

$\alpha(1,3)$ Fucosyltransferases FucT-IV and FucT-VII Control Susceptibility to Atherosclerosis in Apolipoprotein E^{-/-} Mice

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Objective—These studies examine the contributions of $\alpha(1,3)$ fucosyltransferases (FucT) IV and VII to the generation of selectin counter-receptors necessary for selectin-dependent atherogenesis. They also determine the functional contribution of FucT-IV and FucT-VII to shear-dependent tethering of monocytes to P-selectin, a process believed to be required for atherogenesis.

Methods and Results—Atherosclerotic lesion size and histology were determined in apolipoprotein E^{-/-} mice sufficient or deficient in FucT-IV or FucT-VII. Lesion size was subtly reduced in FucT-IV-deficient mice and significantly reduced in FucT-VII-deficient mice. FucT deficiency did not alter lesion histology, plasma total cholesterol, or the lipoprotein distribution profile. Atheroprotection in FucT-IV or FucT-VII deficiency aligned with subtle and profound reductions, respectively, of P-selectin counter-receptor activity on peripheral blood monocytes as determined by tethering to P-selectin-IgG in vitro under shear flow.

Conclusions—FucT-VII-mediated $\alpha(1,3)$ fucosylation of selectin ligands is a necessary concomitant to atherogenesis in apoE^{-/-} mice and is required for P-selectin-dependent peripheral blood monocyte adhesion under shear stress. FucT-IV deficiency yields subtle deficits in monocyte P-selectin counter-receptor activity and is associated with a subtle decrement in atherosclerosis. These studies identify an important role for FucT-VII in atherogenesis, and a subsidiary role for FucT-IV, and implicate leukocyte selectin counter-receptors in the pathogenesis of atherosclerosis. (*Arterioscler Thromb Vasc Biol.* 2004;24:1897-1903.)

Key Words: atherosclerosis ■ apolipoprotein E ■ fucosyltransferase ■ monocyte ■ selectin

Several adhesion molecule systems have been proposed to facilitate the leukocyte trafficking events inherent to the atherosclerotic process. Receptor/ligand pairs thought most likely to participate prominently in this process include the selectin receptor/counter-receptor system, the vascular cell adhesion molecule-1/integrin (VLA-4) system, and the intercellular adhesion molecule-1/integrin (LFA-1, Mac-1) system.^{1,2}

Nonetheless, our understanding of how the selectin adhesion molecules and their ligands contribute to the atherosclerotic process is incomplete. Deficiency of E-selectin and/or P-selectin limits atherosclerosis in apolipoprotein E-deficient (apoE^{-/-})³⁻⁵ and low-density lipoprotein receptor-deficient⁶⁻⁸ mice. The authors of these studies imply that this protection might be attributed to reduced selectin-dependent recruitment of monocytes into the atherosclerotic lesion. This hypothesis is consistent with the observation that rolling of monocytic cells on atherosclerotic endothelium is P-selectin-dependent.⁹ However, decreased monocyte density is not a consistent finding in lesions from P-selectin or E-selectin and P-selectin-deficient mice,^{2,5} and a cause-and-effect relationship remains to be established between P-selectin-dependent monocyte adhesion to endotheli-

um and monocyte recruitment in the pathogenesis of atherosclerosis. Furthermore, there is not yet a clear understanding of the relative contributions made by selectin-dependent adhesion, vascular cell adhesion molecule-dependent adhesion, or other adhesive mechanisms in the recruitment of monocytes to inflammatory lesions of the vasculature.

Alternative and/or additional roles for the selectins and their ligands in the atherosclerotic process are likely. For instance, platelets expressing P-selectin have recently been shown to enhance atherosclerosis,^{10,11} as does procoagulant activity afforded by endothelial-derived soluble P-selectin.¹² In addition, there is evidence that a T-cell-mediated immune response may contribute to the atherosclerotic process.¹³⁻¹⁹ In this context, the initiation and/or progression of atherosclerosis may involve E-selectin and/or P-selectin-dependent recruitment of activated Th1 and Tc1 lymphocytes to nascent and/or established atherosclerotic lesions,²⁰⁻²² and/or P-selectin-dependent platelet-mediated delivery of T lymphocytes to lymph nodes.²³ Clearly, although E-selectin and P-selectin make important contributions to the initiation and/or progression of atherosclerosis, it is evident that their relative and

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absolute contributions to atherosclerosis remain to be precisely defined.

