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Specialized Contributions by α(1,3)-Fucosyltransferase-IV and FucT-VII during Leukocyte Rolling in Dermal Microvessels

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Summary

Noninflamed skin venules support constitutive leukocyte rolling. P-selectin controls the rolling frequency, whereas E-selectin dictates rolling velocity (V_{roll}). Fucosylated selectin ligands are essential for all interactions, as rolling was absent in mice doubly deficient in α1,3-fucosyltransferase (FucT)-IV and FucT-VII. The rolling fraction was reduced in FucT-VII$^{-/-}$ animals but normal in FucT-IV$^{-/-}$ mice. However, V_{roll} was markedly increased in both strains. P-selectin ligands generated by FucT-VII are crucial for initial leukocyte tethering, whereas E-selectin ligands that permit maximum slowing of V_{roll} require simultaneous expression of FucT-IV and FucT-VII. These results demonstrate a role for FucT-IV in selectin-dependent adhesion and suggest that the endothelial selectins and FucTs have distinct but overlapping functions in the immunosurveillance of the skin.

Introduction

Leukocyte recruitment from blood to tissues requires a cascade of distinct molecular events that mediate cell adhesion and extravasation in postcapillary venules (Springer, 1994; Butcher and Picker, 1996). Leukocytes are initially tethered to endothelial cells by adhesion pathways featuring high on-rate and tensile strength but rapid reversibility. This leads to margination and rolling of the tethered cell along the venular wall. Subsequently, rolling cells must encounter a chemoattractant, which triggers engagement of integrins and firm arrest. Leukocytes can enter most extravascular compartments only after they have undergone each of these steps. The molecular components of adhesion cascades vary between different tissues and between physiologic and pathologic situations in the same tissue. This combinatorial diversity was shown to mediate selective homing of distinct leukocyte subsets to Peyer's patches, peripheral lymph nodes, and the bone marrow (Butcher and Picker, 1996; Mazo and von Andrian, 1999).

In most nonlymphoid tissues, adhesion pathways must be upregulated by inflammatory stimuli to allow leukocyte adhesion and extravasation. Inflammation-induced enhancement of leukocyte recruitment operates also in the skin (Walter and Issekutz, 1997; Robert and Kupper, 1999). However, some leukocytes migrate to cutaneous tissues in the absence of inflammation, suggesting that one or more homing pathways are constitutively active in dermal venules. Indeed, several studies have shown that leukocytes roll at high frequency in noninflamed skin (Mayrovitz, 1992; Janssen et al., 1994; Nolte et al., 1994; Milstone et al., 1998). In contrast, rolling is rare in other noninflamed tissues such as the mesentery or cremaster muscle unless endothelial cells are exposed to inflammatory stimuli (Fiebig et al., 1991; Ley et al., 1995).

The adhesion pathways that mediate leukocyte rolling in vivo include the α4 integrins and the selectin family (Carlos and Harlan, 1994; Kansas, 1996). L-selectin (CD62L) is expressed on most leukocytes and binds endothelial ligands in lymphoid organs and inflamed tissues. E-selectin (CD62E) is only found on endothelial cells. It binds ligands on circulating granulocytes, monocytes, and some memory lymphocytes. On endothelial cells in culture and in most tissues, E-selectin is only detectable after several hours exposure to cytokines (TNFα or IL-1) or bacterial endotoxin, which induce transcription of the E-selectin gene. P-selectin (CD62P) is stored in platelet α-granules and in Weibel-Palade bodies of endothelial cells. It is rapidly translocated to the cell surface when cells are exposed to secretagogues such as histamine or thrombin. Some inflammatory cytokines can also induce P-selectin transcription.

Prior reports suggest that rolling in noninflamed skin is mediated by selectins; P-selectin antibodies blocked rolling in ears of hairless mice, whereas antibodies to L- or E-selectin had no effect (Nolte et al., 1994). However, leukocyte rolling was observed in the dorsal skin of P-selectin-deficient (P$^{-/-}$) mice (Yamada et al., 1995). The mechanisms for this P-selectin-independent event have not been determined.

Selectins mediate rolling by binding to sialyl-Lewis^x (sLeX) and related carbohydrates on cell surfaces. These physiologic ligands must be fucosylated by α1,3-fucosyltransferases (FucTs) (Maly et al., 1996; Lowe, 1997; J. B. Lowe et al., personal communication). Leukocytes express two FucTs, namely FucT-IV and FucT-VII. The importance of the latter has been demonstrated in FucT-VII$^{-/-}$ mice (Maly et al., 1996), whereas the role of FucT-IV remains to be defined. FucT-VII$^{-/-}$ mice show blood leukocytosis as well as impaired lymphocyte homing and leukocyte extravasation to sites of inflammation. Intravital microscopy in FucT-VII$^{-/-}$ ear venules revealed a substantial reduction in the number of rolling leukocytes and increased velocities of the remaining rolling cells. The fact that rolling is not abolished in FucT-VII$^{-/-}$

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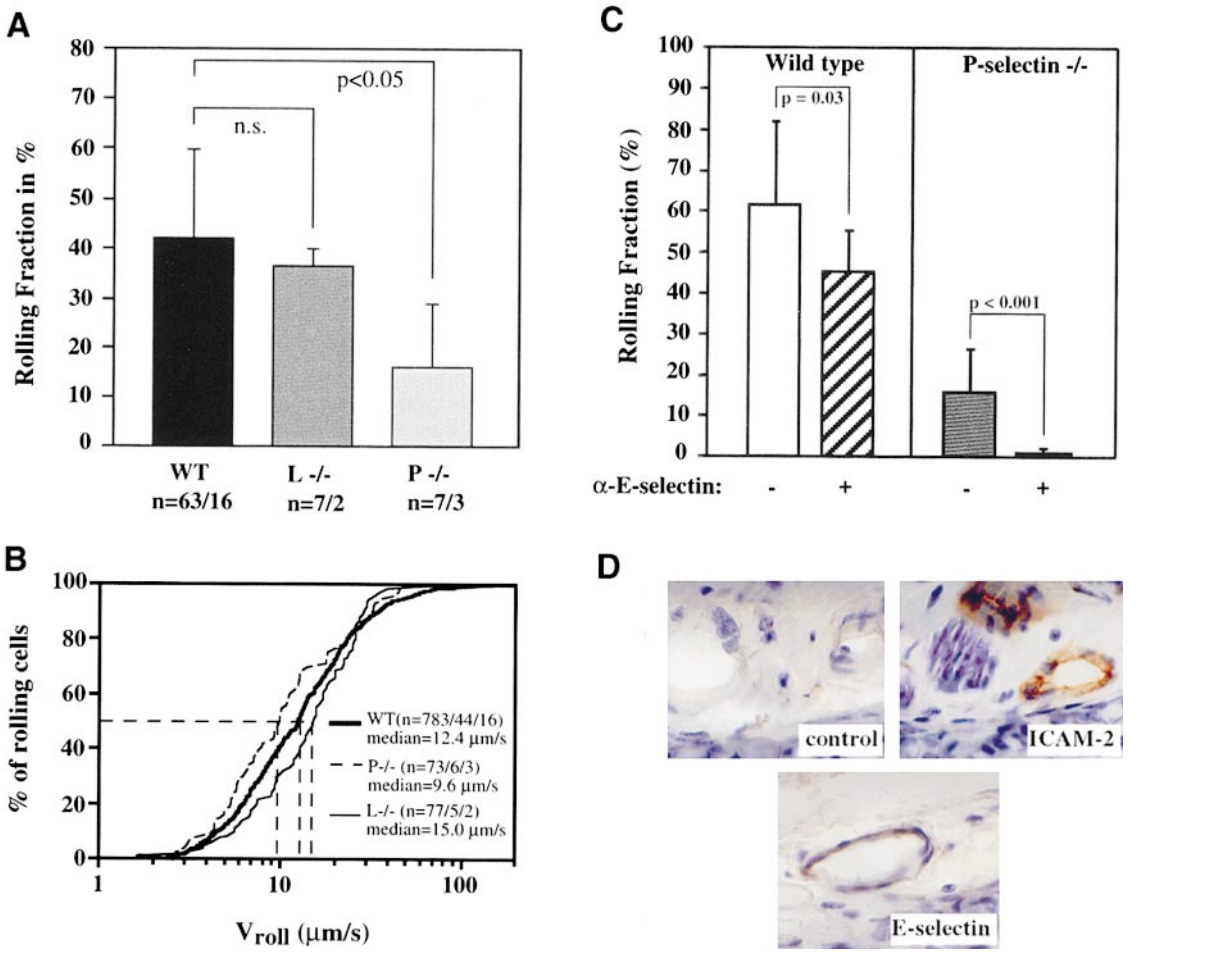

