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European Journal of Pharmacology 296 (1996) 181–187

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Oligotide attenuates leukocyte-endothelial cell interaction via P-selectin in the rat mesenteric vascular bed

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Received 7 August 1995; revised 12 October 1995; accepted 17 October 1995

Abstract

A novel single stranded polydeoxyribonucleotide (oligotide) was studied for its ability to modulate leukocyte-endothelial cell interaction, by means of intravital microscopy in the rat mesenteric microvasculature. Superfusion of the rat mesentery with 50 μM N^G-nitro-L-arginine methyl ester (L-NAME), caused a significant, time-dependent increase in leukocyte rolling and adherence compared to control rats superfused with Krebs-Henseleit solution. However, oligotide (15 mg/kg i.v.) consistently reduced the L-NAME-induced leukocyte rolling (62 ± 14 vs. 23 ± 3 cells/min; $P < 0.02$) and adherence (11 ± 2 vs. 4 ± 1 cells/100 μm length of venule $P < 0.01$), without altering systemic blood pressure or mesenteric venular shear rate. Moreover, immunohistochemical localization of P-selectin expression on mesenteric venules was significantly increased ($P < 0.01$) after exposure to L-NAME, which was significantly attenuated by oligotide ($P < 0.05$). Similar results were also obtained by flow cytometric analysis performed on rat platelets. Stimulation of rat platelets with L-NAME significantly ($P < 0.05$) increased the fluorescence intensity of P-selectin, while the concomitant treatment of isolated rat platelets with L-NAME plus oligotide significantly ($P < 0.005$) attenuated P-selectin fluorescence intensity. Our data demonstrate that in vivo administration of oligotide can reduce leukocyte rolling and adherence in the mesenteric rat microvasculature by attenuating P-selectin expression, and confirming the key role of nitric oxide as an important regulator of leukocyte-endothelial cell interaction.

Keywords: Intravital microscopy; Flow cytometry; Blood pressure, mean arterial; Leukocyte rolling; Leukocyte adherence; Venular shear rate

1. Introduction

Leukocyte-endothelial interaction involves a complex interplay among adhesion glycoproteins (i.e., integrins, immunoglobulin superfamily members, and selectins). One member of the selectin family, P-selectin, is rapidly translocated from the Weibel-Palade bodies to the endothelial cell surface upon activation with thrombin, histamine, hypoxia-reoxygenation, or oxygen-derived free radicals (Lorant et al., 1991; McEver et al., 1989; Patel et al., 1991). P-selectin promotes rolling of leukocytes, the first step in leukocyte-endothelial interaction, thus facilitating polymorphonuclear leukocyte activation and adherence (Lorant et al., 1991, 1993). Once activated, leukocytes adhere to the endothelium and start to transmigrate, thus potentiating endothelial dysfunction and tissue injury (Entman et al., 1991; Aoki et al., 1990). The primary early hallmark of this endothelial dysfunction is the reduced release of nitric oxide (NO), which not only regulates vascular tone, but also plays a significant role in modulating leukocyte-endothelial interaction (Kubes et al., 1991). In this regard, we have previously established a functional relationship between the loss of endothelium-derived NO and the expression of P-selectin (Davenpeck et al., 1994). Moreover, organic nitrates, which release NO and act as NO donors (Carey et al., 1992), are able to attenuate endothelial dysfunction during circulatory pertubations (e.g., ischemia followed by reperfusion) with enhanced leukocyte-endothelial interaction. Similarly, blocking NO synthesis via N^G-monomethyl-L-arginine or N^G-nitro-L-arginine methyl ester (L-

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SSDI 0014-2999(95)00695-8

NAME) increases leukocyte adherence and emigration in the mesenteric microcirculation and increases microvascular permeability (Kubes and Granger, 1992; Kurose et al., 1993).

It has been reported that a family of oligonucleotide molecules, represented by defibrotide, is able to stimulate the release of prostacyclin and NO, thus protecting ischemic tissues (Masini et al., 1995). Defibrotide has been found to exert cytoprotective actions in acute inflammatory disorders (Niada et al., 1986; Lefer et al., 1990; Palmer and Goa, 1993). Interestingly, defibrotide has been shown to inhibit both activation and accumulation of leukocytes in ischemia reperfusion-induced tissue injury (DiPerri et al., 1987; Lefer et al., 1990).

