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Varying Roles of E-Selectin and P-Selectin in Different Microvascular Beds in Response to Antigen¹

Michael J. Hickey,* Samina Kanwar,* Donna-Marie McCafferty,* D. Neil Granger,[†] Michael J. Eppihimer,[‡] and Paul Kubes^{2*}

Expression of E-selectin and P-selectin is critical in the effector phase of leukocyte recruitment in response to Ag. Whether their relative roles differ between tissues in response to the same Ag is unknown. In this study, a type I hypersensitivity response was elicited in C57BL/6 mice by systemic sensitization with OVA. Following local Ag challenge, endothelial selectin expression was examined in the skin and cremaster muscle microvasculature using a dual-radiolabeled mAb technique. Next, the dermal and muscle microcirculations were visualized using intravital microscopy to establish roles for P-selectin and/or E-selectin. In untreated mice, leukocyte recruitment in both skin and skeletal muscle was mediated entirely by P-selectin. Following Ag challenge, leukocyte rolling flux and adhesion were dramatically increased and leukocyte rolling velocity was unchanged in muscle. Only P-selectin expression increased in muscle, and leukocyte recruitment was entirely dependent upon this selectin. In contrast, in Ag-challenged skin, leukocyte rolling flux did not increase, but rolling velocity dropped profoundly. In skin, only E-selectin expression increased, and blockade of either E-selectin or P-selectin had minimal effect on either rolling flux or rolling velocity. Blockade of both selectins reduced rolling flux by 80% and increased rolling velocity sevenfold. These data highlight striking differences in expression of the endothelial selectins in separate microvascular beds in response to the same stimulus and demonstrate that these differences underlie very different patterns of leukocyte recruitment. The data underscore the importance of studying individual microvascular beds to understand tissue-specific leukocyte recruitment in vivo. *The Journal of Immunology*, 1999, 162: 1137–1143.

Leukocyte recruitment is a very precise series of events that is initiated by endothelial selectins including P-selectin and E-selectin (1). These molecules tether leukocytes to the vessel wall to initiate rolling along the endothelial surface. Rolling leukocytes can then be activated to firmly adhere to the endothelium via integrins and members of the Ig superfamily (1). Interest in the endothelial selectins stems from the fact that complete inhibition of leukocyte rolling also prevents induction of adhesion and transmigration (2, 3) and therefore may be a useful anti-inflammatory therapeutic intervention. In vivo, intravital microscopy of the mesentery or cremaster muscle microvasculature has served to reinforce this view; direct visualization of the microvasculature has established that the selectins play an important role in leukocyte rolling leading to subsequent adhesion (4–6). Generic paradigms of leukocyte recruitment are inferred from these tissues, but distinct patterns of selectin expression may in fact occur in different tissues for different stimuli and perhaps even to the same stimulus. Moreover, the possibility also exists that differential expression of endothelial selectins may also underlie

very different patterns of leukocyte recruitment within a particular vascular bed. Quantitation of endothelial selectin expression in combination with visualization of leukocyte recruitment in the same vascular bed are absolute requirements to the understanding of endothelial selectin expression paradigms that mediate patterns of leukocyte recruitment.

In vitro, there is ample evidence that the same endothelium stimulated with different stimuli will induce different patterns of endothelial selectin expression. A very good example to date is TNF- α , which causes profound expression of E-selectin but not P-selectin over the first 24 h in human umbilical vein endothelium (7), whereas IL-4 induces expression of VCAM-1 and perhaps P-selectin but not E-selectin (8, 9) over this same time frame in the same cells. Heterogeneity in the induction of the adhesion molecules can also be seen in different endothelial cells. Activation with protein kinase C induced significant E-selectin expression in human umbilical vein endothelium but not in dermal microvascular endothelium (10). Adhesion molecule expression also differs distinctly between stimulated human umbilical vein endothelium and intestinal microvascular endothelium (11). There is less evidence in vivo that distinct patterns of selectin expression exist in different vascular beds. Eppihimer et al. reported increases in both P-selectin and E-selectin expression in all organs in response to LPS (12). Ley and colleagues have demonstrated that TNF- α induces leukocyte rolling dependent upon both P-selectin and E-selectin in the cremaster muscle (13, 14). Clearly, administration of exogenous LPS and TNF- α appear to induce expression of both endothelial selectins in numerous tissues, and, at least in the cremaster microvasculature, they both contribute to leukocyte rolling.

Although these studies are seminal in demonstrating the importance of both selectins, exogenous application of TNF- α and LPS almost certainly do not reflect all responses in all tissues. In this study, we examined the endogenous immune response to foreign

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Ag in both the cremaster muscle and the skin microvasculature. To achieve this aim, we used a combination of two approaches to quantitate responses to Ag: 1) we quantitatively measured selectin expression using a radiolabeled-mAb technique, and 2) we examined leukocyte recruitment using intravital microscopy in skin and skeletal muscle of sensitized mice. We report diametrically opposing responses in the expression of the two endothelial selectins in the two microvascular beds in response to Ag and demonstrate that these differences underlie striking differences in the basic mechanisms of leukocyte recruitment within a particular vascular bed.

