Annu. Rev. Pharmacol. Toxicol. 2000. 40:283–94

PHARMACOLOGY OF SELECTIN INHIBITORS IN ISCHEMIA/REPERFUSION STATES

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Key Words P-selectin, sialyl Lewis^x, myocardial injury, endothelium, coronary circulation

■ **Abstract** Recently, the selectin family of glycoprotein adhesion molecules (P-selectin, E-selectin, and L-selectin) has been implicated in the pathogenesis of a number of inflammatory disease states. The selectins modulate the early adhesive interactions between circulating neutrophils and the endothelium. Both P-selectin and E-selectin can be expressed on the surface of endothelial cells following stimulation by a number of inflammatory mediators. In contrast, L-selectin is constitutively expressed on the surface of neutrophils at very high levels. In addition, neutrophils also express ligands for the endothelial selectins, including the carbohydrate sialyl Lewis^x and the high-affinity ligand P-selectin glycoprotein ligand 1, which facilitate neutrophil-endothelial interactions. Selectins have been extensively investigated in ischemia/reperfusion injury states. The study of selectin involvement in ischemia/reperfusion injury has been facilitated by the development of highly specific selectin antagonists, including monoclonal antibodies, carbohydrates, small molecule inhibitors, and soluble forms of P-selectin glycoprotein ligand 1. This article reviews the results of current studies of selectin antagonists in experimental models of ischemia/reperfusion injury.

INTRODUCTION

The selectins are a family of glycoproteins that play a significant role in the regulation of cell adhesion as well as in cell signalling. Selectins are expressed on the cell surface of key cell types. There are three members of the selectin family: L-selectin, P-selectin, and E-selectin. The nomenclature is based on (*a*) the fact that there is a lectin domain in their structure, hence the name selectin (1), and (*b*) the cell types on which the selectin is expressed. Thus, L-selectin is found on leukocytes, E-selectin is expressed on the surface of endothelial cells, and P-selectin is expressed on platelets and endothelial cells.

L-selectin is constitutively expressed on several select types of monocytes, lymphocytes, and neutrophils. L-selectin exists on the tips of the pseudopods of these white blood cells and can be shed from these cell surfaces upon their acti-

vation (2). P-selectin, however, exists internally in Weibel-Palade bodies of endothelial cells and α-granules of platelets (3). P-selectin can be translocated to the cell surface by activation with thrombin, histamine, hydrogen peroxide, and inhibitors of nitric oxide synthase (3). This process peaks in 10–20 min. In contrast, E-selectin is expressed on endothelial cells only after de novo synthesis following activation of endothelial cells, a process requiring 4–6 h (4). Activators of E-selectin include the cytokines—tumor necrosis factor alpha,—interleukin 1β, and bacterial lipopolysaccharide (endotoxin).

All three selectins play a key role in the cell adhesion cascade of inflammation. Basically, the selectins initiate leukocyte rolling along the endothelium (5), the first step in leukocyte recruitment. The most important selectin in regulating leukocyte rolling in ischemia/reperfusion and other shock-like states is P-selectin (6). This rolling, or ''capture,'' effectively slows the velocity of leukocyte movement in the microcirculation and enables many of these leukocytes to proceed to the second step, firm adhesion to the endothelium. Some of the adherent leukocytes will undergo transendothelial migration and thus congregate at the site of infection or inflammation (7).

In ischemia/reperfusion, the first step in reperfusion injury, an inflammatory process, is the loss of endothelium-derived nitric oxide, resulting in a rapid (i.e. within 2–5 min) endothelial dysfunction (8). The second step in the reperfusion injury process is up-regulation of P-selectin on the endothelial surface of the affected area 10–20 min following reperfusion (9). This leads to increased adhesion of neutrophils [polymorphonuclear leukocytes (PMNs)] to the dysfunctional/selectin up-regulated endothelium (10). At this point, the process slows down and a gradual infiltration of PMNs occurs, which at 180 minutes postreperfusion becomes significant. Finally, reperfusion injury with its resultant tissue necrosis occurs to a marked extent by 270 min (i.e. in the case of the ischemic/reperfused heart, myocardial necrosis) (11). Thus, key players in the process linking the endothelial dysfunction to the PMN involvement are the selectins, particularly P-selectin. In addition to the translocation of P-selectin to the surface of endothelial cells and platelets, new P-selectin expression occurs via up-regulation of such transcription factors as NF-κB (12, 13), a process that may amplify the role of P-selectin, by invoking a second peak of activation later in time following the early translocation of P-selectin to the cell surface. In contrast to P- and L-selectin, E-selectin does not apparently play a major role in reperfusion injury, at least during the first 4 h postreperfusion.