Oligotide is a newly discovered single-stranded polydeoxyribonucleotide complex isolated by a controlled depolymerization of mammalian DNA, and comprises a cluster of chains of different length and base sequences (Bianchi et al., 1993; Lanzarotti et al., 1993). The chain length of oligotide is distributed in a Gaussian-like fashion, with a mean molecular weight of 8 ± 2 kDa and with a purine/pyrimidine nucleotide molar ratio of 1.0 ± 0.2 (Lanzarotti et al., 1993). Oligotide is also able to inhibit leukocyte-endothelial interactions, thus preventing traumatic intestinal injury in rats in vivo (Skurk et al., 1995). Nevertheless, the mechanism by which oligotide protects against endothelial dysfunction has not yet been determined.

The aim of the present study was to evaluate whether oligotide could influence L-NAME-induced leukocyte-endothelial cell interaction and P-selectin expression, in the rat mesenteric microvasculature, and to ascertain the role of oligotide on the two key steps in leukocyte-endothelial interaction, namely leukocyte rolling and leukocyte adherence.

2. Materials and methods

2.1. Intravital microscopy

Male Sprague-Dawley rats, weighting 250–275 g, were anesthetized with sodium pentobarbital (35 mg/kg) injected intraperitoneally. A tracheotomy was performed to maintain a patent airway throughout the experiment. A polyethylene catheter was inserted in the left carotid artery to monitor mean arterial blood pressure. Mean arterial blood pressure was recorded on a Grass Model 7 oscillographic recorder using a Statham P23AC pressure transducer (Gould, Cleveland, OH). The abdominal cavity was opened via a midline laparotomy, and a second incision was made through the skin and abdominal musculature on the right flank as decribed earlier (Davenpeck et al., 1994).

A loop of ileal mesentery was exteriorized through the midline incision and placed in a temperature-controlled fluid-filled plexiglass chamber for observation of the mesenteric microcirculation via intravital microscopy. A jugular vein was cannulated for administration of sodium pentobarbital (40 mg/kg and as needed to maintain a surgical plane of anesthesia throughout the experiment). The mesentery was placed over a plexiglass pedestal in the superfusion chamber, and the ileum was secured for stabilization of the viewing field. The ileum and mesentery were superfused throughout the experiment with a modified Krebs-Henseleit solution (containing in mM: 118 NaCl, 4.74 KCl, 2.45 $CaCl_2$, 1.19 KH_2PO_4, 1.19 $MgSO_4$, 12.5 $NaHCO_3$) warmed to 37°C and bubbled with 95% N_2 and 5% CO_2. A Microphot microscope, (Nikon Corp., Tokyo, Japan) with a $40\times$ objective lens and a $10\times$ ocular was used to visualize the mesenteric microcirculation. The image was projected by a video camera (Hamamatsu, Hamamatsu, Japan) onto a black and white Sony high resolution video monitor, and the image recorded with a videocassette recorder. Red blood cell velocity was determined on-line using an optical Doppler velocimeter (Borders and Granger, 1984) obtained from the Microcirculation Research Institute, College Station, TX.

The rats were allowed to stabilize for 20–30 min following surgery. Following stabilization, a 30–50 μm diameter post-capillary venule was chosen for observation. A baseline recording was made to establish basal values for leukocyte rolling and adherence. The mesentery was then superfused with L-NAME (50 μM/l) in modified Krebs-Henseleit (K-H) solution for 120 min. Video recordings were made at 30, 60, 90 and 120 min after initiation of superfusion for quantification of leukocyte rolling and adherence. Oligotide was administered as a bolus (15 mg/kg, i.v.), and immediately thereafter L-NAME superfusion was started. Rats were randomly divided into three groups: (1) control rats which received oligotide, (2) L-NAME-superfused rats, (3) L-NAME-superfused rats treated with oligotide. It was established in several rats that control rats receiving 0.9% NaCl responded identically to control rats receiving oligotide, so that there was no further need to study additional untreated control rats.