Figure 1. Rolling Fractions, Cumulative Velocity Curves, and Selectin Expression in Ear Venules of WT, $P^{-/-}$, and $L^{-/-}$ Mice

(A) Rolling fractions were determined as percentage of rolling cells in the total flux of rhodamine 6G–labeled leukocytes. Bars represent mean ± SD. For WT mice, baseline rolling fractions from all mAb inhibition experiments shown in Table 1 were pooled. Rolling fractions between WT and knockout mice were compared using the Kruskal-Wallis test and Dunn's multiple comparison test (ns, not significant). n = number of venules/animals examined.

(B) Cumulative rolling velocity curves in WT, $L^{-/-}$, and $P^{-/-}$ mice. The percentage of cells that rolled at or below a given velocity is shown as a function of V_{roll}. n = total number of cells/venules/mice analyzed in each group.

(C) Rolling fractions were measured in WT and $P^{-/-}$ mice before and after injection of anti-E-selectin mAb 9A9 (50 μg/mouse). Data shown are mean ± SD. Rolling fractions were compared using the Wilcoxon signed rank test.

(D) Immunohistology of murine ear sections were stained with nonbinding rat IgG (negative control), anti-ICAM-2 mAb mIC2/3c4 (positive control), or anti-E-selectin mAb 10E9.6. Positive staining is visible as brown color. Sections were counterstained with hematoxyline. Original magnification in all sections is 400×.

mice is consistent with the idea that FucT-IV may generate selectin ligands. Alternatively, a fucosylation-independent pathway could be involved (Frenette et al., 1998).

Here we examined the molecular pathways on leukocytes and endothelial cells that mediate constitutive rolling in the skin. We show that E-selectin is constitutively expressed at low levels in dermal vessels and enables cells to roll at low velocities, whereas P-selectin determines the frequency of rolling events. L-selectin does not play a role in this setting. Using FucT-deficient strains, we show that both FucTs are necessary for optimal leukocyte rolling. Most P- and E-selectin ligands are generated by FucT-VII, but some carbohydrates modified by FucT-IV can also function as ligands for the two selectins.

Results

L-Selectin Does Not Contribute to Leukocyte Rolling in Ear Venules

To address the role of L-selectin, we studied leukocyte rolling in L-selectin-deficient ($L^{-/-}$) mice (Figure 1A). Consistent with a previous study, which found no effect of anti-L-selectin in murine skin venules (Nolte et al., 1994), the rolling fraction in $L^{-/-}$ ears (36.1% ± 3.3%, mean ± SD, n = 7 venules in 2 mice) did not differ significantly from that in wild-type (WT) mice (40.7% ± 17.8%, $p > 0.05$). The cumulative velocity profile of rolling velocity (V_{roll}) in $L^{-/-}$ mice was also similar to that in WT animals, although the former lacked a small population of cells rolling faster than 50 μm/s, which constitute ~5% of rolling cells in WT mice (Figure 1B). Thus, we

Table 1. Hemodynamics and Leukocyte Rolling Parameters in Ear Venules of WT, FucT-IV$^{-/-}$, and FucT-VII$^{-/-}$ Mice

Strain/mAb		Number of Venules/Animals	Diameter (μm)	V_{free} (μm/s)	WSR (s^{-1})	Rolling Fraction (%)	V_{roll} (μm/s)	$V_{roll} < 15$ (20) μm/s (%)	V_{rel} (%)	$V_{rel} < 4\%$ (%)
Wild-type										
Control	pre	12/3	27 ± 9	337 ± 121	103 ± 48	45 ± 8	11.3	62.9	3.7	51.8
	post	12/3	27 ± 9	390 ± 157	124 ± 61	52 ± 12^{ns}	14.0	54.4	3.9	52.1
ICAM-2	pre	8/3	22 ± 6	399 ± 154	142 ± 61	39 ± 15	13.7	54.9	3.5	57.5
	post	8/3	22 ± 6	548 ± 347	201 ± 115	49 ± 18^{ns}	13.3	55.7	2.9	69.5
P-selectin	pre	6/3	27 ± 4	341 ± 114	114 ± 52	28 ± 16	8.5	81.6	2.5	83.7
	post	6/3	27 ± 4	330 ± 100	136 ± 83	10 ± 5**	16.8	44.4	5.5	44.4
E-selectin	pre	10/3	21 ± 6	455 ± 156	205 ± 65	62 ± 20	14.9	50.3	3.2	71.7
	post	10/3	21 ± 6	391 ± 98	182 ± 55	46 ± 12*	22.6	17.8	5.7	14.0
FucT-IV$^{-/-}$										
P-selectin	pre	9/4	26 ± 6	684 ± 254	202 ± 88	45 ± 20	22.4	42	3.3	62
	post	9/4	24 ± 6	775 ± 470	247 ± 150	5 ± 5**	22.7	50	4.5	40
E-selectin	pre	7/3	23 ± 8	508 ± 210	185 ± 101	61 ± 8	25.3	38.7	4.9	34.6
	post	7/3	23 ± 8	493 ± 195	168 ± 79	62 ± 10^{ns}	34.3	10.0	7.3	13.6
FucT-VII$^{-/-}$										
P-selectin	pre	6/3	18 ± 5	523 ± 251	245 ± 122	7 ± 4	ND	ND	ND	ND
	post	6/3	18 ± 5	560 ± 149	271 ± 115	0	ND	ND	ND	ND
E-selectin	pre	6/3	21 ± 9	619 ± 259	225 ± 117	7 ± 1	32.2	28.7	7.0	25.9
	post	6/3	20 ± 10	641 ± 283	303 ± 164	4 ± 1*	76.9	6.5	15.5	14.5

After assessment of baseline conditions (pre), animals were injected i.v. with 50 μg of one of the respective mAbs. Effects were determined 15 min later (post). The velocity of ≥10 rolling (V_{roll}) and ≥20 noninteracting cells (V_{free}) was measured by frame-by-frame analysis of video tapes. V_{rel} was calculated as V_{roll}/mean of V_{free} *100%. Venular diameters, V_{free}, wall shear rates (WSR), and rolling fractions are shown as mean ± SD. Since V_{roll} and V_{rel} were not normally distributed, median values are given. Rolling fractions after mAb treatment versus baseline values were compared using Wilcoxon signed rank test (ns, not significant; *$p < 0.05$, **$p < 0.001$). Percentage of slow rolling cells in all rolling leukocytes was determined based on data not shown and cumulative velocity curves in Figures 2C and 2F (for WT) and Figures 4B and 4F (for FucT-IV$^{-/-}$ and FucT-VII$^{-/-}$, respectively). Slow rolling in WT mice was defined as $V_{roll} < 15$ μm/s and $V_{rel} < 4\%$. Slow rolling in FucT-deficient mice was defined as $V_{roll} < 20$ μm/s and $V_{rel} < 4\%$. Since anti-P-selectin abrogated rolling in FucT-VII$^{-/-}$ mice, V_{roll} was not determined (ND).

concluded that L-selectin makes a minor, if any, contribution to leukocyte rolling in noninflamed ear venules and so focused our further efforts on E- and P-selectin.