The ligands for E-selectin, P-selectin, and L-selectin maintain optimal functional activity only when modified by certain glycan structures that contain 1 or more $\alpha(1,3)$ -fucose residues.²⁴ The $\alpha(1,3)$ fucosyltransferases (FucT), FucT-IV and FucT-VII, are responsible for the fucosylation reactions that result in physiological selectin ligand activity.²⁴ Analyses of mice with induced mutations of these genes have shown that FucT-IV and FucT-VII deficiency lead to subtle or substantial deficiencies, respectively, of E-selectin and P-selectin ligand activity on neutrophils, monocytes, Th1 lymphocytes, and Tc1 lymphocytes.^{25–27}

The role for FucT-IV and FucT-VII in the generation of active E-selectin, P-selectin, and L-selectin ligands used for leukocyte trafficking in atherosclerosis has not been defined. Therefore, we examined atherosclerosis in apoE^{-/-} mice also genetically deficient in either FucT-IV or FucT-VII. The studies disclose that FucT-VII-mediated $\alpha(1,3)$ fucosylation of leukocyte selectin ligands is a necessary concomitant to atherogenesis in apoE^{-/-} mice and is required for P-selectin-dependent peripheral blood monocyte adhesion under shear stress. By contrast, we observe that FucT-IV deficiency yields subtle deficits in monocyte P-selectin ligand activity and is associated with a subtle decrement in atherosclerosis. Taken together, these studies identify an important role for FucT-VII in atherogenesis, and a minor role for FucT-IV, and implicate leukocyte selectin ligands in the pathogenesis of this disease.

Methods

Generation and Maintenance of

Experimental Mice

Noncongenic FucT-IV^{-/-}/apoE^{-/-} and FucT-VII^{-/-}/apoE^{-/-} strains of mice, and congenic FucT-VII^{-/-}/apoE^{-/-} strains of mice were established as detailed elsewhere (please see <http://atvb.ahajournals.org>) and maintained on a diet of water and normal chow (Prolab RMH 3000) ad libitum. Littermate studies in noncongenic strains were initiated before extensive backcrossing of these strains onto the C57BL/6J background. The initial results obtained in the noncongenic FucT-VII^{-/-} strain were subsequently confirmed when the congenic FucT-VII^{-/-} strain was established. Experimental procedures and animal husbandry conformed to the guidelines of The University of Michigan Committee on the Use and Care of Animals. Veterinary care was provided by The University of Michigan Unit for Laboratory Animal Medicine.

Lipid Analysis

Published techniques and commercially available reagents (Sigma) were used for quantitation of plasma lipids,³ and for determination of cholesterol lipoprotein profile by fast protein liquid chromatography.²⁸

Lesion Size Determination

Aortic root lesion size was quantitated according to the method of Paigen,²⁹ and en face lesion area was determined using image analysis software to analyze fixed and stained aortas as described in detail in the supplemental material (available at <http://atvb.ahajournals.org>).

Leukocyte Counts

Manual leukocyte counts were performed using the Unopette system (Becton Dickinson and Company). Manual leukocyte differential counts were performed on Wright's stained blood smears.

Immunohistochemistry

Frozen sections of aortic root lesions in cross-section were labeled for the presence of monocytes/macrophages (anti-CD11b; Pharmingen 01712D), T lymphocytes (anti-CD4; Pharmingen 09002A), or actin (anti-alpha smooth muscle actin; Dako U7033) according to protocols detailed online.

Monocyte Adhesion Assay

Peripheral blood monocytes from C57BL/6J congenic mice were isolated using a modified negative selection technique³⁰ detailed online and sterile and endotoxin-free reagents. Shear-dependent monocyte-selectin adhesive interactions were analyzed using a parallel-plate flow chamber with the bottom plate coated with P-selectin-human IgG chimera.²⁵

Statistical Analyses

Summarized data are presented as the mean \pm SEM. Two group comparisons were performed using the 2-tailed Student *t* test. Correlations were evaluated with Pearson rank correlation coefficient. For all analyses, significance was accepted at $P \leq 0.05$.

Results

Trend Toward Decreased Atherosclerotic Lesion Size in Noncongenic FucT-IV-Deficient ApoE^{-/-} Littermate Mice

Preliminary studies established the temporal course of atherosclerotic lesion formation and the histological characteristics of the lesions in wild-type, apoE^{-/-}, FucT^{+/+}/apoE^{-/-}, and FucT^{-/-}/apoE^{-/-} strains of mice. These studies disclosed that very small fatty streak lesions were present at 3 months and that well-developed fatty streaks had developed by 4 months of age. By contrast, at 6 months of age, complex lesions had begun to develop, with the appearance of smooth muscle cells in early fibrous regions of the lesions (data not shown). Subsequent determinations of lesion size were performed at 4 and 6 months of age.

In a subset of the male noncongenic FucT-IV^{-/-}/apoE^{-/-} and FucT-IV^{+/+}/apoE^{-/-} littermate mice at 4 and 6 months, we observed that aortic root lesion area correlated with lesion area expressed as percent of total aortic intimal surface area (Figure 1A, available online at <http://atvb.ahajournals.org>). These data indicate that aortic root lesion size predicts the extent of atherosclerosis in the entire aorta.