The number of rolling and adhered leukocytes, as well as the leukocyte rolling velocity were determined off-line by play-back of the videotape. Leukocytes were considered to be rolling if they were moving at a velocity significantly slower than that of red blood cells. Leukocyte rolling is expressed as the number of cells moving past a designated point per minute (i.e., leukocyte flux). A leukocyte was judged to be adherent if it remained stationary for > 30 s (Granger et al., 1989). Adherence is expressed as the number of adherent leukocytes/100 μm of vessel length. Red blood cell velocity (V) and venular diameter (D) were used to

calculate venular wall shear rate (g) employing the formula $g = 8\,(V_{mean}/D)$ ($V_{mean} = V_{rbc}/1.6$), where V = velocity, and D = diameter (Granger et al., 1989)

2.2. Immunohistochemistry

Immunohistochemical localization of P-selectin was determined after intravital microscopy was completed. Both the superior mesenteric artery and superior mesenteric vein were then rapidly cannulated for perfusion fixation of the small bowel. In other experiments, the mesentery was fixed 30 min after addition of L-NAME, and comparable results were obtained as after 120 min of L-NAME superfusion (data not shown). The ileum was first washed free of blood by perfusion with Krebs-Henseleit buffer warmed to 37°C and bubbled with 95% O_2 and 5% CO_2. Once the venous perfusate was free of red blood cells, perfusion was initiated with iced 4% paraformaldehyde mixed in phosphate-buffered 0.9% NaCl for 5 min. A 3–4 cm long segment of ileum was isolated from the perfused intestine and fixed in 4% paraformaldehyde for 90 min at 4°C. The ileum was then cut into rings, and the tissue dehydrated using graded acetone washes at 4°C. Tissue sections were embedded in plastic (Immunobed: Polysciences, Warrington, PA), and 4 μm thick sections were cut and transferred to Vectabond coated slides (Vector Laboratories, Burlingame, CA).

Immunohistochemical localization of P-selectin was accomplished using the avidin-biotin immunoperoxidase technique (Vectastain ABC Reagent: Vector Laboratories, Burlingame, CA) as previously described by Beckstead et al. (1986) and modified by Weyrich et al. (1993). Tissue sections were treated with 0.25% trypsin (Sigma) to improve reagent penetration, and then incubated with 0.3% hydrogen peroxide for 30 min to remove endogenous peroxide. Blocking serum (horse) was applied to the tissue for 30 min to reduce nonspecific binding, and then the tissue sections were incubated with the primary antibody directed against P-selectin (PB1.3) at a dilution of 1/100 for 24 h. PB1.3 was a generous gift from Dr. J.C. Paulson, Cytel Corp., San Diego, CA. The tissue was then incubated with the biotinylated secondary antibody and the peroxidase staining was carried out using 3,3'-diaminobenzidine. Control preparations consisted of omission of the primary antibody or omission of the secondary antibody. Expression of adhesion molecules was determined by microscopic observation of the brown peroxidase reaction product on the venular endothelium of the tissue sections. Positive staining was defined as a venule displaying brown reaction product on greater than 50% of the circumference of its endothelium. 50 venules per tissue section were examined and the percentage of positive staining venules was tallied.

2.3. Flow cytometric analysis of P-selectin expression on platelets

Flow cytometric analysis of P-selectin expression on rat platelets were performed by a method previously described (Weyrich et al., 1993). In brief, four male Sprague-Dawley rats (0.3–0.4 kg) were anesthetized with intraperitoneal sodium pentobarbital injection (30 mg/kg, i.v.). Blood (5–10 ml) was collected by left ventricular puncture from each animal and was anticoagulated with sodium citrate phosphate buffer (Sigma Chemical Co., St. Louis, MO). Platelet-rich plasma was obtained by centrifuging the blood at $300 \times g$ for 20 min. The platelet rich plasma was then centrifuged at $2000 \times g$ for 10 min to form a platelet-rich pellet. This pellet was washed twice in Ca^{2+}-free Tyrode's solution containing 0.2% bovine serum albumin. The final cell pellet was resuspended in Dulbecco's phosphate buffer saline (DPBS) containing 4 mM Ca^{2+}.

