successful in elaborating an immune system capable of perfectly discriminating between circumstances where potent destructive powers must be applied, and where restraint must be exercised. Leukocytes and their trafficking processes, for example, are occasionally prone to unleashing their powers in a tissue-destructive manner. Excessive trafficking of leukocytes to extravascular locations can lead to serious tissue injury and destruction in both acute and chronic circumstances (1), as exemplified by neutrophil-dependent myocardial injury that accompanies cardiac reperfusion injury (2) and by leukocyte infiltration into joints in patients with rheumatoid arthritis (3). In the context of this series, "Cell Adhesion in Vascular Biology," we review the conceptual basis for intervention in leukocyte trafficking through blockade of selectin-dependent leukocyte adhesion, discuss selected examples in which these strategies have been applied in vivo, and address novel molecular approaches to the development of selectin antagonists.

As reviewed in previous articles in this series (4, 5) and elsewhere (6), neutrophil extravasation is enabled by a multistep process initiated by the selectin family of cell adhesion molecules. P-selectin is expressed by activated vascular endothelium and by activated platelets, whereas E-selectin expression is restricted to activated endothelium. L-selectin, by contrast, is expressed constitutively by neutrophils and by other leukocytes. Each of the three selectins maintains an NH_2-terminal, extracellular domain with primary sequence similarity to the C-type family of calcium-dependent lectins. This carbohydrate binding domain, or CRD, plays an essential role in glycan-dependent recognition by selectins of their glycoprotein and glycolipid "counter-receptors." (The term counter-receptor in this context is the complementary molecule that binds to an adhesion molecule in a structurally specific manner.)

Neutrophil–endothelial cell interactions mediated via the selectins in the context of vascular shear flow are characterized by transient tethering of the neutrophil, followed by rolling of the neutrophil along the endothelial surface of the vessel wall. Studies in vitro and in vivo indicate that selectin-dependent neutrophil rolling is essential to subsequent events in the transmigration process, including exposure to locally high concentrations of endothelial cell-derived IL-8, platelet activating factor, and other neutrophil activating molecules (6). These molecules, in turn, promote activation of neutrophil β2 integrins, leading to integrin-dependent firm adhesion to the integrin counter-receptor, ICAM-1, and finally, neutrophil extravasation.

The selectin-dependent rolling phenomenon relevant to neutrophil recruitment can involve endothelial E- and P-selectin recognition of neutrophil counter-receptors. E- and P-selectin counter-receptor activity has been assigned to several specific neutrophil glycoproteins, including one termed P-selectin glycoprotein ligand 1 (PSGL-1), and various neutrophil glycolipids (reviewed in reference 7). These molecules are each posttranslationally modified by specific sialylated, fucosylated lactosamine-type glycans, as represented by the sialyl Lewis x tetrasaccharide molecule and its structural variants. These glycan modifications are essential to functional recognition of these counter-receptors by E- and P-selectin (6, 7) and represent molecular "shapes" with potential for blockade of selectin-dependent leukocyte recruitment.

Selectin-dependent rolling phenomenon involving neutrophils also engages neutrophil L-selectin recognition of inducible "endothelial" counter-receptor(s) (7). There is evidence that endothelial cells proper can express such counter-receptors, but their molecular nature remains unclear (7). Recent work also indicates that leukocytes can roll on previously adherent neutrophils in an L-selectin-dependent manner (8). These observations imply that one component of inducible "endothelial" activity for L-selectin evident in vivo may be accounted for by L-selectin counter-receptors displayed by neutrophils previously recruited to the endothelial cell surface through activation-dependent expression of E- and/or P-selectin, and ICAM-1. PSGL-1 may represent one such neutrophil-borne counter-receptor for L-selectin (9). Taken together, these considerations imply that L-selectin can promote neutrophil recruitment independent of, or synergistically with, E- or P-selectin. The relative contributions of these two mechanisms

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to the L-selectin-dependent component of neutrophil recruitment are not yet precisely defined and may vary with the inflammatory condition.