In the FucT-IV-sufficient or FucT-IV-deficient data set, plasma concentrations of total cholesterol, and triglycerides (Table I, available online at <http://atvb.ahajournals.org>) were similar to those reported previously in apoE^{-/-} mice.³ Relative plasma cholesterol concentrations increased with age and were higher in males than females. However, we found no FucT-IV-dependent differences between age-matched and sex-matched controls. Plasma triglyceride concentrations compared with age matched FucT-IV^{+/+}/apoE^{-/-} mice were lower in FucT-IV^{-/-}/apoE^{-/-} males at 4 months and higher in FucT-IV^{-/-}/apoE^{-/-} females at 6 months of age. These divergent changes in triglyceride concentrations in groups of FucT-IV^{-/-}/apoE^{-/-} mice that both have smaller lesions implicate a mechanism other than triglyceride concentration in the limitation of lesion size.

Aortic root lesion size for 4- and 6-month-old apoE^{-/-} mice either sufficient or deficient for FucT-IV is shown in Figure 1A. At 4 months of age, lesion size was similar in FucT-IV^{-/-}/apoE^{-/-} and FucT-IV^{+/+}/apoE^{-/-} mice

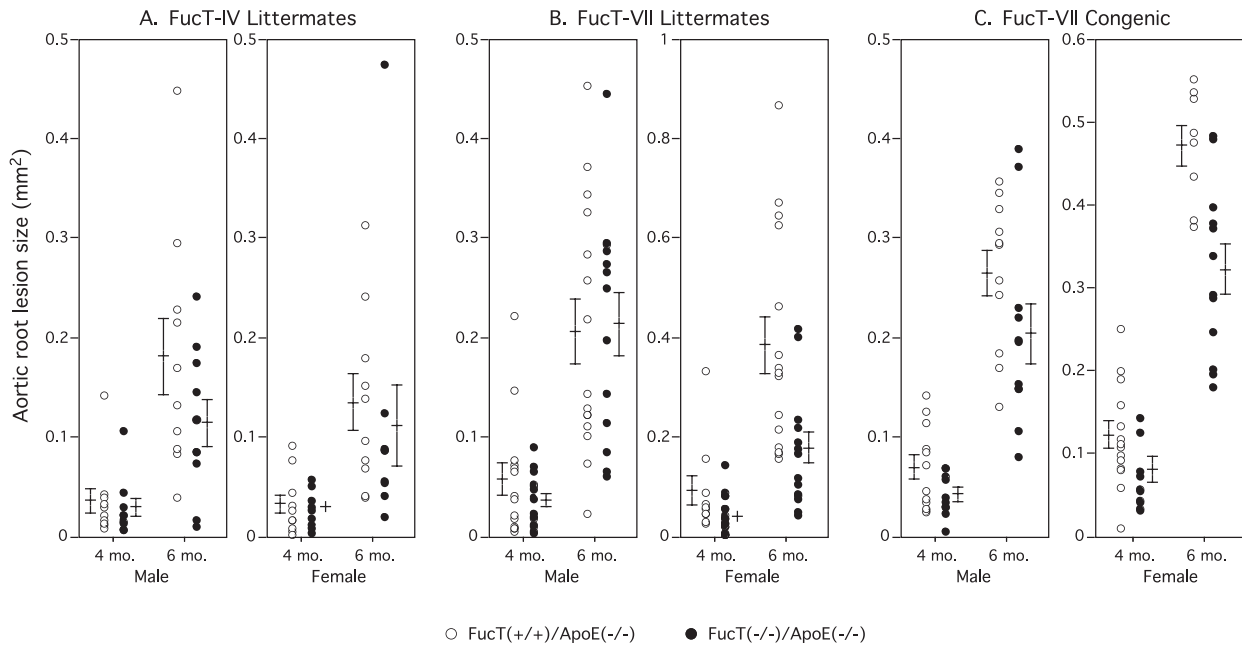


Figure 1. Atherosclerotic lesion size. Aortic root lesion size in (A) FucT-IV^{+/+}/apoE^{-/-} versus FucT-IV^{-/-}/apoE^{-/-} littermate mice (B), FucT-VII^{+/+}/apoE^{-/-} versus FucT-VII^{-/-}/apoE^{-/-} littermate mice, and (C) FucT-VII^{+/+}/apoE^{-/-} versus FucT-VII^{-/-}/apoE^{-/-} C57Bl/6J congenic mice.