E- and P-Selectin Mediate Leukocyte Rolling in Noninflamed Skin of WT Mice

To determine which selectins control the frequency of leukocyte rolling in normal skin, we analyzed effects of neutralizing mAbs (50 μg/mouse i.v.) in WT mice (Table 1). The nonbinding control mAb 2-4A1 and anti-ICAM-2 mAb mIC2/3c4, which we used as a second, binding control, tended to increase rolling fractions. These changes were statistically not significant ($p > 0.05$, Wilcoxon signed rank test). In contrast, anti-E-selectin mAb 9A9 attenuated rolling fractions modestly (26% inhibition, Figure 1C), but the reduction was statistically significant ($p = 0.03$ versus baseline). Thus, most cells were likely to roll via P-selectin. Indeed, anti-P-selectin mAb 5H1 had a pronounced effect on rolling fractions (63% inhibition, $p < 0.001$ versus baseline). Although an earlier study had reported that polyclonal anti-P-selectin completely abolished rolling in ear venules (Nolte et al., 1994), we continued to detect rolling cells in most venules after P-selectin inhibition (rolling fraction: 9.7% ± 5.0%). Treatment of two mice with a larger dose of mAb 5H1 (100 μg/mouse) had no further effect (rolling fraction: 11.5% ± 8.1%), indicating that the residual rolling interactions were not due to subsaturating mAb concentrations. Moreover, when anti-P-selectin-treated mice were additionally injected with mAb 9A9, rolling was completely abolished (data not shown). None of the antibodies in this study induced significant changes in hematocrit or leukocyte counts during at least 45 min after injection (data not shown). Thus, leukocyte rolling in noninflamed skin venules of WT mice depends on the two endothelial selectins: the rolling frequency is largely determined by P-selectin; E-selectin plays a relatively small but statistically significant role.

Rolling in Ear Venules of P-Selectin$^{-/-}$ Mice Is Exclusively Mediated by E-Selectin

Although our experiments with neutralizing mAbs suggested that E-selectin alone may be sufficient to support some leukocyte rolling in skin venules, it remained theoretically possible that mAb 5H1 did not completely abrogate endothelial P-selectin activity in WT animals. Therefore, we also analyzed P$^{-/-}$ mice (Figures 1A and 1B). The baseline rolling fraction in P$^{-/-}$ ear venules was 16.3% ± 13.9% (n = 6/3), considerably lower than in a cohort of WT mice that was analyzed in parallel (40.7% ± 17.8%). mAb 9A9 essentially abrogated rolling in P$^{-/-}$ mice (0.14% ± 0.38%, $p < 0.001$ versus baseline, n = 7/3), suggesting that both E- and P-selectin are constitutively expressed and have overlapping functions in noninflamed skin venules. Each selectin can support rolling in the absence of the other (Figure 1C). Other

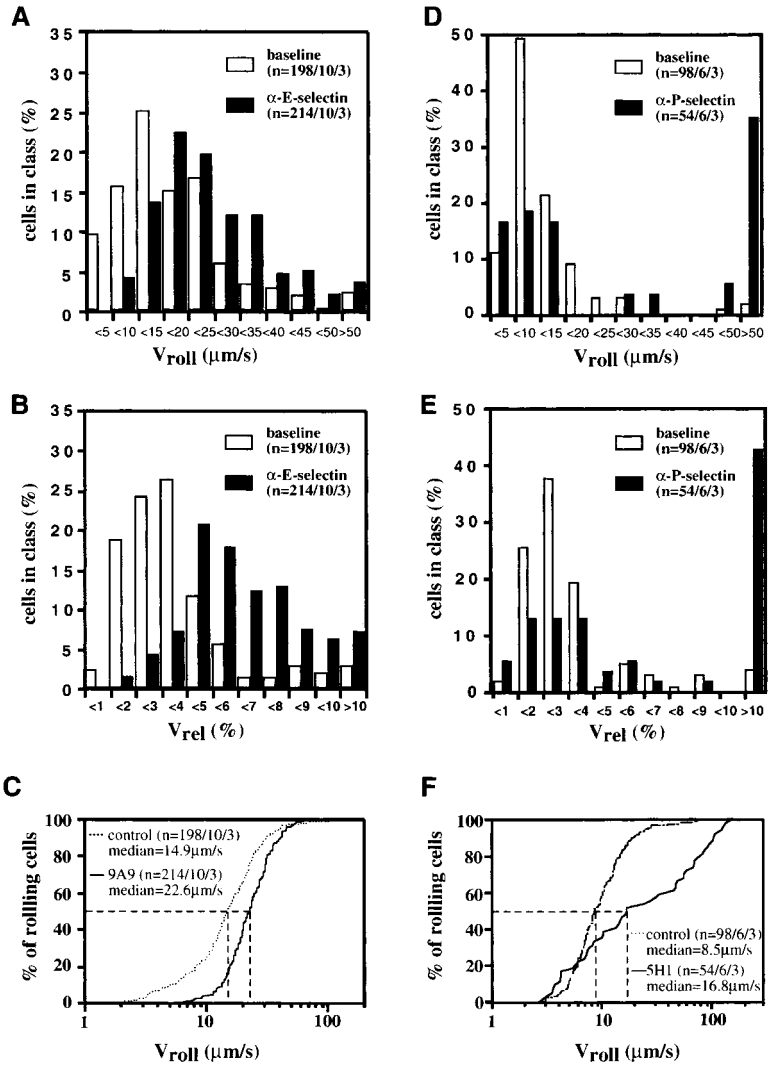

Figure 2. Effect of Anti-E- and P-Selectin mAbs on Rolling Velocities in WT Ear Venules

After assessment of baseline rolling, mice were treated with 50 μg anti-E-selectin (A–C) or anti-P-selectin (D–F). Histograms for V_{roll} (A and D) and V_{rel} (B and E) are shown before (empty bars) and after mAb injection (filled bars).

(A and D) V_{roll} was measured for ≥15 consecutive cells per venule. Cells were assigned to velocity classes ranging from >0 μm/s to <5 μm/s, 5 μm/s to <10 μm/s, and so on.

(B and E) To account for differences in hemodynamic conditions, V_{rel} was calculated by normalizing V_{roll} of each cell to V_{free}, the mean velocity of 20 consecutive free-flowing leukocytes measured in parallel in the same venule. Cells were assigned to velocity groups ranging from >0% of V_{free} to <1%, 1% to <2%, and so on. Cumulative velocity curves (C and F) before and after mAb injection were generated as well. n = total number of cells/venules/mice.

adhesion pathways that mediate leukocyte rolling elsewhere do not appear to contribute in this setting.

Constitutive Expression of E-Selectin in Murine Skin

To confirm the functional data with anti-E-selectin mAb, we performed immunohistochemistry on serial sections of murine ears (Figure 1D). Whereas no specific signal was observed with control rat IgG, essentially all microvessels were stained by anti-ICAM-2 mAb mIC2/3c4. Since mAb 9A9 is relatively insensitive at detecting E-selectin by immunohistochemistry of fixed tissues (our unpublished data and B. Wolitzky, personal communication), we used mAb 10E9.6, a nonblocking mAb to E-selectin (Ramos et al., 1997). mAb 10E9.6 weakly stained a subset of ICAM-2^+ microvessels in normal WT ear sections. When TNFα was preinjected into the pinna of the ear, a marked increase in the number and apparent staining intensity of E-selectin-positive vessels was found (data not shown). Sections of $P^{-/-}$ ears showed a similar staining pattern and intensity as those in WT mice (data not shown). Immunoreactivity with mAb 10E9.6 was specific for microvessels in the skin because no staining was detected in skin-draining lymph nodes (data not shown).

E-Selectin Not P-Selectin Is Required for Slow Rolling in Ear Venules

The rolling fraction is a sensitive parameter to measure the frequency and efficacy of initial adhesive contacts (tethering), but it is less indicative of the quality or strength of established rolling (Stein et al., 1999). To determine the relative contribution by P- versus E-selectin to interactions subsequent to tethering, we compared V_{roll} in ear venules of $P^{-/-}$ and WT mice. The velocity histograms and cumulative velocity curves from both strains were very similar (Figure 1B).