TYPES OF SELECTIN INHIBITORS

There are a variety of approaches that can be taken to inhibit or block one or more of the selectin family members. These are listed in Table 1. The earliest inhibitors of the selectins were monoclonal antibodies (mAbs) directed against a specific selectin molecule. These mAbs have the advantage of a high degree of

TABLE 1 Various selectin antagonists that have been investigated in ischemia/reperfusion injury states

Selectin antagonist	Antagonist type	Cellular target	Dose range	References(s)
PB 1.3 (CY-1747)[a]	Monoclonal antibody	P-selectin	1–2 mg/kg	17–24, 26
DREG-200[b]	Monoclonal antibody	L-selectin	1 mg/kg	27–29
CL-2[b]	Monoclonal antibody	E-selectin	1 mg/kg	31
CY-1787[a]	Monoclonal antibody	E-selectin	1 mg/kg	9
SLe^x-OS (CY-1503)[a]	Carbohydrate	E-selectin, P-selectin	5–40 mg/kg	14, 36–44
TBC-1269[c]	Small molecule	E-selectin, P-selectin	25 mg/kg	47
sPSGL-1 and rsPSGL-1–Ig[d]	Peptide analog	P-selectin	5 μg/rat–1 mg/kg	50–53, 56

[a]Cytel Corporation, San Diego, CA. Sle^x-OS, Sialyl $Lewis^x$ oligosaccharide.
[b]Boehringer Ingelheim, Ridgefield, CT.
[c]Texas Biotechnology, Houston, TX.
[d]Genetics Institute, Cambridge, MA. sPSGL-1, Soluble P-selectin glycoprotein ligand-1; rsPSGL, recombinant analog of sPSGL-1; Ig, immunoglobulin.

specificity, but they cross-react with only a few species. Most of the early mAbs cross-reacted with human selectin molecules but had limited actions among other mammalian species. However, many of these mAbs did cross-react with cat tissues. All mAbs were effective at doses of 1–2 mg/kg.

Once it became known that the three members of the selectin family recognize a common carbohydrate ligand (i.e. sialyl Lewis^x) (13), the race was on to develop analogs of sialyl Lewis^x (SLe^x) that acted as a soluble selectin blocker and blocked the actions of the selectins. The enormous difficulty and cost of synthesizing these carbohydrate analogs delayed progress for a while, but with the synthesis of a SLe^x-oligosaccharide (SLe^x-OS) known as CY-1503, great progress was made. This substance was effective at 10–20 mg/kg in a variety of mammalian species, and it blocked all selectins (14). Unfortunately, it had a circulating $t_{1/2}$ of only 10–30 min. A variety of small-molecule SLe^x-mimetics have been developed and studied as selectin antagonists. These are either sulfatide analogs, manosylated biphenyl derivatives, or dipeptides, and they are discussed later. Several years later, the high-affinity ligand for P-selectin, P-selectin glycoprotein ligand-1 (PSGL-1) (15), was discovered. Although PSGL-1 is a ligand primarily for P-selectin, it can serve as a ligand for all the selectins (16). Recently, a soluble form of PSGL-1 (sPSGL-1) has been used as a functional inhibitor of selectin actions in vivo. Finally, nitric oxide (NO) and NO donors have been found to be effective selectin antagonists (6, 11).

PROTECTIVE ACTIONS OF SELECTIN INHIBITORS IN ISCHEMIA/REPERFUSION

Monoclonal Antibodies Directed Against Selectins

Monoclonal antibodies neutralizing P-selectin were the first significant anti-selectin blockers available. The leading anti–P-selectin mAb is PB1.3, produced by the Cytel Corporation of San Diego, California. This antibody, also known as CY-1747, is an immunoglobulin (Ig) G_1 antibody raised against human P-selectin. However, it cross-reacts with feline, canine, rabbit, and rat P-selectin. Not only is it an effective P-selectin–neutralizing antibody in these species, it also recognizes only surface-expressed P-selectin and, thus, is a very valuable reagent for immunocytochemistry and immunolocalization of P-selectin expressed on the surface of endothelial cells (17). PB1.3 at a dose of 1–2 mg/kg was found to be effective in myocardial ischemia/reperfusion (MI/R) injury when given intravenously at the time of reperfusion (17). Not only was cardiac necrosis reduced by 58%, a highly significant finding ($P < 0.01$), but a comparable degree of coronary vascular endothelial preservation and reduced PMN adhesion to the coronary endothelium was also observed (17). Cardioprotection by PB1.3 was also observed following MI/R in dogs, which also confirmed that PB1.3 attenuated PMN infiltration into the reperfused myocardium (18–20).