Aliquots of platelet suspensions were stimulated with L-NAME (1 mM) at 37°C for 15 min without stirring in the presence or absence of oligotide (0.5 and 1 mg/ml). Some aliquots were incubated with DPBS (non-stimulated control platelets). Subsequently, platelets were fixed with an equal volume of 2% paraformaldehyde in PBS at pH 7.2 and washed twice with DPBS. The platelet suspensions were treated with human block immunoglobulin G (IgG) (4.0 mg/ml, Sigma Chemical Co.) and then the primary anti-P-selectin monoclonal antibody PB 1.3 (20 μg/ml, Cytel Corp., San Diego, CA) was added to the platelet suspensions, and maintained at 4°C for 60 min. The platelets were then washed in DPBS with 0.2% bovine serum albumine to remove any excess of primary antibody. F(ab')2 fragments of a goat anti-mouse IgG-phycoerythrin conjugate (Tago, Burlingame, CA) was used as the secondary antibody at a 1:100 dilution, and the cells were maintained at 4°C for 30 min. The stained platelets were washed twice with DPBS with bovine serum albumine and finally fixed in 1% paraformaldehyde, and then analyzed by flow cytometry (FACScan, Becton-Dickinson, San Jose, CA).

3. Results

3.1. Intravital microscopy

Superfusion of the rat mesentery with 50 μM L-NAME resulted in a time-dependent increase in leukocyte rolling and adherence in postcapillary venules of the rat mesenteric microvasculature (Figs. 1 and 2). There was no significant difference in the initial mean arterial blood pressures among the three groups of rats, and no significant changes in mean arterial blood pressure occurred over the 120 min intravital mi-

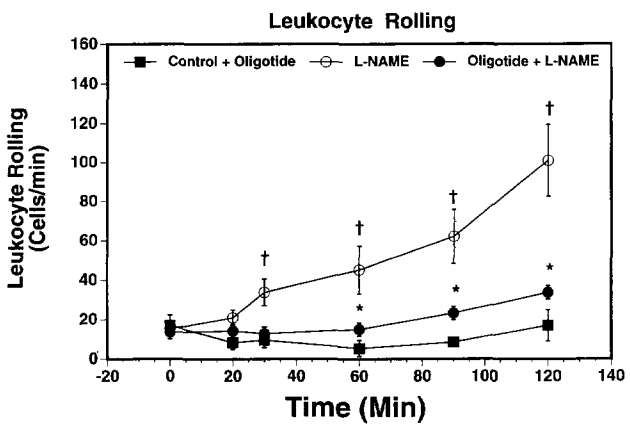

Fig. 1. Leukocyte rolling in rat mesenteric venules observed in control rats, rats subjected to 50 μM N^G-nitro-L-arginine methyl ester (L-NAME), or to L-NAME + oligotide (15 mg/kg bolus). All values are means ± S.E.M. for six rats in each group. L-NAME clearly increased WBC rolling from 30 to 120 min. This was significantly attenuated by oligotide from 60 to 120 min. $^{\dagger}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. L-NAME. Oligotide was given at 0 min.

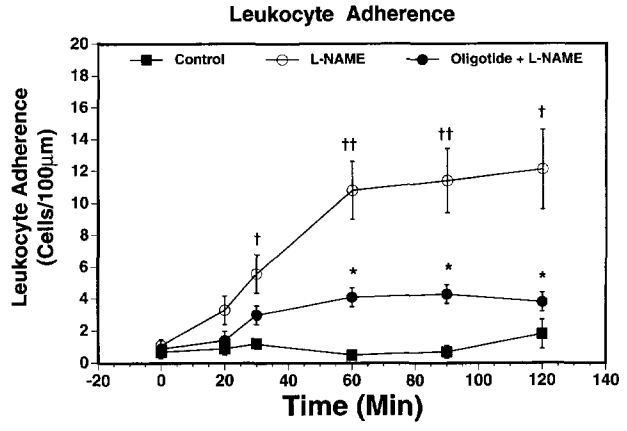

Fig. 2. Leukocyte adherence in rat mesenteric venules from either control rats, rats subjected to 50 μM N^G-nitro-L-arginine methyl ester (L-NAME), or to L-NAME + oligotide (15 mg/kg bolus). All values are means ± S.E.M. for six rats in each group. L-NAME significantly increased WBC adherence from 30 to 120 min. This was significantly attenuated by oligotide at 60–120 min. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. control, $^{*}P < 0.05$ vs. L-NAME. Oligotide was given at 0 min.

croscopy observation period (Fig. 3). Additionally, the venular shear rate was calculated in the three experimental groups (data not shown). There was no significant difference in shear rates among the three groups, indicating that the adhesive interactions observed between leukocytes and endothelial cells were not due to changes in physical hydrodynamic forces, brought about by the infusion of the oligotide, or to spontaneous hemodynamic alterations. Oligotide (15 mg/kg i.v.) consistently reduced the L-NAME-induced leukocyte rolling (62 ± 14 vs. 23 ± 3 cells/min; $P < 0.02$) and adherence (11.4 ± 2 vs. 4 ± 1 cells/100 mm; $P < 0.01$).