It should be cautioned that the composition of leukocytes that roll in $P^{-/-}$ mice may not be identical to the larger population of cells rolling in WT mice. However, if one assumes this to be the case, the similar velocity profiles would suggest that V_{roll} in ear venules is primarily determined by E-selectin, not P-selectin. Thus, we measured the effect of E-selectin inhibition on V_{roll} in WT mice (Table 1; Figure 2). Median V_{roll} increased from 14.9 μm/s before to 22.6 μm/s after anti-E-selectin injection, whereas control mAb 2-4A1 had no such effect (11.3 μm/s versus 14.0 μm/s). Analysis of frequency histograms of V_{roll} confirmed previous observations that this parameter is not normally distributed but skewed toward low rolling

velocities (Stein et al., 1999). The anti-E-selectin mAb caused a significant shift in V_{roll} histograms and the cumulative velocity curve that affected all rolling cells ($p < 0.001$; Figures 2A and 2C) and was not observed with mAb 2-4A1 nor with mAb mIC2/3c4 ($p > 0.05$ for both).

As shown in Table 1, hemodynamic parameters in ear venules varied considerably between different venules. Random variations in blood flow were also sometimes detected in the same venule. Since changes in wall shear rate can affect rolling, we calculated V_{rel} (i.e., V_{roll} as percent of V_{free}, the mean velocity of ≥ 20 noninteracting cells measured in parallel in the same venule). After thus accounting for hemodynamic variability, the effect of anti-E-selectin was even more apparent (Figure 2B). In untreated venules, >70% of all leukocytes rolled at a velocity <4% of V_{free}, whereas 15 min after anti-E-selectin injection, only 14% rolled below that velocity. In contrast, slow rolling was not altered by treatment with nonbinding control mAb or anti-ICAM-2 (data not shown). Although V_{roll} was normal in $P^{-/-}$ mice (see above), inhibition of P-selectin in WT animals increased V_{roll} and V_{rel} (Figures 2D and 2E). However, unlike anti-E-selectin, anti-P-selectin did not accelerate the entire rolling population but primarily affected cells that moved at higher velocities; the frequency of slow rolling cells (below 5 μm/s) remained unchanged (Figure 2F). Together, these data suggest that P-selectin determines the overall frequency of rolling in the skin, whereas E-selectin is crucial for rolling at very low velocities.

FucT-IV Is Crucial for Slow Leukocyte Rolling

Having clarified the role of P- and E-selectin in leukocyte rolling in normal skin, we set out to characterize the leukocyte-expressed carbohydrate ligands that interact with these molecules. Previous work has shown that rolling is markedly reduced in the skin of FucT-VII$^{-/-}$ mice, but some venules support rolling, albeit at a higher velocity than in WT ears (Maly et al., 1996). We therefore tested whether carbohydrates that are fucosylated by FucT-IV can function as selectin ligands. As shown in Figure 3A and Table 1, rolling fractions in FucT-IV$^{-/-}$ ears did not differ from those in WT mice (44.5% ± 19.9% versus 40.7% ± 17.8%). In contrast, virtually no rolling was observed in mice deficient in both FucTs, suggesting that FucT-IV alone can generate functional selectin ligands on circulating FucT-VII$^{-/-}$ leukocytes.

To investigate whether FucT-IV or FucT-VII affects the quality of rolling, we measured V_{roll} (Table 1). There was a substantial increase in median V_{roll} in ear venules of FucT-IV$^{-/-}$ versus WT mice (23.4 μm/s versus 12.4 μm/s; Figure 3B). Velocity histograms (data not shown) and cumulative velocity curves in FucT-IV$^{-/-}$ mice showed a marked shift toward higher rolling velocities that was most apparent in the slowest rolling cells. Analysis of FucT-VII$^{-/-}$ mice confirmed increased V_{roll} (Maly et al., 1996), which was somewhat more pronounced in fast rolling cells than in FucT-IV$^{-/-}$ animals (median = 38.7 μm/s). Most venules in FucT-IV$^{-/-}$ and FucT-VII$^{-/-}$ mice contained few or no cells that rolled very slowly ($V_{roll} \leq 10$ μm/s), whereas the majority of WT venules supported such slow rolling at variable frequency (Figure 3C).

FucT-IV Generates Ligands for Both P- and E-Selectin

The paucity of slow rolling FucT-IV$^{-/-}$ leukocytes resembled the effect of anti-E-selectin in WT ears. However, while anti-E-selectin did not alter rolling fractions in FucT-IV$^{-/-}$ animals (Figure 4A; Table 1), it caused a significant shift in V_{roll} and V_{rel} (Figures 4B–4D). Thus, although FucT-IV is an important determinant of V_{roll}, fucosylation by FucT-IV is not obligatory for molecules that are recognized by E-selectin during established rolling.

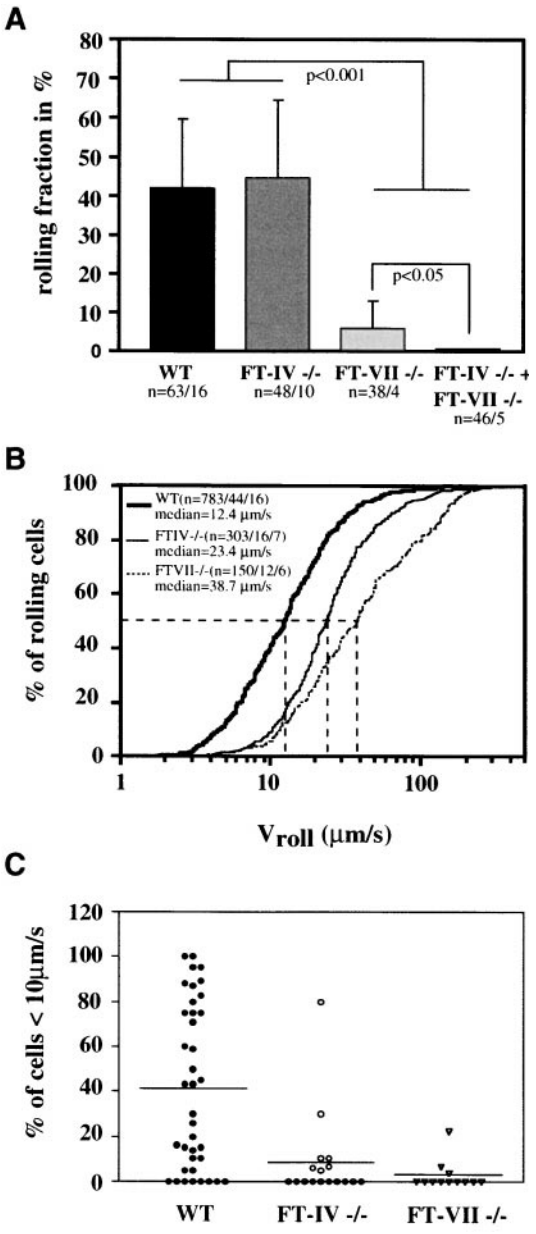

Figure 3. Rolling Fractions and Rolling Velocities in FucT-IV$^{-/-}$, FucT-VII$^{-/-}$, and FucT-IV + FucT-VII$^{-/-}$ Mice

(A) Rolling fractions and statistics were determined as described in Figure 1. Data from FucT-VII$^{-/-}$ mice are based on experiments described previously (Maly et al., 1996). n = number of venules/animals.

(B) Cumulative rolling velocity curves in WT, FucT-IV$^{-/-}$, FucT-VII$^{-/-}$ mice. The percentage of cells that rolled at or below a given velocity is shown as a function of V_{roll}. n = total number of cells/venules/mice in each group.