Not only does PB1.3 protect the ischemic-reperfused myocardium in rats, it also protects against reperfusion injury in the splanchnic region by reducing intestinal injury (21) and by reducing mesenteric microvascular leakiness to albumin (22, 23). Furthermore, PB1.3 was found to attenuate reperfusion injury to the rabbit ear (24) and concomitantly reduce PMN adherence to the ischemic-reperfused endothelium. This reduced necrosis was also confirmed in the rabbit ear with a different antibody against P-selectin, a P-selectin–IgG chimera (25). In all cases, the P-selectin antibodies did not significantly induce any circulating leukopenia.

Finally, PB1.3 was also effective in total body I/R (i.e. hemorrhage-reinfusion) in rabbits (26). PB1.3 protected the microvasculature from fluid loss and maintained cardiac output and arterial blood pressure.

Monoclonal antibodies have also been produced against L-selectin. One of these, DREG-200, an IgG1 antibody, effectively blocks L-selectin on leukocytes (27). DREG-200 was found to significantly attenuate myocardial reperfusion injury in cats (28) by limiting cardiac necrosis, reducing endothelial dysfunction, and attenuating neutrophil infiltration. On the basis of these results, a humanized form of DREG-200 was prepared and tested in this same feline model of MI/R (29). This Hu DREG-200 attenuated postreperfusion cardiac necrosis by 52% compared with 60% for the nonhumanized form of DREG-200. Moreover, in addition to preserving coronary endothelial function and limiting PMN infiltration, Hu DREG-200 was found to significantly reduce left ventricular contractile dysfunction occurring in the first few hours of reperfusion (29). Employing another mAb against L-selectin, Ramasworthy et al (30) reported salutary effects in rabbits subjected to total body hemorrhage for 2 h followed by reinfusion of shed blood. As with the other L-selectin studies, there was no significant change in circulating leukocyte counts in antibody-treated animals.

With regard to mAbs directed against E-selectin, only a few reports have been published, presumably because of the lack of positive effects of these mAbs in several models of I/R injury. The first such report was published by Winquist et al (31), who showed that CL-2, an anti–E-selectin mAb, did not reduce infarct size in cynomolgus monkeys in a model of MI/R injury in which at 4 h postreperfusion an anti–intercellular adhesion molecule-1 mAb (R6.5) had been effective. Moreover, Weyrich et al (9) found a similar lack of effectiveness of an E-selectin mAb (CY-1787) in the same feline model of MI/R, in which both P-selectin and L-selectin mAbs were effective. Similarly, Kurose et al (32) found that CL-3, another anti–E-selectin mAb, was ineffective in splanchnic I/R in rats, although PB1.3 was effective in this same model system. One is forced to conclude that E-selectin does not play a significant role in reperfusion injury, at least during the first 4–6 h postreperfusion. This may be related to the important regulatory role of P-selectin on leukocyte rolling following acute tissue trauma, with a lesser role for L-selectin and virtually no influence on rolling by E-selectin (33).