3.2. Immunohistochemistry

Localization of P-selectin was accomplished using a modified avidin biotin immunoperoxidase technique. Positive staining was observed only on the venular endothelium in the rat ileum. The percentage of venules staining positive for P-selectin in ileal sections from sham-operated control rats was consistently low (16% ± 3%) (Fig. 4). Superfusion of the mesentery and ileum with 50 μM L-NAME for 120 min resulted in an increase in P-selectin expression as quantified by the percentage of venules staining positive for P-selectin

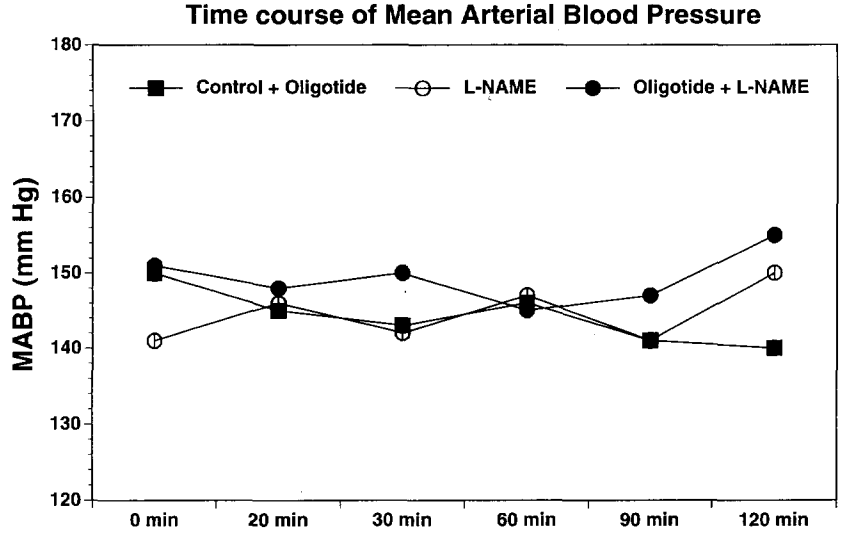

Fig. 3. Mean arterial blood pressures of the three experimental groups of rats. All values are means ± S.E.M. for five to six rats in each group. Oligotide was given at 0 min.

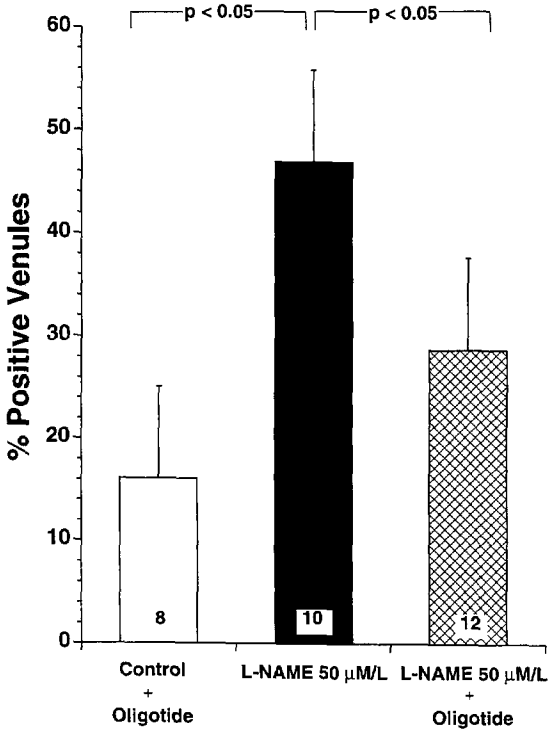

Fig. 4. Percent of positive staining venules for P-selectin in the three experimental groups of rats. Bar heights represent mean values; brackets indicate ± S.E.M.; numbers at base of bars indicate numbers of histological preparations studied from three to four rats in each group.