(C) For each individual venule (symbols) in WT, FucT-IV$^{-/-}$, and FucT-VII$^{-/-}$ strains, the percentage of cells that rolled slower than 10 μm/s is shown. Means of each group are shown as horizontal lines.

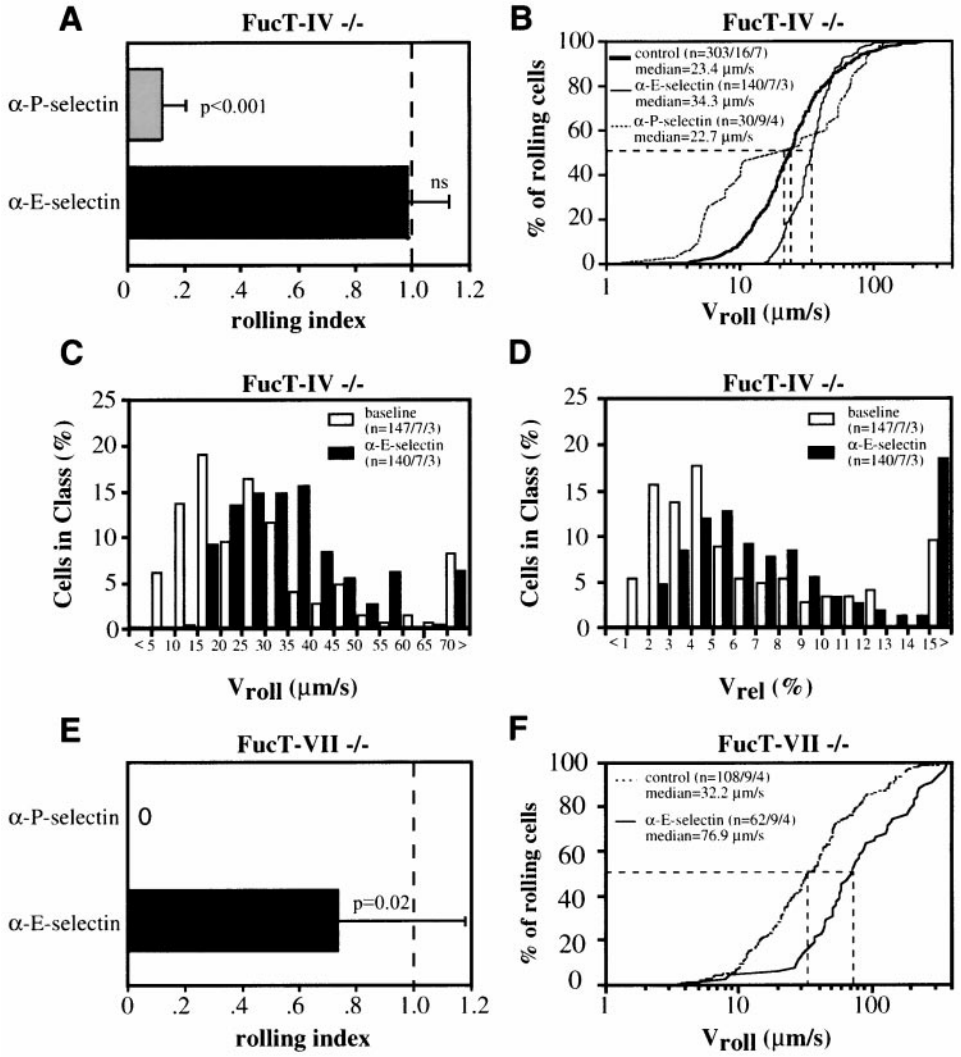

Figure 4. Effect of Anti-E- and -P-Selectin mAbs on Leukocyte Rolling in Ear Venules of FucT-IV$^{-/-}$ and FucT-VII$^{-/-}$ Mice

(A and E) Rolling fractions in FucT-IV$^{-/-}$ (A) and FucT-VII$^{-/-}$ (E) mice were measured before and 15 min after injection of anti-E-selectin or anti-P-selectin. Results are given as rolling index, i.e., the rolling fraction in each venule after mAb treatment was normalized to baseline rolling, which was given a value of 1. Mean ± SD are shown. Rolling fractions were compared using Wilcoxon signed rank test (ns, not significant).

(B and F) Effects of anti-E- and -P-selectin on cumulative velocity curves in FucT-IV$^{-/-}$ (B) and FucT-VII$^{-/-}$ (F) mice. For the control curve, baseline data from both antibody inhibition groups were pooled. Histograms for V_{roll} (C) and V_{rel} (D) in FucT-IV$^{-/-}$ mice were generated from data obtained before (empty bars) and after (filled bars) injection of anti-E-selectin. The small number of residual rolling cells in anti-P-selectin-treated FucT-IV$^{-/-}$ animals was insufficient to generate meaningful velocity histograms for this group. n = the total number of cells/venules/mice analyzed in each group.

Anti-P-selectin had qualitatively similar effects in FucT-IV$^{-/-}$ and WT mice (Table 1). The rolling fraction was dramatically reduced (to 4.5% ± 5.2%, $p < 0.001$ versus baseline; Figure 4A) but not abolished, and residual rolling was abrogated by addition of anti-E-selectin (data not shown). After anti-P-selectin, V_{roll} of 13 out of 30 FucT-IV$^{-/-}$ cells (43.3%) was below 10 μm/s (median: 22.7 μm/s; Figure 4B), suggesting that mainly the fast rolling cells were affected. Since anti-E-selectin accelerated slow rolling cells much more than anti-P-selectin, E-selectin determines the lowest V_{roll} even in the absence of FucT-IV. However, the cumulative rolling velocity curve in FucT-IV$^{-/-}$ ears is shifted a priori toward higher V_{roll} than in WT mice. Thus, ligands generated by both FucTs are necessary for E-selectin to exert its full physiologic function. From these findings, we conclude that both P- and E-selectin support rolling of FucT-IV-deficient leukocytes; the absence of FucT-IV does not diminish interactions that determine the frequency of rolling, but the strength of established interactions is significantly reduced. In other words, carbohydrates on leukocytes generated by FucT-VII alone confer a normal ability to form tethers in skin venules (predominantly mediated by P-selectin), but they are insufficient for maximum slowing of rolling cells (largely E-selectin dependent).

Role of P- and E-Selectin in Ear Venules of FucT-VII$^{-/-}$ Mice

To determine whether FucT-IV dependent carbohydrates interact with P- or E-selectin, we analyzed the effects of neutralizing mAbs in FucT-VII$^{-/-}$ ear venules that supported significant baseline rolling (Table 1). Anti-P-selectin abolished rolling in nearly all venules indicating that, at least under conditions prevalent in normal skin, E-selectin alone cannot form functional tethers with leukocytes that do not express FucT-VII (Figure 4E). Nevertheless, E-selectin contributed to rolling in FucT-VII$^{-/-}$ mice because anti-E-selectin reduced rolling fractions by 35% ($p = 0.02$) and significantly increased V_{roll} and V_{rel} of the residual interacting cells (Figure 4F and data not shown).

Discussion

Here we demonstrate that essentially all observable leukocyte rolling interactions in normal murine skin are mediated by two endothelial adhesion molecules, P- and E-selectin. Both are constitutively expressed in dermal venules and interact with leukocyte-expressed ligands that require fucosylation by FucT-IV and/or FucT-VII. In WT mice, the two selectins serve distinct functions: P-selectin is the chief determinant of overall rolling frequency, whereas E-selectin is responsible for maximal slowing of rolling cells. In skin venules of P$^{-/-}$ mice, a smaller but sizeable fraction of leukocytes underwent rolling that was entirely E-selectin dependent and occurred with similar V_{roll} as in WT mice. Together, these findings indicate that there is differential emphasis on P- versus E-selectin as determinants of rolling fraction and V_{roll}, respectively, but their function in the skin is partially overlapping. P-selectin contributes to V_{roll}, especially at intermediate to high velocities, but some leukocytes can tether and roll on E-selectin without requiring P-selectin.