(FucT-IV^{-/-}/apoE^{-/-} = 0.028 ± 0.005 , $n=20$; versus FucT-IV^{+/+}/apoE^{-/-} = 0.034 ± 0.008 , $n=20$; $P=0.573$) even when the data were segregated by sex (male FucT-IV^{-/-}/apoE^{-/-} = 0.028 ± 0.009 , $n=10$; versus FucT-IV^{+/+}/apoE^{-/-} = 0.036 ± 0.012 , $n=10$; $P=0.617$; female FucT-IV^{-/-}/apoE^{-/-} = 0.029 ± 0.006 , $n=10$; versus FucT-IV^{+/+}/apoE^{-/-} = 0.032 ± 0.010 , $n=10$; $P=0.809$). By contrast, at 6 months of age, average lesion size was 28% smaller in FucT-IV^{-/-}/apoE^{-/-} mice relative to FucT-IV^{+/+}/apoE^{-/-} mice (FucT-IV^{-/-}/apoE^{-/-} = 0.113 ± 0.023 , $n=20$; versus FucT-IV^{+/+}/apoE^{-/-} = 0.158 ± 0.024 , $n=20$; $P=0.184$), although this decrement did not reach statistical significance. Segregation by sex at 6 months of age revealed that average lesion size was 18% and 37% smaller in FucT-IV^{-/-}/apoE^{-/-} females and males, respectively, although again these decrements did not reach statistical significance (female FucT-IV^{-/-}/apoE^{-/-} = 0.111 ± 0.042 , $n=10$; versus FucT-IV^{+/+}/apoE^{-/-} = 0.135 ± 0.028 , $n=10$; $P=0.644$; male FucT-IV^{-/-}/apoE^{-/-} = 0.114 ± 0.024 , $n=10$; versus FucT-IV^{+/+}/apoE^{-/-} = 0.181 ± 0.039 , $n=10$; $P=0.159$). Analyses of this data set from noncongenic animals imply that a FucT-IV deficiency may lead to a subtle decrement in atherogenesis that is not attributed to changes in the plasma lipids.

Decreased Atherosclerotic Lesion Size in Noncongenic FucT-VII-Deficient Littermates

As in the FucT-IV noncongenic data set, aortic root lesion size correlated with lesion area expressed as percent of total aortic intimal surface area (Figure 1B, available online at <http://atvb.ahajournals.org>) in a subset of both FucT-VII^{-/-}/apoE^{-/-} and FucT-VII^{+/+}/apoE^{-/-} male littermate mice. These data indicate that aortic root lesion size predicts the extent of atherosclerosis in the entire aorta. Relative plasma total cholesterol concentrations increased with age and were higher in

males than females (Table I). A decrease in total cholesterol was present only in FucT-VII^{-/-}/apoE^{-/-} females at 6 months of age. This decrease was associated with a smaller lesion size, but smaller lesions in other groups of FucT-VII^{-/-}/apoE^{-/-} mice were not associated with decreases in total cholesterol. Plasma triglyceride concentration within genotype and sex was stable over time except for an increase in the average concentration in FucT-VII^{+/+}/apoE^{-/-} males at 6 months. This resulted in a relatively lower triglyceride concentration in the 6-month-old FucT-VII^{-/-}/apoE^{-/-} males, which, however, was not associated with smaller lesion size.

At 4 months of age, lesion size independent of sex was 52% smaller in FucT-VII^{-/-}/apoE^{-/-} mice compared with FucT-VII^{+/+}/apoE^{-/-} mice (FucT-VII^{-/-}/apoE^{-/-} = 0.037 ± 0.006 , $n=34$; versus FucT-VII^{+/+}/apoE^{-/-} = 0.071 ± 0.016 , $n=24$; $P=0.025$). Reduction in lesion size (Figure 1B) was 58% in female FucT-VII^{-/-}/apoE^{-/-} mice (FucT-VII^{-/-}/apoE^{-/-} = 0.038 ± 0.009 , $n=19$; versus FucT-VII^{+/+}/apoE^{-/-} = 0.091 ± 0.029 , $n=10$; $P=0.040$), and 37% in male FucT-VII^{-/-}/apoE^{-/-} mice (FucT-VII^{-/-}/apoE^{-/-} = 0.036 ± 0.007 , $n=15$; versus FucT-VII^{+/+}/apoE^{-/-} = 0.057 ± 0.016 , $n=14$; $P=0.239$). At 6 months of age, lesion size independent of sex was 34% smaller in FucT-VII^{-/-}/apoE^{-/-} mice compared with FucT-VII^{+/+}/apoE^{-/-} mice (FucT-VII^{-/-}/apoE^{-/-} = 0.194 ± 0.022 , $n=28$; versus FucT-VII^{+/+}/apoE^{-/-} = 0.295 ± 0.037 , $n=30$; $P=0.024$). At this age, smaller lesion size was again observed in female FucT-VII^{-/-}/apoE^{-/-} mice (FucT-VII^{-/-}/apoE^{-/-} = 0.178 ± 0.031 , $n=15$; versus FucT-VII^{+/+}/apoE^{-/-} = 0.385 ± 0.058 , $n=15$; 54% smaller, $P=0.004$). By contrast, the reduction in lesion size observed in males at 4 months of age was no longer evident at the 6-month evaluation (FucT-VII^{-/-}/apoE^{-/-} = 0.214 ± 0.032 , $n=13$; versus FucT-VII^{+/+}/apoE^{-/-} = 0.206 ± 0.033 , $n=15$;

Plasma Lipid Concentrations in C57BL/6J Congenic FucT-VII Mice*

Age	Sex	Genotype	Total Cholesterol (mg/dL)	Triglycerides (mg/dL)
4 mo	F	Fuc-TVII+/+/ApoE-/-	377±12 (n=14)	53±5 (n=14)
		Fuc-TVII-/-/ApoE-/-	394±19 (n=11)	71±18 (n=11)
	M	Fuc-TVII+/+/ApoE-/-	610±33 (n=12)	120±7 (n=12)
		Fuc-TVII-/-/ApoE-/-	528±28 (n=10)	110±22 (n=10)
6 mo	F	Fuc-TVII+/+/ApoE-/-	412±27 (n=8)	75±17 (n=8)
		Fuc-TVII-/-/ApoE-/-	376±27 (n=11)	55±7 (n=11)
	M	Fuc-TVII+/+/ApoE-/-	540±25 (n=12)	112±13 (n=12)
		Fuc-TVII-/-/ApoE-/-	520±30 (n=10)	123±21 (n=10)

*No significant differences between age-matched and sex-matched FucT-VII genotypes.