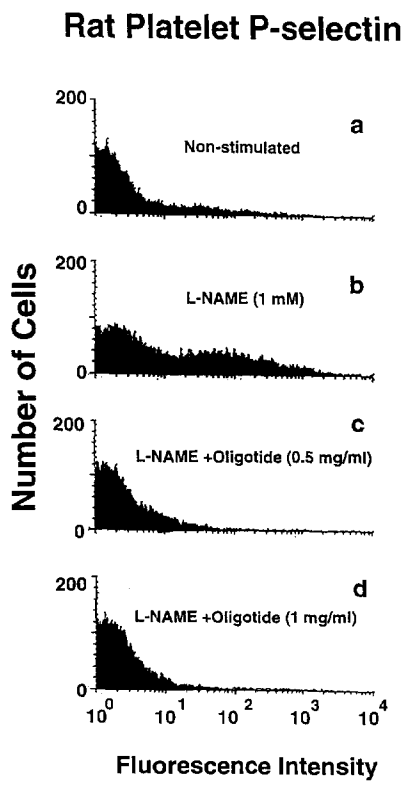

Fig. 5. Representative fluorescence histograms of L-NAME-stimulated rat platelets incubated with anti-P-selectin monoclonal antibody (PB 1.3) and labeled with phycoerythrin conjugated secondary antibody. L-NAME (50 μM) markedly increased the number of P-selectin positive cells as well as the mean channel fluorescence. Preincubation of oligotide (0.5 or 1 mg/ml) with platelets attenuated both the number of cells positive for P-selectin and the mean channel fluorescence after stimulation with L-NAME. The control histogram in the upper panel represents a histogram obtained omitting the primary antibody.

(47% ± 9.6%; $P < 0.05$). This represents a significant increase in the surface expression of P-selectin under these conditions. This increase in expression of P-selectin on mesenteric venules was significantly attenuated by the simultaneous i.v. infusion of 15 mg/kg oligotide (Fig. 4). Thus, oligotide suppressed P-selectin expression on the endothelial cell surface of the rat mesenteric microvasculature.

Table 1
Flow cytometric analysis of P-selectin expression on rat platelets

Condition	% positive cells	Mean channel fluorescence
Without stimulation	6 ± 2	4.4 ± 0.8
L-NAME (1 mM)	43 ± 7 [a]	25 ± 4 [a]
L-NAME + oligotide (0.5 mg/ml)	25 ± 8 [b]	12 ± 3 [b]
L-NAME + oligotide (1 mg/ml)	2 ± 0.1 [b]	3 ± 0.1 [b]

Rat platelets were stimulated with L-NAME for 20 min at 37°C. Effects of oligotide (0.5 and 1 mg/ml) on L-NAME-induced P-selectin expression are shown. [a] $P < 0.01$ vs. control non-stimulated platelets; [b] $P < 0.05$ vs. L-NAME. Data are means ± S.E.M. of the two independent experiments, and each one consisted of triplicate determinations.

3.3. Flow cytometric analysis of P-selectin expression

We examined the in vitro effects of oligotide on rat platelet P-selectin expression after stimulation with 1 mM L-NAME. Fig. 5 shows representative fluorescence histograms of P-selectin expressed on rat platelets. Non-stimulated rat platelets exhibited very little platelet surface binding of the P-selectin monoclonal antibody (Fig. 5a and Table 1). However, after incubation with 1 mM L-NAME, the binding of MAb PB 1.3 to platelets was significantly increased (Fig. 5b). Ten min preincubation of rat platelets with oligotide (0.5–1 mg/ml) significantly attenuated L-NAME-induced P-selectin expression (Fig. 5b,c; Table 1). Thus oligotide specifically inhibited P-selectin expression on cell surfaces, a finding which may be of considerable significance in explaining the mechanism of oligotide's effect on the mesenteric microvasculature.