Given that neutrophil recruitment can be the sum of events mediated by L-selectin alone, by P- or E-selectin alone, and by L-selectin operating synergistically with E- and/or P-selectin, and since the ligands for these interactions are incompletely defined and likely overlap in their recognition specificities, design and interpretation of experiments involving in vivo blockade of selectin-dependent adhesion can be difficult. Furthermore, there can be quantitative differences in the spectrum of E- and P-selectin expression in different vascular beds and in different inflammatory circumstances, and the densities of their endothelial cell surface expression can vary dramatically over time through the duration of the inflammatory insult. These considerations add to the complexity. Nonetheless, the apparently early and pivotal role for selectin-dependent neutrophil adhesion in the recruitment process implies that effective in vivo blockade of selectin-dependent interactions can, in principle, diminish leukocyte recruitment and the intensity of the inflammatory process. The validity of this concept is implied by genetic experiments involving the construction and testing of mice made deficient in each of the three selectins. This work, reviewed in a previous article in this Perspectives Series (10), clearly demonstrates that the three selectins provide overlapping, yet crucial, contributions to neutrophil recruitment in acute inflammation.

Studies that have examined the effects of genetic deletion of key steps in the biosynthesis of the glycan components of neutrophil E- and P-selectin ligands have yielded conclusions complementary to those derived from the selectin-deficient animals. In the mouse, for example, genetic ablation of a specific fucosyltransferase locus deletes expression of neutrophil E- and P-selectin ligand activity. Deletion of selectin ligand activity is accompanied by faulty neutrophil recruitment to extravascular compartments, with apparently compensatory elevations in circulating blood leukocyte counts (11). Rare deficits in neutrophil selectin ligand expression due to a metabolic defect in the selectin ligand synthetic pathway also yield faulty neutrophil trafficking in humans suffering from the Leukocyte Adhesion Deficiency II syndrome (12). These genetic studies confirm that the selectin ligands and associated adhesive processes are essential to neutrophil recruitment in acute inflammation and provide experimental support for efforts to diminish neutrophil recruitment and inflammation via blockade of selectin-dependent adhesion.

Antagonism of selectin-mediated leukocyte recruitment in vivo has been approached with anti-selectin antibodies, selectin-IgG chimeras, peptides derived from selectin sequences, glycan moieties derived from endogenous selectin ligands, and pharmacophores that mimic such endogenous glycans. Antibody blocking studies have been informative because these reagents bind with high affinity to their cognate antigens, because they can block each selectin in a relatively specific manner, and because doses of 1–2 mg/kg yield can yield circulating concentrations capable of blocking the cognate epitope for up to 12 h. Selectin-IgG chimeras have similar virtues, including even longer half lives (up to 50 h) (13). By contrast, the other types of inhibitory molecules are generally smaller, have intravascular half lives that are much shorter, or less well-defined, and may have lower affinities and/or specificities for the cognate receptor. Most interventional efforts have made use of acute inflammatory models that assess neutrophil recruitment, due to the lack of long-acting selectin blocking reagents, difficulties arranging continuous, long-term intravascular delivery of the available short acting inhibitors, and the paucity of information concerning the functional relevance of selectin-dependent leukocyte recruitment in chronic inflammatory conditions.