$P=0.866$). Analysis of this data set from noncongenic animals indicates that FucT-VII deficiency results in a clear decrement in atherosclerosis that is not attributed to changes in the plasma lipids.

Atherosclerotic Lesion Size Is Dependent on FucT-VII Genotype in C57BL/6J Congenic Mice

To extend the observation of limited lesion size in FucT-VII-/-/apoE-/- noncongenic littermates, aortic root lesion area was quantified in C57BL/6J congenic FucT-VII-/-/apoE-/- mice constructed from FucT-VII-/- mice backcrossed to be C57BL/6J congenic²⁵ (Figure 1C). At 4 months of age, lesion size independent of sex was 36% smaller in FucT-VII-/-/apoE-/- mice compared with FucT-VII+/+/apoE-/- mice (FucT-VII-/-/apoE-/- = 0.062 ± 0.010 , $n=21$; versus FucT-VII+/+/apoE-/- = 0.097 ± 0.012 , $n=26$; $P=0.028$). Smaller lesion sizes were manifest in both male FucT-VII-/-/apoE-/- (FucT-VII-/-/apoE-/- = 0.042 ± 0.022 , $n=10$; versus FucT-VII+/+/apoE-/- = 0.069 ± 0.002 , $n=12$; 39% smaller, $P=0.081$) and female FucT-VII-/-/apoE-/- mice (FucT-VII-/-/apoE-/- = 0.080 ± 0.054 , $n=11$; versus FucT-VII+/+/apoE-/- = 0.122 ± 0.062 , $n=14$; 34% smaller, $P=0.089$). At 6 months of age in congenic mice, lesion size independent of sex was 25% smaller in FucT-VII-/-/apoE-/- animals compared with FucT-VII+/+/apoE-/- animals (FucT-VII-/-/apoE-/- = 0.265 ± 0.024 , $n=23$; versus FucT-VII+/+/apoE-/- = 0.352 ± 0.029 , $n=19$; $P=0.026$). Smaller lesion sizes were manifest in female FucT-VII-/-/apoE-/- mice (FucT-VII-/-/apoE-/- = 0.322 ± 0.105 , $n=12$; versus FucT-VII+/+/apoE-/- = 0.472 ± 0.069 , $n=8$; 32% smaller, $P=0.002$), and male FucT-VII-/-/apoE-/- mice (FucT-VII-/-/apoE-/- = 0.204 ± 0.099 , $n=11$; versus FucT-VII+/+/apoE-/- = 0.265 ± 0.076 , $n=11$; 23% smaller, $P=0.119$).

In congenic FucT-VII+/+/apoE-/- versus FucT-VII-/-/apoE-/- mice, there were no differences in plasma total cholesterol in either male or female mice at 4 or 6 months of age. Likewise, there were no differences in plasma triglycerides in either male or female mice at 4 or 6 months of age (Table). These data confirm in C57BL/6J congenic mice that FucT-VII deficiency in apoE-/- mice does not alter plasma total cholesterol or triglycerides, and imply that the isolated alterations of these parameters measured in some groups of littermate animals were a result of their mixed genetic background. To ensure that there were no differences in the lipoprotein fractions between FucT-VII-sufficient and FucT-VII-defi-

cient strains that might modulate atherogenesis in these animals, we performed plasma fast protein liquid chromatography analysis (Figure 2). The profiles for lipoprotein distribution were identical in FucT-VII-/-/apoE-/- mice and FucT-VII+/+/apoE-/- mice, and were typical for apoE-/- mice in that they were rich in very-low-density lipoprotein and low-density lipoprotein, with very little high-density lipoprotein.³

Atherosclerotic Lesion Histology Is Not Altered by FucT Deficiency

Aortic root atherosclerotic lesions in FucT+/+/apoE-/- mice at 4 months of age consisted of well-developed fatty streak lesions. Lesions progressed to the early fibrous plaque stage with small necrotic cores by 6 months of age. Thus lesion development was temporally delayed but otherwise histologically similar compared with aortic root lesions in P-sel+/+/apoE-/- mice.⁵ The temporal difference in lesion development is most likely caused by environmental variation. Despite smaller lesion size, lesion histology at either 4