Materials and Methods

Animals

All animals used in this study were male C57BL/6 mice weighing between 20–35 g and were used between 6 and 10 wk of age.

Sensitization and challenge protocol

To develop a model of acute, allergic inflammation, a type I hypersensitivity reaction was elicited by systemically (i.p. injection) sensitizing animals with 10 μ g chicken OVA (Sigma, St. Louis, MO) and 10 mg Grade V aluminum hydroxide (AIOH; Sigma) in a total volume of 0.2 ml saline. Two weeks later, animals were locally challenged, either intradermally or intrascrotally, with the sensitizing Ag. Intrascrotal injection localizes the Ag solution beneath the scrotal skin directly adjacent to the cremaster muscle and results in the Ag solution directly bathing the cremaster tissue. In the skin, challenge with 10 μ g OVA had no effect on leukocyte rolling flux; therefore, another series of animals were challenged with 100 μ g OVA. Sham sensitization and sham challenge involved systemic and local injection of 0.2 ml saline, respectively. The animals were prepared for either fluorescent or light intravital microscopy to visualize the dermal (skin flap) or skeletal (cremaster) muscle microcirculations, respectively. Leukocyte kinetics were quantified in untreated mice and at 4 h post-Ag challenge in the skin flap and cremaster muscle.

Intravital microscopy

Animals were anesthetized by i.p. injection of a mixture of 10 mg/kg xylazine (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and 200 mg/kg ketamine hydrochloride (Rogar/STB, London, Ontario, Canada). The left jugular vein was cannulated to administer anesthetic, fluorescent dyes, and various drugs. Animals were then prepared as follows to view either the dermal (skin flap) or skeletal muscle microcirculation.

Skin flap preparation. A midline abdominal incision was made beginning at the level of the diaphragm and extending to the pelvic region. The skin was carefully separated from the underlying tissue, remaining attached laterally. Blood supply to the skin flap remained intact. The skin flap was then extended over a viewing pedestal, secured along the edges using 4-0 suture exposing the dermal microvasculature. The exposed skin was continuously superfused with bicarbonate-buffered saline to avoid tissue dehydration. Due to the thickness of the skin flap, leukocyte-endothelial cell interactions were not visible by transillumination. Therefore, for this protocol, animals were injected with the fluorescent dye, rhodamine 6G (0.3 mg/kg i.v.; Sigma), immediately before microscopic visualization. Rhodamine 6G at the dose used labels leukocytes and platelets and has been shown to allow detection of the same number of rolling leukocytes as transmitted light and have no effect on leukocyte kinetics (15, 16). Therefore, it allows for quantification of leukocyte rolling flux, leukocyte rolling velocity, and leukocyte adhesion via epifluorescence microscopy. Rhodamine 6G-associated fluorescence was visualized by epi-illumination at 510–560 nm using a 590-nm emission filter (16, 17). Although window chambers have been used to study the dermal microcirculation, this is the first documentation of this acute method of examination of the skin microcirculation.

Cremaster muscle preparation. An incision was made in the scrotal skin to expose the left cremaster muscle, which was then carefully removed from the associated fascia. A lengthwise incision was made on the ventral surface of the cremaster muscle. The testicle and epididymis were separated from the underlying muscle and reintroduced into the abdominal cavity. The muscle was then spread out over an optically clear viewing pedestal and secured along the edges with 3-0 suture. The exposed tissue was superfused with warm bicarbonate-buffered saline (pH 7.4).

The dermal and cremasteric microcirculations were observed through an intravital microscope (Nikon-Optiphot-2, Japan) with a 40 \times water immersion lens (skin flap; 40/0.55 WI; Nikon, Tokyo, Japan) or a 25 \times objective lens (skeletal muscle; Leitz Wetzlar L25/0.35) and a 10 \times eyepiece. The

image of the dermal microcirculatory bed was recorded using a silicon-intensified fluorescent camera (model C-2400-08; Hamamatsu Photonics, Hamamatsu City, Japan), and the cremasteric microcirculation was recorded using a video camera (Panasonic-Digital 5100, Panasonic, Secaucus, NJ). Images of the dermal and skeletal muscle microcirculation were recorded before and after administration of various Abs in untreated mice and 4 h after Ag challenge. All experimental parameters were quantified at these time points.