Although our findings clearly establish a role for E-selectin, P-selectin is required for most cells to roll in WT ear venules. This is consistent with previous intravital microscopy studies in murine skin (Nolte et al., 1994; Yamada et al., 1995; Milstone et al., 1998; Hickey et al., 1999). A polyclonal serum to P-selectin was found to block rolling in ear skin of hairless mice (Nolte et al., 1994). In contrast, Yamada et al. observed residual leukocyte rolling in a dorsal skin chamber in P$^{-/-}$ mice (1995). The authors concluded that P-selectin was important but not requisite for rolling in the skin. However, the molecular mechanism(s) of P-selectin-independent interactions remained to be defined. Also, it cannot be ruled out that rolling in this model resulted from a subtle inflammatory response to the surgically implanted observation chamber. More recently, an intravital microscopy study of E-selectin$^{-/-}$ mice found similar rolling fractions in the intact ear skin of mutant and WT mice (Milstone et al., 1998). In the present experiments, neutralization of E-selectin in WT mice had a subtle but statistically significant effect on rolling fractions that might be easily missed. Furthermore, our experiments in P$^{-/-}$ mice demonstrate that P-selectin-independent rolling does indeed occur in normal skin and that this interaction is E-selectin mediated. Thus, E-selectin might be an important tethering receptor for skin-homing leukocyte populations that cannot interact with P-selectin.

On cultured endothelial cells and in many nondermal vascular beds such as the mesentery or cremaster muscle, E-selectin is only detected several hours after exposure to inflammatory cytokines or endotoxin (Kansas, 1996; Vestweber and Blanks, 1999). In contrast, E- as well as P-selectin are constitutively expressed in bone marrow venules and sinusoids (Mazo and von Andrian, 1999). The present results confirm that both selectins are also expressed in normal dermal microvessels. E-selectin was detected by immunohistochemistry in sections of normal skin, but not of lymph nodes. The nonuniform distribution of E-selectin in microvessels is consistent with the observation that rolling is only seen in venules; arterioles or capillaries do not support detectable interactions. Thus, the regulatory mechanisms that restrict E-selectin expression to venules appear to be as selective in resting skin as they are in inflamed vascular beds of many other tissues (Olofsson et al., 1994; Jung and Ley, 1997).

We did not examine skin sections for expression of P-selectin, because this molecule, unlike E-selectin, is stored in endothelial Weibel-Palade bodies (Kansas, 1996). The resolution achieved by immunohistochemistry using light microscopy is insufficient to distinguish unambiguously between luminal and intracellular P-selectin. However, the functional data presented here and in earlier studies (Nolte et al., 1994; Yamada et al., 1995) indicate that P-selectin is luminally expressed in most skin venules. This is also supported by studies on the accumulation of i.v. injected radiolabeled mAbs in murine skin (Hickey et al., 1999). In these experiments, constitutive P-selectin expression was apparent in the absence of inflammation. The same study also reported a 10-fold increase of anti-E-selectin accumulation in inflamed skin compared to baseline accumulation, which was very low, suggesting that E-selectin expression is normally sparse.

P- and E-selectin appear to fulfill distinct functions during rolling; anti-P-selectin markedly reduced rolling fractions, but had only a modest effect on slow rolling, whereas the opposite was true for anti-E-selectin. This observation is consistent with studies in inflamed cremaster muscle venules where inflammatory stimuli are necessary to upregulate endothelial selectins and L-selectin ligands whose expression and relative contribution to rolling varies over time (Ley et al., 1995; Jung and Ley, 1997). Although circumstances in this tissue are thus different from the ear model where selectins are continually expressed a priori, studies in the cremaster muscle found that cytokine-induced E-selectin was similarly necessary for slow rolling but had little influence on rolling fluxes, whereas P-selectin determined rolling frequencies (Ramos et al., 1997; Ley et al., 1998).

The reduction in V_{roll} afforded by E-selectin may facilitate constitutive leukocyte recruitment to the skin. As V_{roll} decreases, the time that is spent by a rolling cell in a venule increases proportionally. This may allow certain cells to detect and respond to low levels of chemoattractants in skin venules. Indeed, a recent study has shown that the transit time of rolling leukocytes in inflamed cremaster muscle venules is inversely correlated with

Table 2. Effects of Selectin Inhibition and FucT Deficiency on Rolling Fractions and Rolling Velocities in Murine Ear Venules

Mouse Strain	Anti-E-Selectin	Anti-P-Selectin	Rolling Fraction[a]	V_{roll}	Slow Rolling[c]
Wild-type	+	—	↓[b]	↑↑[b]	↓↓[b]
	—	+	↓↓[b]	↑↑[b]	(↓)[b]
	+	+	Ø	Ø	Ø
L-selectin$^{-/-}$	—	—	no Δ[a]	no Δ[a]	no Δ[a]
P-selectin$^{-/-}$	—	—	↓↓[a]	no Δ[a]	(↑)[a]
	+	—	Ø	Ø	Ø
FucT-IV$^{-/-}$	—	—	no Δ[a]	↑↑[a]	↓↓[a]
	—	+	↓↓↓[b]	(↑)[b]	↑↑[b]
	+	—	no Δ[b]	↑↑↑[b]	↓↓[b]
	+	+	Ø	Ø	Ø
FucT-VII$^{-/-}$	—	—	↓↓↓[a]	↑↑[a]	↓↓[a]
	—	+	Ø	Ø	Ø
	+	—	↓[b]	↑↑[b]	↓↓↓[b]
FucT-IV + -VII$^{-/-}$	—	—	Ø	Ø	Ø

Arrows indicate a significant increase (↑) or decrease (↓) compared to rolling parameters obtained in untreated WT mice[a] or compared to control parameters obtained in the same preparation before MAb treatment[b]. [c] Slow rolling was defined as the frequency of cells with $V_{roll} <$ 15 μm/s. Ø: indicates that no rolling was detectable.

leukocyte arrest and emigration (Jung et al., 1998). Consequently, although leukocytes roll at a normal frequency in E-selectin$^{-/-}$ mice, their ability to undergo integrin-dependent arrest in inflamed tissues is reduced (Ley et al., 1998; Milstone et al., 1998).

Our study provides a molecular basis for earlier reports of leukocyte rolling occurring at high frequency in noninflamed skin of mice, rats, and hamsters (Mayrovitz, 1992; Menger and Lehr, 1993; Janssen et al., 1994). This phenomenon has also been noted in the skin of frogs (Wagner, 1839), suggesting that it has evolved relatively early and may be present in many species, presumably because it serves an important physiologic purpose. Many tissues do not support rolling in the absence of inflammation, suggesting that dermal venules are highly specialized (Fiebig et al., 1991; Ley et al., 1995). Since the skin is arguably the most vulnerable organ of the body, constitutive expression of endothelial selectins may reflect a skin-specific stage of increased alertness to permit rapid and efficient leukocyte recruitment to sites of injury.