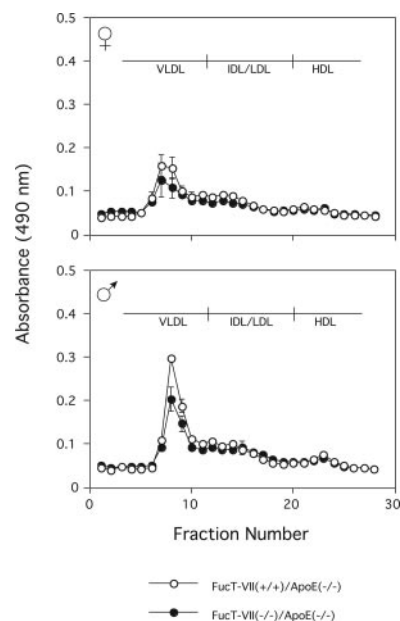


Figure 2. Plasma lipoprotein distribution. Plasma lipoprotein distribution profiles obtained by fast protein liquid chromatography analysis are identical in 6-month-old female or male FucT-VII+/+/apoE-/- and FucT-VII-/-/apoE-/- C57BL/6J congenic apoE-/- mice. Data represent 4 to 5 pooled plasma samples from 2 mice each.

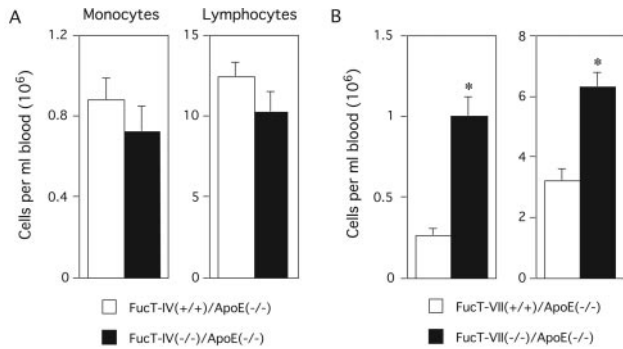


Figure 3. Peripheral blood leukocyte counts. A, Differential monocyte and lymphocyte counts were not different between FucT-IV+/+apoE^{-/-} (n=11) and FucT-IV^{-/-}/apoE^{-/-} (n=10) littermate mice. B, A monocytosis and lymphocytosis was present in congenic C57BL/6J FucT-VII^{-/-}/apoE^{-/-} mice (n=21) compared with FucT-VII+/+apoE^{-/-} mice (n=23). There were ≈50% males and females in all groups. **P*≤0.05.

or 6 months of age was not qualitatively different between FucT (IV or VII)-deficient and FucT-sufficient mice. Immunohistochemical staining of frozen sections revealed scattered CD4⁺ T cells and numerous CD11b⁺ macrophages in all lesions, and α -actin in fibrous regions of lesions at 6 months of age, with no qualitative differences observed between respective FucT-deficient and FucT-sufficient genotypes. By contrast, in similarly aged mice, P-selectin deficiency limited lesions to fatty streaks in both the aortic root⁵ and the aorta.⁴ The development of early fibrous plaques in FucT^{-/-}/apoE^{-/-} mice may reflect the presence of a yet to be discovered FucT-independent but physiologically relevant selectin ligand or, more likely, residual FucT-dependent selectin ligand activity contributed by the remaining FucT.

FucT-VII^{-/-}/ApoE^{-/-} Mice Maintain a Peripheral Blood Leukocytosis

Consistent with previous studies,²⁵ FucT-IV deficiency did not alter leukocyte counts in apoE^{-/-} mice (Figure 3A), although the total white blood cell count in these strains on a mixed genetic background were slightly higher than those reported in corresponding apoE^{+/+} congenic strains.²⁵

Previous studies documented a peripheral blood leukocytosis in mice deficient in either P-selectin³¹ or FucT-VII-dependent selectin ligands.^{25–27} As compared with FucT-VII+/+apoE^{-/-} mice, congenic FucT-VII^{-/-}/apoE^{-/-} mice also maintain this leukocytosis, which is comprised in part by a monocytosis (3.8-fold increase) and a lymphocytosis (2.0-fold increase; Figure 3B). Based on the proposed roles for monocytes and lymphocytes in the development of atherosclerosis, an increase in their circulating numbers could be postulated to enhance lesion formation. However, atherosclerotic lesion sizes were smaller in FucT-VII^{-/-}/apoE^{-/-} mice despite the presence of this monocytosis/lymphocytosis.