Sialyl Lewis^x Analogs in Reperfusion Injury

The earliest information on the selectin ligand indicated that is was a carbohydrate structure (34). This led to the notion that sialyl Lewis^x was the selectin ligand (35). The first available chemical substance that was able to block the selectin ligand was a sialyl Lewis^x oligosaccharide (SLe^x-OS). SLe^x-OS (CY-1503) was first tested in an I/R model by Buerke et al (36). SLe^x-OS protected against reperfusion injury–induced cardiac necrosis by 83%, a value significantly greater than that observed with any single anti-selectin mAb. Moreover, CY-1503 also preserved cat coronary endothelium and markedly inhibited PMN adhesion to the endothelium, while not influencing circulating leukocyte counts. In an effort to determine whether these cardioprotective effects resulted in improved cardiac performance, the maximum velocity of the rise in left ventricular pressure, dP/dt_{max}, was assessed. CY-1503–treated cats subjected to MI/R exhibited 100% recovery of dP/dt_{max} compared with 71% recovery for MI/R cats receiving either saline or a nonsialylated Lewis^x oligosaccharide (Le^x-OS). A similar cardioprotective effect of CY-1503 was observed in MI/R dogs reperfused for 4.5 h (37). This study also was the first to show that in vivo endothelial preservation by CY-1503 is an important component of the cardioprotection. One of the problems with SLe^x-OS is the short half-life and the need to use a dose of approximately 5–10 mg/kg. This was partially overcome by using a liposome-conjugated SLe^x-OS (38). This study indicated that the liposome-conjugated SLe^x-OS at 400 μg/kg was as effective as 10 mg/kg of the nonliposomal compound in cat MI/R, an increase in potency of 25-fold. In order to answer the important question of whether SLe^x-OS significantly protects the ischemic-reperfused myocardium or whether it merely delays injury, Flynn et al (39) studied dogs subjected to MI/R for 48 h treated with CY-1503. CY-1503 treatment reduced infarct size by 55% after 48 h of reperfusion, which correlated with a 55% reduction in PMN infiltration. Others also confirmed a cardioprotective effect of CY-1503 in dogs subjected to MI/R (40), but these investigators employed an extremely large dose (40 mg/kg). The only reports that failed to show a protective effect of CY-1503 in MI/R were those of Birnbaum et al (41), who treated rabbits with the SLe^x-OS, and Gill et al (42), who treated dogs with CY-1503. In contrast, Yamada et al (43) demonstrated that CY-1503 exerted a cardioprotective effect in rabbits subjected to MI/R. No obvious explanation exists for the differences in the studies of CY-1503 in rabbits and dogs. The SLe^x-OS clearly protected rats against I/R injury because CY-1503 was found to exert several beneficial effects in traumatic shock in rats, a form of whole body I/R (44), and in MI/R injury (45).

In the interval after the clear-cut effects of CY-1503 were reported, other small-molecule selectin inhibitors have been studied in I/R states. Seko et al (46) showed that synthetic oligopeptides corresponding to portions of the N-terminal lectin domain of human selectins significantly attenuated infarct size in rats subjected to MI and to 48 h of reperfusion, consistent with the report by Flynn et al in dogs (39). Other small-molecule anti-selectin agents are also effective in I/R models. Thus, TBC-1269, a synthetic mannosylated-biphenyl analog, was effective in

hepatic I/R (47), and a SLe^x-mimetic (a serine-glutamic acid peptide analog) protected mice subjected to a form of cutaneous I/R. Similar results in mice skin were reported by Rao et al (48) using a glucaronic acid substituted analog of glycyrrhizin.

PSGL-1 Analogs in Reperfusion Injury

One of the great achievements in selectin research was the discovery of the high-affinity ligand for the selectins by Moore and coworkers (16). This substance isolated from human neutrophils was termed P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 serves as a high-affinity ligand for P-selectin (and to a lesser extent L-selectin and E-selectin) because it displays oligosaccharide sequences recognized by the calcium-dependent lectin domain of the selectins. PSGL-1 is 10,000 times more potent than SLe^x in binding to P-selectin (49). Leukocyte trafficking is mediated by selectin–PSGL-1 interactions and is a key molecule responsible for recruitment of neutrophils into areas of inflammation (50).

Recently, peptide analogs of PSGL-1 have been constructed. A soluble form of PSGL-1 (sPSGL-1) has been found to be effective in I/R. This sPSGL-1 was reported to markedly protect against renal I/R (51) at doses of 5–50 μg in rats. sPSGL-1 significantly protected against renal necrosis due to PMN infiltration. More recently, a recombinant analog of sPSGL-1, rsPSGL-1-Ig, exerted remarkable cardioprotective effects at 1 mg/kg in intact cats subjected to MI/R (52) and in isolated, PMN-perfused rat hearts at 200–500 μg/kg (53) subjected to global I/R. The rsPSGL-1–Ig attenuated feline myocardial necrosis by 62% in cat heart and preserved myocardial contractility by 55% in isolated rat heart. In both cases, there was dramatic attenuation of infiltrated PMNs. A low-affinity mutant of rsPSGL-1–Ig was inactive in all cases (52, 53), whereas a tetramer form of rsPSGL-1–Ig exhibited a potency twice that of the native form. In addition, a metalloproteinase isolated from cobra venom (i.e. mocarhagin) has been shown to cleave a decapeptide from the N terminus of the PSGL-1 receptor (54). Mocharagin also shows significant cardioprotective effects at 200 ng/mL in the same PMN-perfused rat heart model of I/R as reported for rsPSGL-1–Ig (55). PSGL-1–Ig also works in hemorrhage/reinfusion in mice subjected to hemorrhagic shock (56), a form of total body I/R.