Anti–L-selectin agents might be expected to be unable to completely block neutrophil recruitment in vivo in many inflammatory conditions, since von Andrian et al. (14) have demonstrated that neutrophil rolling in vivo in rabbit mesenteric venules, as assessed by intravital microscopy techniques, is inhibited only partially by intravascular administration of an adhesion blocking anti-L-selectin monoclonal antibody. Indeed, agents that inhibit L-selectin–dependent adhesion generally have shown incomplete effectiveness in blocking acute neutrophil recruitment in most in vivo models of acute inflammation. Studies in a mouse model of peritonitis, for example, showed that a monoclonal anti–L-selectin antibody diminished neutrophil recruitment by roughly 70% (15). An L-selectin-IgG chimera has yielded similar results in the mouse peritoneal exudate model ($\sim$ 70% and $\sim$ 50% reductions in neutrophil accumulation 2 h and 4 h, respectively, after initiation of the inflammatory insult) (13). These results are generally consistent with a 50% reduction in neutrophil accumulation in the peritoneal space in L-selectin–deficient mice subjected to the same peritonitis model (16). Quantitatively similar reductions in neutrophil recruitment have also been observed with an anti-L-selectin antibody in a cat myocardial reperfusion model (17). Considered together these results imply that in these acute inflammatory models, L-selectin appears not to be absolutely required for neutrophil recruitment, presumably because a substantial degree of the requisite rolling type of adhesion is contributed by E- and/or P-selectins, or because, as discussed elsewhere, neutrophil recruitment can be selectin-independent under some circumstances (18). Nonetheless, L-selectin may amplify E- and/or P-selectin-initiated neutrophil recruitment in these models through L-selectin-dependent, neutrophil-assisted capture of other, flowing neutrophils (8, 9), or may contribute to neutrophil recruitment directly through interactions with endothelial cell ligands.

Incomplete reductions in tissue neutrophil accumulation have also been observed using the L-selectin-Ig chimera (19), or anti–L-selectin antibody (20) in well-characterized rat models of neutrophil accumulation in lung injury induced by cobra venom factor (CVF) or by deposition of IgG immune complexes. By contrast, similar approaches using E- and P-selectin-specific reagents clearly demonstrated a requirement for E-selectin, but not P-selectin, in the immune complex model. For example, neutrophil recruitment in the immune complex model was attenuated by slightly more than 50% with an anti-E-selectin monoclonal antibody (21). An E-selectin-Ig chimera was slightly less effective in the same model ($\sim$ 35% reduction; reference 19) and achieved inhibition roughly comparable with that observed with an L-selectin-Ig chimera. By contrast, the P-selectin–Ig chimera was without protective effect in immune complex lung injury (19). Conversely, in the CVF model, P-selectin clearly contributed to neutrophil recruitment, as did L-selectin, whereas E-selectin blocking reagents did not significantly reduce neutrophil accumulation (19–22). The participation of distinct pairs of selectins in each lung injury model illustrates the potential for redundancy in selectin-dependent

contributions to neutrophil recruitment, and implies that different combinations of selectins will be employed in different inflammatory circumstances.

While many of the inferences regarding the role of selectins in inflammatory responses have come from observations regarding the rolling phenomenon in post capillary venules (in the mesentery or in the cremaster muscle), it is important to emphasize that in some vascular beds, such as in the lung, neutrophil extravasation can occur in capillaries, and not in postcapillary venules. Since the physical constraints of the pulmonary capillary bed do not permit neutrophils the spatial freedom to "roll" in these capillaries (23), the initial selectin-dependent capture of neutrophils by activated endothelium may not always operate in the lung. Accordingly, selectin requirements for neutrophil transmigration in the lung may need a different explanation, under some circumstances. Finally, while inhibition of neutrophil recruitment was always incomplete in these studies, it is important to point out that these reductions were typically accompanied by more substantial, and in some instances, nearly complete, reductions in tissue injury parameters. These observations imply that it may not be necessary to achieve complete reduction in neutrophil recruitment via simultaneous blockade of two or three selectins in order to obtain the desired anti-inflammatory effect. Whether this concept will apply broadly to acute inflammatory diseases in laboratory animals, and in humans, remains to be determined.