FucT Genotype Dictates Selectin Ligand Activity on Peripheral Blood Monocytes

The importance assigned to monocytes in the generation of the atherosclerotic lesion^{13,32} and the observation that mice genetically deficient in P-selectin and/or E-selectin are rela-

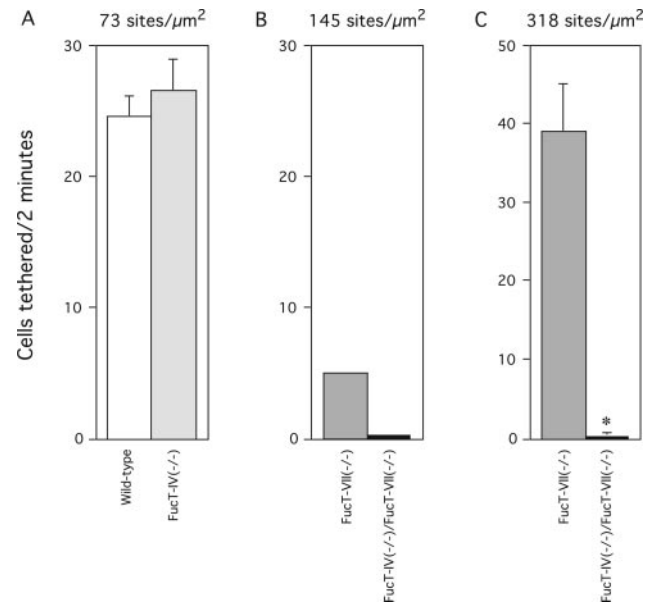


Figure 4. FucT-dependent P-selectin counter-receptor activity on peripheral blood monocytes. Tethering efficiency of C57BL/6J congenic peripheral blood monocytes at a shear stress of 0.77 dynes/cm² on P-selectin-IgG chimera at a density of (A) 73 sites/μm², (B) 145 sites/μm², or (C) 318 sites/μm². **P*≤0.05.

tively protected from atherosclerosis^{4,5,7,8} have led others to discover that P-selectin and its ligand, PSGL-1, mediate monocyte rolling on atherosclerotic endothelium,⁹ and contribute to neointimal formation after injury in apoE^{-/-} mice.³³ In this context, and prompted by the atheroprotective effect we observed in the fucosyltransferase-deficient strains, we characterized P-selectin ligand activities on peripheral blood monocytes isolated from these mice by quantifying shear-dependent rolling adhesion (tethering) to recombinant P-selectin-IgG in a flow chamber-based assay. Cell tethering efficiency was determined at a shear stress (0.77 dynes/cm²) chosen to reflect the mean shear stress measured at the lateral wall region of the internal carotid artery. This region is susceptible to atherosclerosis and is characterized by bidirectional flow of a low shear stress from +4 to -4 dynes/cm².³⁴ At a P-selectin-IgG density of 73/μm², the tethering efficiency of wild-type and FucT-IV^{-/-} monocytes was the same (24.5±1.6, n=4, versus 26.5±2.4, n=4; respectively; Figure 4A). By contrast, even at an intermediate P-selectin-IgG site density of 145/μm², there was little or no detectable tethering of monocytes from FucT-VII^{-/-} or FucT-IV^{-/-}/FucT-VII^{-/-} mice (5 [n=2] versus 0 [n=1]; respectively; Figure 4B). However, at a higher P-selectin-IgG site density (318/μm²), significant numbers of FucT-VII^{-/-} monocytes were observed to tether whereas FucT-IV^{-/-}/FucT-VII^{-/-} monocytes did not (39.0±6.1 [n=3] versus 0.3±0.3 [n=3]; respectively; Figure 4C). These data indicate that FucT-VII accounts for the majority of P-selectin ligand activity on peripheral blood monocytes. Tethering of FucT-VII^{-/-} monocytes is reflective of FucT-VII-independent P-selectin ligand synthesis. This residual activity is accounted for by FucT-IV-dependent synthesis, because such residual activity is absent from FucT-IV^{-/-}/FucT-VII^{-/-} monocytes. The substantial and minor contributions of FucT-VII and FucT-IV to P-selectin ligand

activity on monocytes reflect the magnitude of limitation of atherosclerotic lesion size observed in FucT-VII^{-/-}/apoE^{-/-} mice and FucT-IV^{-/-}/apoE^{-/-} mice, respectively.

Discussion

Previous analysis of mice with disrupted FucT-IV and/or FucT-VII genes has confirmed a primary although partial contribution of FucT-VII to E-selectin, P-selectin, and L-selectin ligand activity.^{25,26} Those studies suggest the possibility that the selectin-mediated cellular adhesion and recruitment events known and/or thought to be important in atherogenesis will be dependent primarily on FucT-VII activity, and secondarily on FucT-IV activity. Limitation of lesion size was evident in FucT-VII-deficient mice at 4 and 6 months of age, in both noncongenic littermate mice and in C57BL/6J congenic mice that were backcrossed to remove possible strain-dependent variability.^{35,36} The protection from atherosclerosis afforded by FucT-VII deficiency was pronounced in female mice, a finding consistent with previous studies of atherosclerosis in chow-fed apoE^{-/-} mice lacking P-selectin or E-selectin.^{4,5} The trend toward a statistically significant protection from atherosclerosis observed in FucT-IV-deficient littermate mice aligns with previous observations that assign a less prominent role for FucT-IV in selectin ligand synthesis relative to the more prominent role for FucT-VII.^{25,27}