Single unbranched venules (20–40 μ m in diameter) were selected for each study. Venular diameter (D_v) was measured using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). Rolling leukocytes were defined as those leukocytes that rolled at a velocity slower than that of RBC. Leukocyte rolling velocity was measured for the first 20 leukocytes entering the field of view at the time of recording and was determined as the time required for a leukocyte to traverse a given length of venule. Leukocyte adhesion was quantified as the number of leukocytes that adhered to the vessel wall for 30 s or more within a given segment of the vessel. RBC velocity (V_{rbc}) was measured online using an optical Doppler velocimeter (Microcirculation Research Institute) and was only measured for the skeletal muscle preparation, as determination of RBC velocity using fluorescence in the skin flap was not possible. Venular blood flow in the skeletal muscle was calculated from the product of cross-sectional area and mean RBC velocity ($V_{mean} = V_{rbc}/1.6$), assuming cylindrical geometry. Venular wall shear rate ($\dot{\gamma}$) was calculated based on the Newtonian definition, $\dot{\gamma} = 8(V_{mean}/D_v)$, and venular wall shear stress was $\dot{\gamma} \times$ blood viscosity, where blood viscosity was assumed to be 0.025 poise (18).

Experimental protocol

In the first series of experiments leukocyte kinetics were established in the dermal microcirculation of untreated mice. In a separate series of mice, an Ag-induced late-phase response was elicited in the skin, and leukocyte rolling, leukocyte rolling velocity, and leukocyte adhesion in the challenged area were examined upon exteriorization 4 h later. A role for selectins in leukocyte recruitment in the skin flap was studied in both untreated skin and following Ag-challenge. After an initial recording, animals were treated i.v. with an anti-P-selectin Ab (RB40.34; PharMingen, San Diego, CA; 20 μ g/animal), an anti-E-selectin Ab (9A9; 100 μ g/animal, generously provided by Dr. Barry Wolitzky, Hoffmann-La Roche Pharmaceuticals, Nutley, NJ), or both Abs simultaneously. Leukocyte kinetics were assessed before and after administration of these Abs. An identical protocol was followed for the mouse cremasteric microvasculature. Analysis of blood samples from animals treated with the Abs showed that Ab administration did not affect circulating leukocyte counts.

Passive cutaneous anaphylaxis (PCA)³ reaction

Serum was obtained from all OVA-sensitized animals at the end of the experiment by intracardiac puncture. Serial dilutions (1/8–1/64) of the serum samples were prepared, and 200 μ L of each sample was injected intradermally into the shaved backs of control, untreated mice and Sprague-Dawley rats. Serum from sensitized mice elicited the same response in Sprague-Dawley rats as it did in C57BL/6 mice. Therefore, rats were used for all subsequent PCA reactions primarily because PCA was easier to detect. After 72 h, animals were challenged with an intracardiac injection of a solution containing 2.5 mg Evan's Blue dye and 5 mg chicken OVA in a total volume of 1.5 ml (saline). The final reaction was read 60 min later as the highest dilution that produced a distinct blue region (Evan's Blue dye extravasation) at the center of the injection site (19). Sensitized animals had serum anti-OVA Ab titers of at least 1/64, whereas sham-sensitized animals had no anti-OVA Abs.

P- and E-selectin expression in the skin and skeletal muscle

In an additional series of experiments, expression of P-selectin and E-selectin was quantified in the skin and skeletal muscle of untreated mice and at 4 h post-Ag challenge. This was accomplished using a modified dual-radiolabeled Ab technique (12). Briefly, animals were injected i.v. with a mixture of either 10 μ g ¹²⁵I-anti-P-selectin (RB40.34) or 10 μ g ¹²⁵I-anti-E-selectin (10E6, generously provided by Dr. Barry Wolitzky, Hoffmann-La Roche Pharmaceuticals) Abs, and a variable dose of ¹³¹I-labeled nonbinding Ab (P-23) calculated to achieve a total injected ¹³¹I activity of 400,000–600,000 cpm (total volume 200 μ L). The Abs were allowed to circulate for 5 min, then the animals were heparinized. A blood sample was obtained from a carotid artery catheter, then the mice were

³ Abbreviations used in this paper: PCA, passive cutaneous anaphylaxis; LTC₄, leukotriene C₄; PAF, platelet-activating factor.