Identification of important glycan components of selectin counter-receptors on neutrophils (6, 7) has provided the opportunity to test key structural subsets of these molecules, and their derivatives, for their neutrophil recruitment inhibitory activity, and anti-inflammatory effect, in vivo. Most of the in vivo inhibitory experiments to date have used monovalent, low molecular weight forms of the sialyl Lewis x tetrasaccharide. This molecule is a key component of substantially more complex leukocyte glycan moieties that function as selectin counter-receptors. The sialyl Lewis x tetrasaccharide and close structural analogues inhibit E- and P-selectin-dependent adhesion of leukocytes in static adhesion assays in vitro with IC_{50} values of $\sim$ 0.5–1.0 mM (reviewed in reference 24). Such relatively high IC_{50} values may be accounted for, in part, by the inherently low affinity of a selectin-carbohydrate interaction. Furthermore, it is likely that the structurally complex endogenous leukocyte glycoprotein counter-receptors for the selectins exhibit greater inherent affinities for selectins. It is also likely that endogenous leukocyte selectin ligands are clustered on the leukocyte surface and are thus effectively polyvalent, adding to the apparent affinity between a leukocyte and a selectin and to the difficulty in inhibiting this interaction with a small, monovalent component of an endogenous counter-receptor. Nonetheless, IC_{50} data derived from in vitro static adhesion assays may not be relevant to the in vivo situation, where blood concentrations below the in vitro-derived IC_{50} values seem adequate to inhibit leukocyte rolling. IC_{50} values derived from in vitro shear flow assays, or from in vivo experiments, are not yet widely available, however. This remains an important area for future study.

The sialyl Lewis x-type oligosaccharides have been tested in the rat lung injury models discussed above, where neutrophil recruitment and tissue injury are functions of either E- and L-selectins (the IgG complex-induced injury model) or dependent upon P- and L-selectins (the CVF model). In these experiments (25, 26), specific reductions in neutrophil recruitment, as measured by lung myeloperoxidase content, plateaued at approximately 35% in dose response studies using the monovalent, tetra and/or pentasaccharide formulations of the sialyl Lewis x structure administered intravenously. The most effective doses (generally 200 μg or greater) yielded equivalent or greater reductions (between $\sim$ 35 and 65%) in hemorrhage and vascular permeability. The incomplete reductions in the levels of myeloperoxidase, and partial, if still substantial, protection against tissue injury in these experiments, roughly parallel reductions obtained with anti-selectin antibodies, or selectin-Ig chimeras, again suggesting that selectin-independent mechanisms (18, 23) may contribute to neutrophil recruitment in these models. Given that antibody blocking studies imply an important role for L-selectin in these models, and in consideration of the evidence for sialylated, fucosylated L-selectin counter-receptors displayed by adherent neutrophils (8, 9), it is important to consider the possibility that the oligosaccharide may be inhibiting L-selectin-dependent capture of rolling neutrophils by adherent neutrophils (described above), in addition to inhibition of E- and/or P-selectin-dependent leukocyte rolling.

Peak blood concentrations of the oligosaccharide at the 200 μg dose in the CVF experiments ($\sim$ 0.67 mg/kg total oligosaccharide) were estimated to be less than 1 μM (26). Assuming that the in vivo protection observed in these experiments is due solely to inhibition of leukocyte-selectin adhesive interactions, these data imply that in vivo IC_{50} values are substantially less than those determined by in vitro static adhesion assays. It is also possible, however, that oligosaccharide-dependent reductions in neutrophil recruitment and tissue injury may be a function of inhibitory effects (at low in vivo concentrations) of these molecules on signal transduction events mediated by the selectins and their ligands (4).

The pentasaccharide formulation of the sialyl Lewis x oligosaccharide has also been shown to protect myocardium from neutrophil-dependent injury in a cat model of myocardial reperfusion injury (27). A single 10 mg/kg intravenous dose in these experiments significantly reduced myocardial necrosis as measured directly, or as reflected by postmyocardial ischemia/reperfusion plasma creatine kinase measurements and preserved cardiac contractility and endothelial cell function. Similar results with sialyl Lewis x type oligosaccharides have been reported in dog (28) and rat (29) myocardial reperfusion injury models, where, like in the cat, P- and L-selectin–dependent neutrophil recruitment contributes substantially to injury. Reductions in tissue injury have also been achieved with this glycan in a rat model of E-selectin–dependent cerebral ischemia and reperfusion injury (30). It remains to be determined if the protective effects of selectin antagonism will be as effective in humans, especially in the context of reperfusion injury associated with thrombolytic therapy for stroke or myocardial infarction.