In principle, it may be possible to refine the understanding of the roles for P-selectin and E-selectin and their ligands in atherogenesis by comparing lesion size and leukocyte content in FucT null mice, relative to these same parameters in E-selectin and P-selectin null mice. P-selectin deficiency in apoE^{-/-} mice is associated with a 3.5-fold decrease in lesion size at 4 months of age.⁵ By comparison, FucT-VII deficiency yields a 1.6-fold decrease in lesion size at the same age. The lesser degree of protection observed in FucT-VII-deficient mice may be accounted for by environmental, experimental, or genetic variation, a yet to be discovered FucT-IV/VII-independent but physiologically relevant selectin ligand, or residual FucT-IV-dependent selectin ligand activity. Such residual FucT-IV-dependent ligand activity on FucT-VII-null monocytes would enable atherogenesis that is intermediate between that observed in P-selectin^{-/-}/apoE^{-/-} mice and apoE^{-/-} mice. Future studies of atherosclerosis prone mice deficient in both FucT-IV and FucT-VII will shed light on the degree to which lesion development is accounted for by the collaborative contributions of FucT-VII and FucT-IV to selectin ligand activity.

Recruitment of monocytes to the nascent or established lesion may involve P-selectin-dependent adhesion processes because P-selectin and its ligand PSGL-1 are important in tethering of monocytic cells to atherosclerotic carotid endothelium,⁹ and because P-selectin deficiency reduces atherosclerosis.^{4,5} New support for this hypothesis is provided by our observation that atherosclerotic lesion size is reduced in FucT-VII^{-/-}/apoE^{-/-} mice in the face of a 4-fold peripheral blood monocytoysis, and by our observation of a prominent deficiency of P-selectin ligand activity on FucT-VII^{-/-} peripheral blood monocytes under shear stress *in vitro*. However, our observations do not yet exclude a contribution to atherosclerosis by

E-selectin and/or P-selectin and/or L-selectin ligand-dependent processes involving lymphocytes or platelets.

FucT-VII deficiency deletes E-selectin and P-selectin ligand activities from Th1 and Tc1 T lymphocyte subsets, and thereby disables selectin-dependent recruitment of these T cell subsets to sites of inflammation.²⁷ Even though T-lymphocyte-dependent adaptive immune mechanisms are clearly implicated in the development of atherosclerotic lesions,¹³⁻¹⁹ there is uncertainty as to the degree to which lesion development may be consequent to T lymphocyte recruitment, or whether such recruitment is selectin-dependent. It is therefore unclear as to whether any of the protection we observe here, or that others have observed in selectin-deficient mice,^{4,5,7,8} can be accounted for by quantitative reductions in T cell recruitment to atherosclerotic lesions, or alterations in the subsets of T lymphocytes that are attracted to such lesions.

FucT-VII-null mice also exhibit deficits in homing of naive T cells to peripheral nodes^{25,26} because of a partial deficit in HEV-borne L-selectin ligand activity. Nonetheless, residual homing of naive T cells in FucT-VII-null mice allows them to mount a meaningful adaptive cellular immune response,²⁷ suggesting that the protection from atherosclerosis we observe in these animals is not accounted for by deficits in the proximal arms of their cellular immune response. Likewise, the atheroprotective mechanisms assigned to B lymphocytes and the humoral arm of the adaptive immune response³⁷⁻³⁹ are most likely not deficient in FucT-VII^{-/-} mice because the number of circulating B cells is normal in these animals,²⁷ and these mice are competent to make antibodies (B. Petryniak and J. B. Lowe, unpublished data, 2003).

L-selectin ligands induced on inflamed vascular endothelium, such as overlying the atherosclerotic lesion, have been implicated in T cell recruitment.⁴⁰ Likewise, leukocyte-borne L-selectin and its ligands, implicated in L-selectin-dependent tethering of rolling leukocytes on leukocytes previously adhered to the vessel wall,^{41,42} may in principle provide a means for delivering monocytes and/or T cells to the atherosclerotic lesion. However, we do not know if these types of adhesive and recruitment mechanisms are relevant to the protection we observe in the fucosyltransferase-deficient mice, because it is not known if these L-selectin-dependent adhesive mechanisms contribute to atherosclerosis, nor if FucT-VII might contribute to the expression of such ligands. In future studies, these issues may be addressed by studying atherosclerosis-prone FucT-deficient mice after reconstitution of their hematopoietic system with wild-type or FucT-deficient bone marrow, and by using similar approaches involving L-selectin null mice.

In conclusion, our observations imply that $\alpha(1,3)$ fucosylation of selectin counter-receptors provides a major contribution to selectin-mediated leukocyte recruitment events implicated in atherogenesis and identifies dominant and subsidiary roles, respectively, for FucT-VII and FucT-IV in this synthetic contribution to atherogenesis. These data suggest that future studies involving genetic manipulation of FucT expression in a leukocyte-specific or endothelial cell-specific manner will more precisely define the contributions of each selectin-dependent process to atherogenesis. Finally, these observations identify FucT-VII and FucT-IV as potential targets for pharmaceutical intervention in atherosclerosis

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