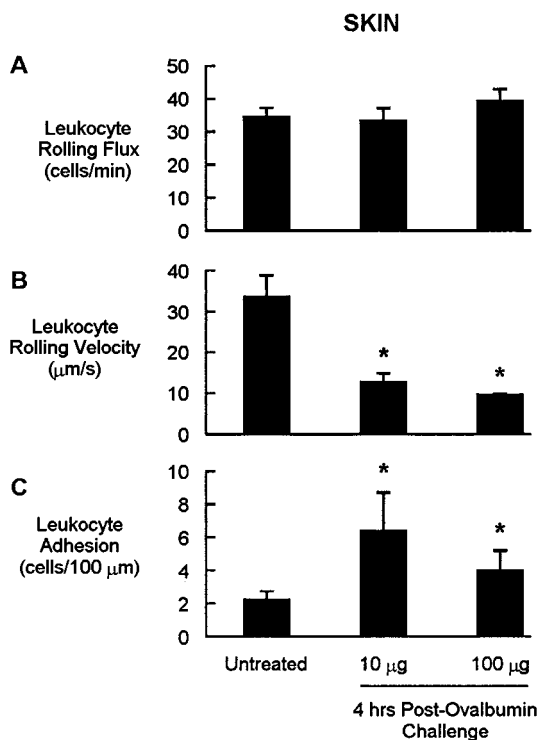


FIGURE 1. Leukocyte rolling flux (A), leukocyte rolling velocity (B), and leukocyte adhesion (C) in dermal postcapillary venules in untreated animals ($n = 9$) and in sensitized animals that were intradermally challenged 4 h previously with either 10 ($n = 12$) or 100 μg ($n = 3$) OVA. *, $p < 0.05$ relative to untreated value.

exsanguinated by blood withdrawal through the carotid artery catheter and simultaneous i.v. infusion with bicarbonate-buffered saline. Whole organs (cremaster muscle and dorsal skin flap) were harvested and weighed. Both ^{131}I and ^{125}I activity were measured in plasma and tissue samples. Both P- and E-selectin expression were calculated per gram of tissue by subtracting the accumulated activity of the nonbinding Ab (^{131}I P-23) from the accumulated activity of the binding Ab (^{125}I RB40.34 or ^{125}I 10E6). Data for P- or E-selectin expression was represented as the percent of the injected dose of Ab per gram of tissue. We have previously demonstrated that this approach provides reliable quantitative values of adhesion molecule expression, that radiolabeled binding Ab can be displaced specifically with sufficient amounts of unlabeled Ab, and that values not different from zero are obtained for P-selectin in P-selectin-deficient mice and for E-selectin in E-selectin-deficient mice (12).

Circulating leukocyte counts

At the end of each experiment, whole blood was drawn via cardiac puncture. Total leukocyte counts were performed using a Bright-line hemocytometer (Hausser Scientific, Horsham, PA) in untreated and sensitized mice.

Statistical analysis

Data are presented as mean \pm SEM. A student's t test with bonferroni correction was used for multiple comparisons. Statistical significance was set at $p < 0.05$.

Results

Ag challenge in skin reduces leukocyte rolling velocity and increases leukocyte adhesion

All Ag-sensitized animals in this study had anti-OVA Ab titers of at least 1/64, as assessed by PCA reaction. Fig. 1 illustrates the kinetics of leukocyte recruitment in the dermal microvasculature of untreated and Ag-sensitized mice at 4 h post-Ag challenge. In untreated animals, there were ~ 30 rolling cells per min in dermal postcapillary venules (Fig. 1A). Intradermal OVA challenge (10

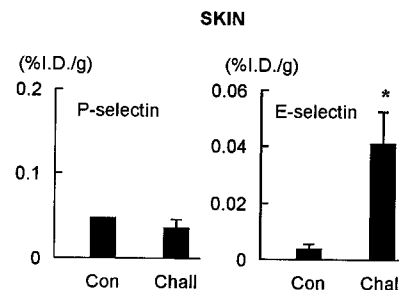


FIGURE 2. Expression of P-selectin (left) and E-selectin (right) in skin in untreated mice (Con) and 4 h after local Ag challenge in sensitized animals (Chall). *, $p < 0.05$ relative to untreated value (Con: $n = 3$; Chall: $n = 5-8$).

μg) in sensitized animals caused no change in leukocyte rolling flux. To determine whether a higher concentration of Ag would induce an increase in rolling flux, another series of animals was challenged with 100 μg of OVA. This concentration also had no effect on the leukocyte rolling flux (Fig. 1A), suggesting either that Ag challenge in the skin does not increase rolling flux or that insufficient stimulus was being used. The latter seems unlikely as Ag challenge at both doses induced dramatic changes in leukocyte rolling velocity (Fig. 1B). In untreated animals, leukocytes rolled at a velocity of ~ 35 $\mu\text{m/s}$. Intradermal Ag challenge with 10 μg OVA dramatically reduced (60–70%) leukocyte rolling velocity ($p < 0.05$). A 10-fold higher dose of Ag did not further reduce leukocyte rolling velocity. Leukocyte adhesion in dermal postcapillary venules was significantly increased 4 h post-Ag challenge (10 μg) relative to untreated animals (Fig. 1C). As 10 μg induced a higher number of adherent cells than the 100 μg concentration, 10 μg was used for all additional experiments including those in muscle. Accumulation of platelets within the challenged dermal microvasculature was undetectable by intravital microscopy. Comparison of circulating leukocyte counts in control mice ($6.3 \pm 2.8 \times 10^6/\text{ml}$) vs sensitized mice 4 h after challenge ($9.5 \pm 3.4 \times 10^6/\text{ml}$) suggests that Ag challenge may have induced release of leukocytes from the noncirculating pool.