4. Discussion

The present study clearly demonstrates that 15 mg/kg oligotide given intravenously is able to attenuate L-NAME-induced leukocyte-endothelial cell interaction, via a P-selectin dependent mechanism. A decrease in NO production by the vascular endothelium has been shown to increase P-selectin expression and, consequently enhance P-selectin-mediated leukocyte-endothelial interaction (Davenpeck et al., 1994). L-NAME has been effectively used as an upregulator of leukocyte-endothelial interaction, since the increased adherence mediated by L-NAME is not due to any direct leukocyte activation. This is because NO synthase inhibitors do not result in increased expression of leukocyte β_2 integrins (i.e., CD11/CD18) (Kubes et al., 1991). Despite the fact that L-NAME inhibits NO synthase primarily in endothelial cells, the up-regulation of P-selectin leads to enhanced leukocyte-endothelial interaction which only later leads to leukocyte activation. Activated leukocytes are able to release cytotoxic oxygen metabolites, proteolytic enzymes, and cytokines such as tumor necrosis factor alpha (TNF-α). Superoxide radicals released from leukocytes have been shown to inactivate NO (Rubanyi and Vanhoutte, 1987), induce vasoconstriction (Murohara et al., 1994), and disrupt cellular membranes through lipid peroxidation. In addition, leukocyte aggregates may participate in microvascular plugging leading to further tissue injury (Kloner et al., 1974; Engler et al., 1986).

In our study, oligotide significantly reduced L-NAME-induced P-selectin expression in both mesenteric endothelial cells and isolated platelets. Recently, it has been demonstrated that oligotide-like molecules can increase NO and prostacyclin release in the guinea pig coronary circulation. This mechanism can help explain why oligotide inhibits P-selectin expression in our model. In fact, several investigators have shown that exogenously administered NO (Gauthier et al., 1994) or prostacyclin (Rosen et al., 1994) can attenuate leukocyte rolling and adherence by decreasing P-selectin expression. The contribution of prostacyclin to the attenuation of P-selectin expression is probably only of secondary significance since prostacyclin exerts only a small direct effect but synergizes with NO in terms of its endothelial actions (Radomski et al., 1987). Moreover, a polydeoxyribonucleotide-based drug has been reported to act as a thrombin inhibitor via an aptamer sequence (e.g. 5′-GGTTGGATTGGTTGG-3′) (Bracht and Schrör, 1994). Since thrombin has been shown to promote P-selectin-mediated leukocyte adherence to the coronary endothelium (Weyrich et al., 1993; Murohara et al., 1994), it is conceivable that oligotide attenuates P-selectin expression and this may be related to potential antagonistic activity of oligotide on thrombin receptors.

Activated leukocytes release superoxide radicals which can directly quench endogenous NO released by endothelial cells (Rubanyi and Vanhoutte, 1987; Ma et al., 1993), a process known to result in endothelial dysfunction. Interestingly, oligotide has been shown to inhibit direct Ca^{2+}-dependent neutrophil activation and subsequent release of oxygen free radicals (DiPerri et al., 1987; Cirillo et al., 1991). Thus, it is also likely that oligotide may downregulate P-selectin expression on the endothelial cell by inhibition of leukocyte release of superoxide radicals (e.g., hydrogen peroxide). Taken together, these in vivo and in vitro data clearly support the concept that oligotide inhibits surface expression of P-selectin on both endothelial cells and platelets. This may be a key mechanism by which oligotide inhibits leukocyte-endothelial interaction, thus contributing to the attenuation of endothelial dysfunction in several different models of ischemia-reperfusion injury, including mesenteric and myocardial ischemia.

In conclusion, this is the first study to demonstrate that in vivo administration of oligotide, a newly developed single stranded polydeoxyribonucleotide complex, inhibits leukocyte rolling and adherence in the rat mesenteric circulation. This activity was found to be mediated via down-regulation of P-selectin present in both endothelial cells and platelets. Polydeoxyribonucleotides may represent a new class of pharmacological agents for studying leukocyte-endothelial cell interaction, and may also represent a new strategy for modulating the pathophysiology of leukocyte-induced endothelial dysfunction in circulatory disorders.

Acknowledgements

Supported by Research Grant No. GM45434 from the National Institute of General Medical Sciences of the N.I.H. T.M. was supported in part by the Japan Heart Foundation, Tokyo, Japan. We thank Drs. Marisa Mantovani, Rodolfo Pescador and Roberto Porta (Crinos Biological Research Laboratories, Villa Guardia, Como, Italy) for the generous supply of the oligotide, and Dr. James C. Paulson (Cytel Corp., San Diego, CA) for supplying the anti-P-selectin MAb PB 1.3. The authors gratefully acknowledge Robert Craig for his technical assistance.

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