It is becoming evident that interference with PSGL-1, the high-affinity ligand for the selectins or its receptor, offers an effective strategy for counteracting tissue necrosis and other effects of reperfusion injury. Moreover, using PSGL-1 as the target rather than SLe^x allows the use of doses two orders of magnitude lower than those required to block with SLe^x.

Nitric Oxide

The lipid-soluble low-molecular-weight gas, NO among its many actions, also inhibits the adherence of leukocytes to the endothelium (57, 58). NO exerts this antiadhesion effect to a large extent by inhibiting the up-regulation of P-selectin

(58). Moreover, NO releasing agents (i.e. NO donors) have been found to markedly inhibit P-selectin up-regulation in the mesenteric microvasculature in rats subjected to splanchnic I/R (59). The inverse relationship between NO and P-selectin has been reviewed in detail elsewhere (60).

P-Selectin Gene-Deficient Animals

One of the most exciting developments in recent years is the generation of specific gene deletions in mice. These so-called gene-knockout mice represent a valuable tool in that they lack a specific endogenous protein. These gene deletion animals therefore do not require receptor antagonists, synthesis inhibitors, or any other modulator of the protein of interest. Moreover, the ''blockade'' is lifelong, usually commencing during the prenatal development of the animal. Thus, it was exciting to have P-selectin–deficient mice available several years ago. These mice, developed by several investigators (61), are devoid of leukocyte rolling along the microvascular endothelium despite an elevation in the number of circulating neutrophils. These mice were found to be protected from postischemic endothelial dysfunction characterized by a loss of vasorelaxant effect to endothelium-dependent dilators (62). These findings serve to validate the earlier reports that there is a reciprocal relationship between endothelium-derived NO and endothelial cell P-selectin expression.

Early studies on the properties of P-selectin–deficient ($-/-$) mice were soon followed by studies in which P-selectin $-/-$ mice were observed to exhibit a very mild response to platelet activating factor at doses that usually produce intestinal necrosis and death (63). Survival was 100% in the P-selectin $-/-$ mice. Following up on these seminal investigations, Scalia et al (56) showed that P-selectin $-/-$ mice experienced a mild reaction to hemorrhage to a 40 mmHg for 45 min followed by reinfusion of all shed blood. Leukocyte rolling and adhesion were absent in response to the total body I/R, and few leukocytes infiltrated into splanchnic tissues. Moreover, the mean arterial blood pressure was well maintained postreperfusion in P-selectin $-/-$ animals. In addition, it has also been demonstrated recently that mice deficient in P-selectin are protected against the effects of MI/R injury induced by coronary artery occlusion and reperfusion (64). Myocardial infarct size and neutrophil accumulation in the myocardium were dramatically reduced in P-selectin null animals compared with wild-type controls (64). These results point to the vital role of P-selectin in mediating the pathophysiology of I/R. Other gene deletions (i.e. E-selectin) are now available and will provide interesting insights in further understanding the role of selectins in I/R injury.

SUMMARY

This brief review has attempted to evaluate and summarize the current evidence for the role of the selectin family of adhesion glycoproteins in ischemia/reperfusion (I/R) disease states. The overwhelming evidence points to an impor-

tant role of the selectins in mediating the early phase of leukocyte-endothelium interaction in reperfusion injury. This mediation is exemplified by the stimulation of leukocyte rolling along the postcapillary venular endothelium triggering an inflammatory cascade, which eventually leads to neutrophil infiltration into ischemic/reperfused tissues, whereupon these neutrophils release a variety of cytotoxic mediators that contribute in a major way to the tissue injury and necrosis that is observed in reperfusion injury.

The selectins may also play a role in cell signaling among various cell types in orchestrating responses to I/R. P-selectin plays a preeminent role among the selectins as the major contributor involved in leukocyte rolling and probably is the key selectin mediator of reperfusion injury. L-selectin appears to play a significant role in the early phase of reperfusion injury, but E-selectin does not appear to be involved in the early phases. However, E-selectin may be active in responses that occur much later than 6 h postreperfusion. Finally, anti-selectin agents remain an important target for design of drugs to treat reperfusion injury states.

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Annual Review of Pharmacology and Toxicology
Volume 40, 2000

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