Molecular mechanisms underlying leukocyte rolling in dermal venules post-Ag challenge

Fig. 2 demonstrates that there is some P-selectin expression in skin under basal conditions, and that this did not increase following Ag stimulation. In direct contrast, E-selectin expression 4 h after Ag challenge increased ~ 10 -fold (Fig. 2). Blockade of E-selectin alone had no effect on the leukocyte rolling flux at 4 h post-Ag challenge in skin (Fig. 3A). The effect of P-selectin blockade was also unremarkable and reduced leukocyte rolling flux by only 30% in dermal postcapillary venules (Fig. 3A). However, tandem inhibition of both E- and P-selectin function reduced leukocyte rolling flux in the skin by 80% at 4 h post-Ag challenge. Interestingly, a small amount of rolling persisted in the skin even in the presence of E-selectin and P-selectin Abs, suggesting an additional rolling pathway in the Ag-challenged dermal microvasculature.

The very low leukocyte rolling velocity in the skin microvasculature following Ag challenge was not affected significantly when either P-selectin or E-selectin function alone was blocked (Fig. 3B); only a 1.5- to 2.0-fold increase in rolling velocity was noted (NS). Blockade of both endothelial selectins caused a striking sevenfold increase in leukocyte rolling velocity, suggesting that E-selectin and P-selectin were working in tandem to induce the dramatic reduction in rolling velocity in Ag-challenged dermal microvessels.

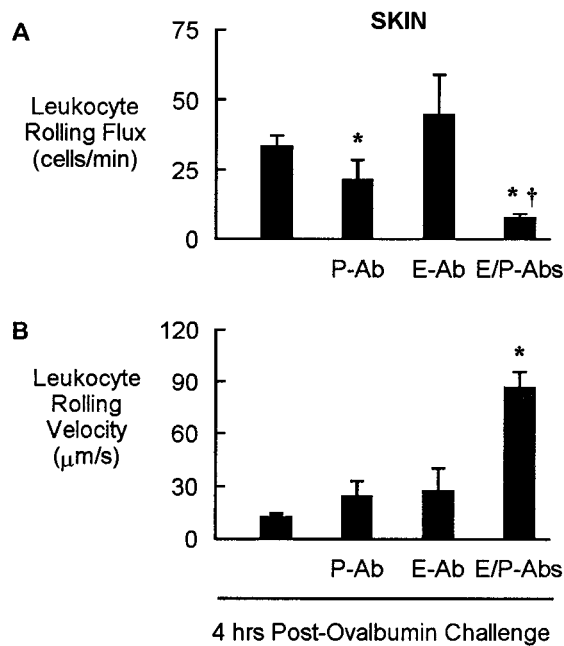


FIGURE 3. The effect of an anti-P-selectin ($n = 4$), anti-E-selectin ($n = 4$), or a combination of anti-E- and P-selectin Abs ($n = 6$) on the leukocyte rolling flux (A) and leukocyte rolling velocity (B) in dermal postcapillary venules 4 h after local Ag challenge in sensitized mice. *, $p < 0.05$ relative to untreated value. †, $p < 0.05$ relative to P-Ab alone.

Ag-induced leukocyte rolling profile in cremaster microvasculature differs from skin

The kinetics of leukocyte recruitment in the cremaster microvasculature of untreated and Ag-sensitized mice at 4 h post-Ag challenge are shown in Fig. 4. In untreated animals, there were ~50 cells/min rolling in muscle postcapillary venules. Local challenge with chicken egg OVA ($10 \mu\text{g}$) in sensitized mice caused a large increase (200 cells/min) in leukocyte rolling flux (Fig. 4A). This is in direct contrast to the small flux of rolling cells (<40 cells/min) in Ag-challenged dermal postcapillary venules. However, unlike the response in dermis, leukocyte rolling velocity was not significantly altered in the cremasteric microvasculature (Fig. 4B). Leukocyte adhesion was increased significantly in this microvasculature 4 h post-Ag challenge (Fig. 4C). Similar to the response in dermal vessels, no platelet accumulation was discernible in the cremasteric microvasculature following challenge.