In addition, it seems likely that the skin supports constitutive homing of leukocytes. Several bone marrow–derived subsets can be found in normal skin, including dendritic cells (DC), Langerhans cells, mast cells, γδ T cells, and some memory T cells (Steinman et al., 1995; Alaibac et al., 1997; Robert and Kupper, 1999). These resident populations are probably maintained by a constant influx of precursors and/or differentiated cells from the blood. Constitutively expressed selectins in the skin may serve as vascular addressins during physiologic homing to that organ. Indeed, E-selectin is known to play a key role in inflammatory skin disease (Springer, 1994; Butcher and Picker, 1996), and there is ample evidence for the ability of skin-homing cells to interact with endothelial selectins. For example, bone marrow–derived mast cells roll via P-selectin in murine skin (Sriramarao et al., 1996a) and human as well as murine DC express α1,3-fucosylated PSGL-1, which allows these cells to roll on P- and E-selectin in vitro and in vivo (Robert et al., 1999). Most γδ T cells also express selectin ligands (Diacovo et al., 1996; Alaibac et al., 1997), and skin-homing T cells are characterized by the cutaneous lymphocyte antigen (CLA), an isoform of PSGL-1 that is a potent E-selectin ligand (Robert and Kupper, 1999). Several studies have demonstrated that myeloid cells and T cells, in particular Th1 cells, must interact with P- and E-selectin to home to inflamed skin (Austrup et al., 1997). Studies in selectin-deficient mice lend further support to this concept; the induction of cutaneous hypersensitivity (CHS) responses are moderately reduced or normal in P$^{-/-}$ mice (Subramaniam et al., 1995; Staite et al., 1996) and unaltered in E-selectin$^{-/-}$ animals (Labow et al., 1994). In contrast, CHS responses are much more severely reduced after inhibition of P-selectin in E-selectin$^{-/-}$ mice (Labow et al., 1994) and in P/E-selectin doubly deficient animals (Staite et al., 1996). Similarly, double knockout mice but not their singly deficient counterparts suffer from spontaneous skin infections (Frenette et al., 1996; Bullard et al., 1996). This phenotype supports the idea that P- and E-selectin are critical for efficient immune surveillance of the skin, but either selectin alone is sufficient to defend dermal integrity and barrier function, at least under SPF/VAF vivarium conditions.

Rolling mediated by either selectin requires α1,3-fucosylated ligands on leukocytes that must be generated by FucT-IV and FucT-VII (Lowe, 1997). In the absence of both enzymes, rolling in ear venules is completely abolished. Collectively, the data in this study (summarized in Table 2) show that most ligands for E- and P-selectin depend on FucT-VII, but occasional rolling is still present in FucT-VII$^{-/-}$ mice but not in FucT-IV+VII$^{-/-}$ animals, indicating that FucT-IV generates selectin ligands that support in vivo rolling of some leukocytes. However, for most leukocytes FucT-VII-dependent carbohydrates determine the rolling fraction (i.e., tethering efficiency), whereas FucT-IV has minor, if any, influence on this parameter. In contrast, products of both FucT-IV and FucT-VII are important during established rolling

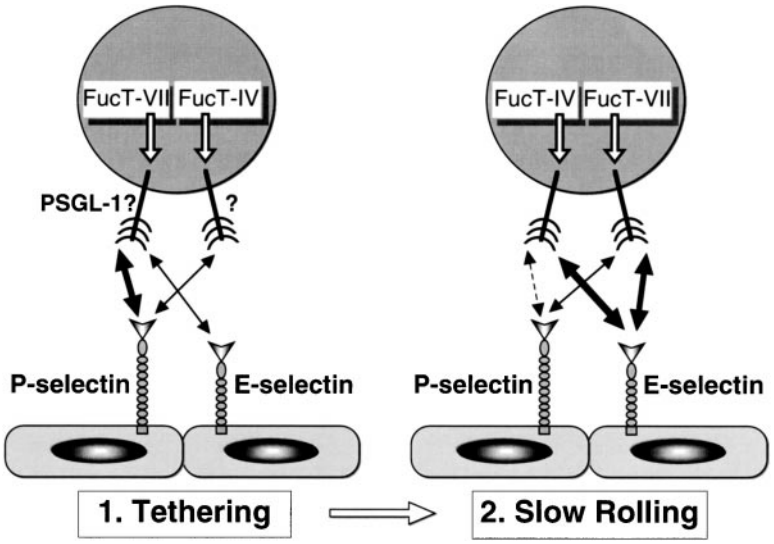

Figure 5. Schematic Diagram of the Relative Contribution by P- and E-Selectin and Carbohydrates Generated by FucT-IV and FucT-VII to Leukocyte Tethering and Rolling in Noninflamed Skin Venules

The estimated relative importance of individual selectin–carbohydrate interactions is indicated by the thickness of arrows. Leukocyte surface glycoconjugates generated by FucT-IV and -VII are shown as separate molecules for clarity. In reality, some glycoproteins such as PSGL-1 may be simultaneously decorated with carbohydrates modified by either enzyme. The contribution of individual adhesion pathways to the magnitude of rolling fractions in postcapillary venules is largely equivalent to the tethering efficiency, i.e., their ability to initiate rolling. Deficiency in P-selectin or FucT-VII had the greatest impact on rolling fractions, whereas E-selectin played a minor role, and absence of FucT-IV did not affect rolling fractions. In FucT-VII$^{-/-}$ mice, only P-selectin can support rare tethering events in a subset of venules, presumably via interactions with FucT-IV-dependent ligands. Once a leukocyte has become tethered, E-selectin is the major determinant of the velocity at which the cell moves. V_{roll} was significantly increased in venules of FucT-IV- and FucT-VII-deficient mice, and inhibition of E-selectin in either strain increased V_{roll} even further. Thus, E-selectin ligands generated by both enzymes are important here. While P-selectin had a modest impact on V_{roll} in animals that expressed both FucTs (i.e., WT and P-selectin$^{-/-}$), P-selectin inhibition in FucT-IV$^{-/-}$ mice increased V_{roll} somewhat. Thus, P-selectin interaction with FucT-VII-dependent ligands can contribute to V_{roll}.

since deficiency in either FucT significantly accelerates V_{roll}. Thus, P-selectin interactions with ligands generated predominantly by FucT-VII control the magnitude of rolling fractions, whereas E-selectin determines V_{roll} by interacting with carbohydrates modified by FucT-VII as well as FucT-IV.

Figure 5 illustrates the relative contribution of P- and E-selectin and FucT-IV and -VII to tethering and slow rolling. It should be cautioned that the diagram in Figure 5 is based in part on the assumption that rolling leukocytes in ear venules are a homogeneous population that is sufficiently similar in treated and untreated strains to allow a direct comparison. However, the rolling cells probably belonged to several different subsets, some of which might differ from others in their relative or absolute expression of FucTs and/or their ability to interact with P- or E-selectin. Indeed, leukocyte subsets have been described that interact preferentially with E- but not P-selectin and vice versa (Diacovo et al., 1996; Sriramarao et al., 1996b). Such subsets might be more or less affected by the lack of an individual selectin or FucT than the population at large. Since fluorescent labeling of leukocytes with rhodamine 6G does not allow us to distinguish between leukocyte families in ear venules, we cannot exclude this possibility. However, these specialized leukocytes probably constitute a relatively small fraction in the total population of interacting cells. Most leukocytes that are likely to roll in ear venules are neutrophils, which express both FucT-IV and -VII and ligands for P- as well as E-selectin (Maly et al., 1996; J. B. Lowe et al., personal communication).

Another assumption is that the density of P- and/or E-selectin was similar in WT and mutant mice. Subtle differences between strains cannot be excluded with certainty, although immunohistology of ear sections revealed no apparent difference in E-selectin expression in WT, P-selectin$^{-/-}$, or FucT-IV$^{-/-}$ mice, at least within the limits of sensitivity of this nonquantitative method (data not shown). Moreover, our results in mutant strains were consistent in quality and quantity with those in mAb-treated WT mice. Overall, the consistency and reproducibility of our data in different settings were not suggestive of any dramatic change in the regulation of selectins. It is possible, however, that genetic deficiency in one FucT resulted in changes in the chemistry and/or abundance of carbohydrates generated by the remaining enzyme. Both FucT-IV and -VII can use the same substrate, N-acetyllactosamine, but FucT-VII can only fucosylate this precursor after sialylation by α(2,3)sialyltransferases. Thus, the products generated by FucT-VII are sLeX-like selectin ligands (Maly et al., 1996). When FucT-IV and FucT-VII are present in the same cell, FucT-IV is thought to fucosylate primarily nonsialylated N-acetyllactosamine resulting in LeX, which is not recognized by selectins. This distinct substrate preference may lead to competition between the two FucTs if the availability of N-acetyllactosamine is limited. In the absence of FucT-IV more substrate might be available for FucT-VII, which could result in abnormally abundant sLeX-like carbohydrates on FucT-IV$^{-/-}$ leukocytes. However, FucT-IV can also utilize sialylated N-acetyllactosamine to generate limited amounts of sLeX (Lowe, 1997). While this property of FucT-IV can explain the occurrence of rolling in FucT-VII$^{-/-}$ mice, it remains to be shown that FucT-IV has equivalent activity in WT leukocytes.