A series of sulfate-containing compounds, including sulfatides (31) and heparin-derived oligosaccharides (32) have also been used successfully to antagonize leukocyte-selectin interactions in vivo. These studies are an extension of work demonstrating the inhibitory activity of such molecules in vitro in L- and P-selectin-dependent leukocyte adhesion assays. Inhibition in vitro and in vivo is consistent with the discovery that the endogenous ligands for L- and P-selectins contain sulfate residues essential for function (6), suggesting that these compounds interfere with the sulfate-dependent component of L- and P-selec-

tin adhesion. Peptides derived from selectin CRDs have been shown to block neutrophil adhesion to selectins in vitro; recent experiments suggest that such peptides can diminish neutrophil recruitment in vivo (33). Peptides derived from selectin counter-receptors, especially sulfated peptides known to be critical for in vitro function (6), have yet to be examined in vivo.

Whether designed, discovered, or selected in vitro, sialyl Lewis x mimics represent a series of promising alternatives to the current repertoire of selectin antagonists. For example, a computer-based pharmacophore search, using energetically-preferred conformations of the sialyl Lewis x structure and its known structure-activity relationships, has been used to identify a natural product from licorice, termed glycyrrhizin, with low micromolar IC_{50}s in an ELISA assay that measures P-selectin–dependent interactions. A fucosylated derivative of glycyrrhizin is an effective selectin antagonist in vitro and blocks neutrophil recruitment in an in vivo mouse model that is presumed to be selectin-dependent (34). In another example, in vitro "evolution," via the polymerase chain reaction, has been used to develop a series of 39 base pair long single stranded DNA aptamers with low nanomolar Kds for L-selectin (35). These molecules and their derivatives exhibit Kds two- to fourfold higher (i.e., two- to fourfold lower affinities) for E- and P-selectins, inhibit L-selectin–dependent rolling type cell adhesion in vitro, and block L-selectin–dependent lymphocyte trafficking in vivo. Chemically modified aptamers circulate with a half life of $\sim$ 18 min in the laboratory rat, suggesting that similar such molecules may find utility as specific, high affinity in vivo antagonists for E-, P-, and L-selectins in inflammatory conditions.

The initial success of this approach suggests that we can also anticipate the use of combinatorial molecular library approaches to identify small molecule selectin antagonists. Indeed, phage display technology has been used to identify a peptide sequence that interacts with the plant lectin concanavalin A, presumably through its carbohydrate recognition domain (36). The organic and medicinal chemistry literature is also replete with synthetic schemes and in vitro inhibition data (if not yet in vivo) for structural mimics of the glycan portions of the selectin counter-receptors (37). We can also anticipate other approaches to diminish selectin-dependent leukocyte recruitment, through pharmacological inhibition of induced selectin expression, for example, or via molecules that inhibit the activity of the glycosyltransferases required for leukocyte selectin ligand synthesis, or that block synthesis of nucleotide sugar substrates for these enzymes.

These advances imply that the future may bring a large array of highly selective, high affinity molecular antagonists of the interactions between the selectins and their counter-receptors. However, our ability to effectively utilize these molecules as therapeutics will require a more complete understanding of the biology of the selectins, and as discussed by Ajit Varki in this Perspectives series (38), their authentic counter-receptors. There is a need for a more detailed exploration of the in vivo consequences of selectin ligand antagonism to the complex signal transduction processes associated with selectin-dependent cell adhesion. These processes have been reviewed recently by Zimmerman et al. in this Perspectives Series (4). There is also a need to better understand the functional relevance of circulating, soluble forms of selectins and some of their counter-receptors, especially since the levels of these molecules can fluctuate in concert with inflammatory conditions, and may, at least in principle, regulate selectin-dependent adhesion and signal transduction processes in vivo. The possibility that these molecules will interact in vivo with pharmacologically-produced selectin antagonists obviously must be considered. Finally, the implied relevance of selectin-dependent leukocyte recruitment in chronic inflammatory conditions must be experimentally confirmed if antagonism of selectin-dependent interactions is likely to be useful in these circumstances. Mice with genetic deficiencies in the selectins, or their counter-receptors, should prove useful for this work, although caution in interpreting data from such mice needs to be exercised (39).

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