Molecular mechanisms underlying leukocyte rolling in cremaster venules post-Ag challenge

Similar to the skin microvasculature, there were constitutive levels of P-selectin expression but very low amounts of E-selectin expression in muscle (Fig. 5). In striking contrast to skin, the muscle microvasculature responded to Ag challenge by significantly increasing P-selectin expression without an increase in E-selectin expression (Fig. 5). Consistent with this observation were the functional data that blockade of P-selectin function eliminated leukocyte rolling (Fig. 6). Because an anti-P-selectin Ab alone completely inhibited leukocyte rolling in the cremaster, the combination of E-selectin and P-selectin was not performed. E-selectin blockade did not affect leukocyte rolling flux (Fig. 6) and leukocyte rolling velocity remained unchanged (data not shown). Blockade of P-selectin and/or E-selectin had no effect on the existing leukocyte adhesion in either tissue (data not shown) consistent with the view that the selectins mediate rolling but not adhesion.

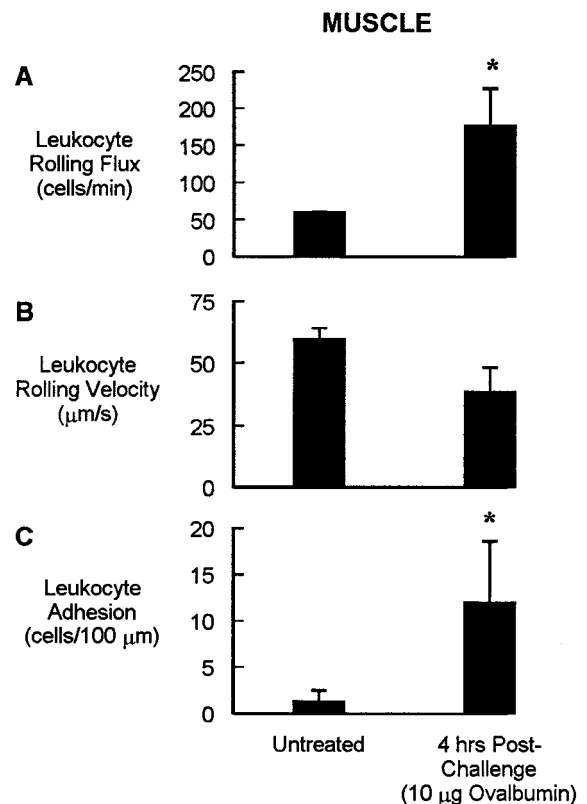


FIGURE 4. Leukocyte rolling flux (A), leukocyte rolling velocity (B), and leukocyte adhesion (C) in skeletal muscle (cremaster) postcapillary venules in untreated animals ($n = 4$) and in sensitized animals that were intracrotally challenged 4 h previously with $10 \mu\text{g}$ ($n = 4$) OVA. *, $p < 0.05$ relative to untreated value.

Constitutive levels of P-selectin impart a functional role in skin and muscle

Leukocyte kinetics in sham-sensitized and sham-challenged animals were identical to untreated animals in both tissues (data not shown). Inhibition of P-selectin function in untreated mice eliminated leukocyte rolling flux in both skin and muscle (Fig. 7). This is consistent with previous work showing that constitutive values for P-selectin expression in various tissues of wild-type mice were greater than P-selectin values obtained from P-selectin-deficient

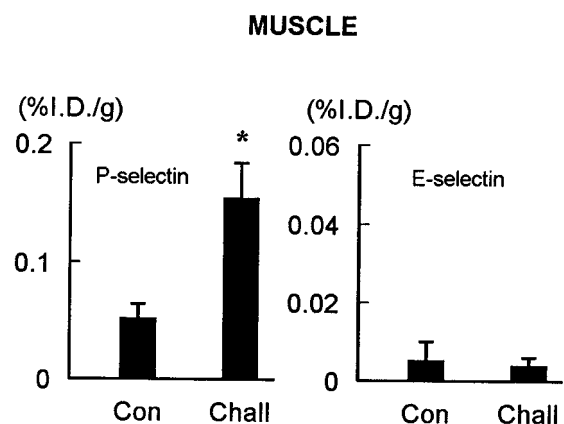


FIGURE 5. Expression of P-selectin (left) and E-selectin (right) in cremaster muscle in untreated mice (Con) and 4 h after local Ag challenge in sensitized animals (Chall). *, $p < 0.05$ relative to untreated value (Con: $n = 3$; Chall: $n = 5-8$).

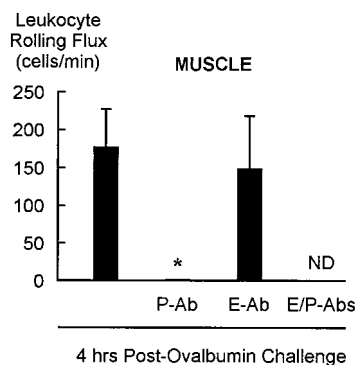


FIGURE 6. The effect of an anti-P-selectin or anti-E-selectin Ab on the leukocyte rolling flux in cremasteric postcapillary venules 4 h after local Ag challenge in sensitized mice. *, $p < 0.05$ relative to untreated value.

mice in the absence of stimulus (12). These data suggest that this low level of constitutive P-selectin expression is capable of mediating leukocyte rolling. In contrast, E-selectin blockade imparted no effect on any of the leukocyte parameters (data not shown). Again, this supports earlier studies in which E-selectin expression values in unstimulated wild-type mice were shown to be approximately the same as those in E-selectin-deficient mice, suggesting no constitutive expression of this selectin (12).