Despite these uncertainties, our data clearly show that neither FucT can completely substitute for the other. Even though substrates for FucT-VII should be abundant in FucT-IV$^{-/-}$ mice, FucT-VII seems incapable of synthesizing selectin ligands for slow rolling. Factors that are intrinsic to each selectin's lectin domain and/or its ligand(s) influence V_{roll}. For example, chemical modifications of the L-selectin ligand PNAd can change the

kinetics and mechanical properties of the bond and drastically alter V_{roll} (Puri et al., 1998). The precise structure of the physiologic P- and E-selectin ligands generated by FucT-IV and/or FucT-VII on murine leukocytes is unknown. However, it is likely that FucT-IV-dependent ligands have distinct binding kinetics from those generated by FucT-VII. This could explain why rolling fractions were normal in FucT-IV$^{-/-}$ mice, whereas V_{roll} was increased and depended more on P-selectin than in WT animals. One hypothetical scenario would be that the bond lifetime between E-selectin- and FucT-IV-dependent CHO is longer than with carbohydrates generated by FucT-VII. This would result in a disproportional contribution of E-selectin and FucT-IV in lowering V_{roll}. In the absence of FucT-IV, E-selectin can still interact with FucT-VII-dependent ligands but perhaps with a shorter bond lifetime resulting in higher V_{roll}.

Of note, recent biochemical analyses of PSGL-1 and E-selectin ligand (ESL)-1 have shown that both are substrates for FucT-IV as well as FucT-VII (Niemelä et al., 1998). PSGL-1 is the most prominent ligand for P-selectin but can also interact with E-selectin, whereas ESL-1 binds selectively and with high affinity only to E-selectin (Vestweber and Blanks, 1999). Our data are consistent with a scenario where slow rolling is mediated by E-selectin binding to carbohydrates generated by both FucTs and presented by ESL-1, PSGL-1, and possibly numerous other structures. In contrast, FucT-IV-dependent glycoconjugates are probably of little importance for the formation of initial contacts between leukocytes and skin venules. This event appears to be dominated by microvillous PSGL-1 modified by FucT-VII to interact with P-selectin. A more profound understanding of the specific role of ESL-1 and PSGL-1 during interactions with P- and E-selectin in the skin awaits further experimentation.

Experimental Procedures

Antibodies

mAb 9A9 (rat IgG2b) and mAb 5H1 (rat IgG1), which neutralize mouse E-selectin and P-selectin, respectively, and the nonbinding control mAb 2-4A1 (rat IgG2b) were kindly provided by Dr. Barry Wolitzky (Hoffman LaRoche, Nutley, NJ). mAb mIC2/3c4 against mouse ICAM-2 (rat IgG2a) was a gift from Dr. Timothy Springer (CBR, Boston, MA). For use in intravital microscopy experiments, these mAbs were HPLC purified from sterile culture supernatants and stored at −70°C in endotoxin-free phosphate-buffered saline (PBS). Immunohistochemistry was performed using culture supernatants of mAb mIC2/3c4, anti-E-selectin mAb 10E9.6 (kindly provided by Dr. Dietmar Vestweber, Münster, Germany), and rat IgG (Southern Biotechnology, Birmingham, AL).

Mice

All animals used in this study were on a C57BL/6 × 129SV/J1 background. FucT-IV$^{-/-}$ and FucT-VII$^{-/-}$ mice were generated using standard gene-targeting techniques (Maly et al., 1996; J. B. Lowe et al., personal communication). Homozygous deficient mice from these two strains were mated to generate mice deficient in both FucT-IV and FucT-VII (FucT-IV+VII$^{-/-}$) as described elsewhere (J. B. Lowe et al., personal communication). L-selectin$^{-/-}$ and P-selectin$^{-/-}$ mice were kindly provided by Dr. Mark Siegelman (University of Texas Southwestern Medical Center, Dallas, TX) and Dr. Denisa Wagner (CBR, Boston, MA), respectively. Age- and sex-matched WT littermates of L-selectin-, P-selectin-, and FucT-deficient strains were used as controls and in mAb inhibition studies. Since there was no detectable difference between WT strains (data not shown), data from these animals were pooled in Results. Experimental animals were bred and housed in a specific pathogen-free/viral antibody-free (SPF/VAF) animal facility and provided with sterilized chow and water ad libidum. All experimental protocols were approved by the Standing Committees on Animals of both Harvard Medical School and the Center for Blood Research.

Animal Preparation and Intravital Microscopy

Animal preparation as well as technical and experimental aspects of our customized intravital video microscopy setup have been described recently (von Andrian and M'Rini, 1998; Robert et al., 1999). In brief, young adult (6–12 weeks old) mice of both sexes were anesthetized by intraperitoneal injection of physiologic saline (10 ml/kg) containing ketamine HCL (5 mg/ml) and xylazine (1 mg/ml). The right jugular vein was catheterized with PE-10 polyethylene tubing. The fluorescent dye rhodamine 6G (20 mg/kg in PBS; Molecular Probes) was administered intravenously to visualize circulating leukocytes by video-triggered stroboscopic epi-illumination. Several superficially located postcapillary and small collecting venules in the left ear were randomly chosen and recorded during 1–3 min intervals to assess baseline rolling interactions. Subsequently, a bolus of 300 μl Ringer's injection solution containing 50 μg of mAb (9A9, 5H1, mIC2/3c4, or 2-4A1) was slowly injected through the jugular vein catheter. Fifteen minutes later, the same venules were again recorded for 1–3 min intervals until ~45 min after mAb injection. Some animals were first treated with mAb 5H1 (50 μg) and 30 min later with mAb 9A9 (50 μg), and rolling in ear venules was recorded before and after each injection.

Peripheral Blood Leukocyte Counts

To determine whether the mAbs used for in vivo experiments affected the number or composition of circulating leukocytes, whole blood was drawn from the retroorbital plexus before and 15 and 45 min after injection of 50 μg mAb 9A9, 5H1, mIC2/3c4, or 2-4A1. Duplicate differential leukocyte counts were performed using a hemocytometer. In some animals, the hematocrit before and after mAb injection was also measured.

Image Analysis

A detailed description of leukocyte rolling and velocity analysis has been published previously (von Andrian and M'Rini, 1998; Stein et al., 1999). Irrespective of the animal strain, treatment protocol, or duration of the experiment, <1% of leukocytes entering ear venules were observed to stick (defined as stationary adhesion for ≥30 s). Thus, leukocyte sticking was not included as a parameter.

Immunohistochemistry

A standard protocol for immunohistochemistry was followed. In brief, paraformaldehyde (2% in PBS, pH 7.5) fixed tissue samples were incubated over night at 4°C with supernatants of mAbs to E-selectin (10E9.6) or ICAM-2 (mIC2/3c4). Control sections were treated with PBS containing 10 μg/ml rat IgG or 1% normal rat serum. Subsequently, slides were incubated with biotinylated rabbit-anti-rat Ig followed by streptavidin-coupled horseradish peroxidase. Specific mAb binding was detected with diaminobenzidine (DAB; Vector Laboratories) followed by counterstaining with hematoxylin.

Statistics

Data in graphs and tables are shown as mean ± SD, unless otherwise indicated. For statistical analysis a commercially available software pack was used (GraphPad Prism, 2.0b). For comparison of two samples, a two-tailed Wilcoxon signed rank test was used when applicable. Multiple samples were compared using the Kruskal-Wallis test and Dunn's multiple comparison test was used to calculate p values. Velocity histograms were compared using the Mann-Whitney U test and the Kolmogorov-Smirnov test. Differences were considered statistically significant when $p < 0.05$.

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