Discussion

The results of this study demonstrate that the immune response to Ag challenge is associated with strikingly different patterns of selectin expression in two different tissues and that this underlies very different modes of leukocyte recruitment. These data were obtained by using a very sensitive quantitative assessment of expression of each of the endothelial selectins in combination with intravital microscopy to directly visualize leukocyte function in each of the microvascular beds. In the skin, P-selectin expression did not rise from constitutive levels, whereas E-selectin expression was significantly increased. Interestingly, despite an increase in E-selectin in skin, an increase in leukocyte rolling was not noted following Ag challenge in this tissue. However, leukocyte rolling velocity was markedly reduced, suggesting that E-selectin may be instrumental in slowing rolling cells. In direct contrast to the skin, following Ag challenge in the muscle microvasculature there was a significant increase in P-selectin expression and a remarkable increase in the leukocyte rolling flux. E-selectin expression remained at very low levels in this tissue and leukocyte rolling ve-

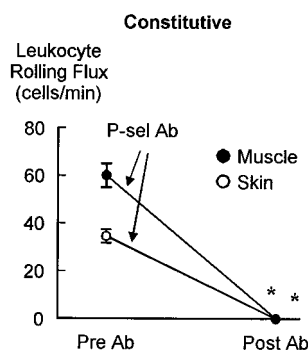


FIGURE 7. The effect of an anti-P-selectin Ab on leukocyte rolling flux in cremaster muscle (solid circles, $n = 3$) or skin postcapillary venules (open circles, $n = 3$) in untreated mice. *, $p < 0.05$ relative to untreated value.

locity did not drop significantly. These data clearly demonstrate the potentially heterogeneous nature of immune responses in different tissues, even in response to an identical Ag. Moreover, these findings suggest that the leukocyte recruitment paradigm of selectin-dependent rolling being important for adhesion holds true in various vascular beds including cremaster and skin, but differences in selectin expression profiles underlie differences in modalities of leukocyte recruitment.

Putting together the quantitative expression data and functional rolling data reveals very important information. First, in untreated mice there is minimal constitutive expression of E-selectin in skin and cremaster, and E-selectin does not appear to support any basal leukocyte rolling in either tissue. This is consistent with the work of Eppihimer et al. (12) that E-selectin expression in heart, intestine, and lungs was not different between wild-type and E-selectin-deficient animals in the absence of stimulus. On the other hand, this study reveals that there is constitutive expression of P-selectin in both skin and cremaster muscle and this accounts for all of the rolling in untreated animals. We have previously seen P-selectin expression in unperturbed lung, mesentery, and intestine, but not heart or brain, suggesting that some tissues, including in this study skin and cremaster muscle, can constitutively express sufficient P-selectin to support functional rolling. The presence of constitutive P-selectin-dependent rolling has been documented in the dorsal skin chamber and dermal venules of mice, further supporting this view (15).

Following Ag challenge, the ratio of P-selectin to E-selectin in skin approaches 1:1, whereas the ratio in muscle was greater than 40:1. Functionally, in muscle we observed a dramatic increase in the number of rolling cells without a change in rolling velocity, whereas in the skin there was no increase in the number of rolling cells but a very significant reduction in rolling velocity. At first glance, it is tempting to conclude that in these systems increases in P-selectin expression elevate the flux of rolling cells, whereas an increase in E-selectin expression is associated with a reduction in rolling velocity. Indeed, there is some strong evidence to support the latter: Kunkel and Ley (14) demonstrated that slow rolling induced by TNF- α was evident in L-selectin-deficient and P-selectin-deficient, but not E-selectin-deficient, mice. These data support the view that E-selectin is an absolute requirement for the slow rolling observed with TNF- α . Although our data are consistent with a need for E-selectin for slow rolling, they do not support an exclusive role for E-selectin as a mediator of slow rolling. In the skin, blockade of E-selectin or P-selectin alone was not sufficient to significantly affect the extremely slow rolling velocity, whereas combined administration of the Abs increased rolling velocity almost sevenfold.

It is intriguing that a very significant increase in P-selectin up-regulation could occur independent of a change in rolling velocity and that this increase was sufficient to recruit more rolling cells. Clearly, this is an example of dissociation between an increase in adhesion molecule expression and an decrease in rolling velocity and belies in vitro work that demonstrates that increasing the concentration of P-selectin protein incorporated into lipid bilayers decreases rolling velocity (2). It is possible that nonrandom selectin expression on microvascular endothelium in vivo, such as clustering or dimerization, may not be entirely replicated by adhesion molecule distribution on glass coverslips or in transfected cell systems. These data also differ from the rat mesentery, where within minutes of exogenous application of leukotriene C_4 (LTC_4) both increased leukocyte rolling and a dramatic reduction in rolling velocity were apparent, and both were dependent upon P-selectin

(20). However, in that model, nonselectin molecules also contributed to the slow rolling with LTC₄. Both inhibitors of platelet-activating factor (PAF) and the β_2 integrin increased rolling velocity, suggesting that P-selectin alone was not sufficient to decrease rolling velocity. Therefore, the data presented in this study highlight the complexity of factors that underlie leukocyte rolling velocity in vivo and also suggest that increased expression of a single selectin does not necessarily reduce the speed of a rolling leukocyte. However, the presence of two selectins at least in the skin was sufficient to reduce rolling velocity in a synergistic manner.

We believe that rolling velocity is an extremely important parameter inasmuch as slow rolling leukocytes respond to chemotactic stimuli with at least 10-fold greater sensitivity than fast rolling cells (20). To illustrate this point, we have previously shown in the rat mesentery that the velocity of leukocytes induced to roll by LTC₄ is 50% lower than those induced by histamine. Subsequently, the histamine-induced fast rolling cells were able to undergo firm adhesion in response to 10 nM PAF but not to 1 nM PAF. In contrast, LTC₄-induced slow rolling cells were able to respond to PAF at the lower concentration and adhere (20). In this study, a similar concept is presented: in the cremaster microvasculature the milieu is such that many fast rolling cells induce a small proportion of cells to adhere, whereas in the skin a small number of slow rolling cells is sufficient to also increase cell adhesion.

Patel et al. (21) have shown that the length of the P-selectin molecule is critical in its ability to induce leukocyte rolling. Shortening of P-selectin by deletion of several of the consensus repeat regions caused P-selectin to become unable to tether free-flowing leukocytes and initiate rolling. This raises some interesting questions about whether a cell would roll exclusively upon the longer P-selectin and not interact with the much shorter E-selectin molecule if both molecules are present. Indeed, Lusinskas et al. (22–23) demonstrated in vitro that when both P-selectin and E-selectin were present, monocytes and CD4⁺ T cells preferred P-selectin, whereas if only E-selectin was available then leukocytes would roll on this molecule. Our results suggest in a system wherein the expression of both P-selectin and E-selectin exists, as is the case in skin, cells use both selectins. Therefore, the longer P-selectin does not preclude leukocyte interactions with E-selectin. These data are consistent with the work of other investigators who have demonstrated an overlapping role for both P-selectin and E-selectin in allergy-induced leukocyte recruitment in the skin (24–26). However, our own work extends those observations to suggest that not all tissues use both endothelial selectins to recruit leukocytes. Second, the use of intravital microscopy of skin and muscle revealed that the E-selectin and P-selectin expression ensemble may dictate a pattern of leukocyte recruitment not seen with P-selectin alone.

Although the elucidation of the mechanism underlying differences in endothelial selectin expression between tissues was not within the scope of this study, at least two explanations are possible. First, the endothelium from cremaster muscle may not have the machinery to express E-selectin, whereas the skin microvasculature can express both P-selectin and E-selectin. This is very unlikely in light of work by Ley and colleagues that demonstrates that TNF- α can induce E-selectin expression in the cremaster muscle (14, 27). It is also unlikely that 4 h was not sufficient for E-selectin synthesis inasmuch as TNF- α could induce much E-selectin expression in the cremaster vasculature within this time frame. Although one could argue that the stimulus was too weak to induce E-selectin in the cremaster, the same stimulus caused strong expression of E-selectin in the skin and was sufficient to activate cremasteric endothelium to express P-selectin. Finally, the same amount of Ag was able to induce E-selectin expression in the cre-

master muscle of P-selectin-deficient mice, suggesting that sufficient amounts of Ag were used but in the presence of P-selectin the system did not express E-selectin (5). The second more likely explanation is that the immune response is differentially regulated by the local environment in each tissue, and that this dictates whether or not E-selectin is expressed. Although there is no direct evidence to support this view, Galli and coworkers have shown that leukocyte recruitment during cutaneous IgE-dependent late-phase responses is mast cell-dependent (28). Furthermore, in human skin grafted onto SCID mice, mast cell degranulation can induce E-selectin-dependent leukocyte recruitment (29). These observations are noteworthy as our preliminary data show no impairment of leukocyte recruitment in mast cell-deficient mice in the cremaster muscle, where all of the leukocyte recruitment is dependent upon P-selectin (P.K., unpublished observations). Whether these extravascular immunocytes dictate the adhesion molecule profile in different microvascular beds during the immune response to Ag is an important future consideration.

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