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Quantitative trait locus analysis of circulating adhesion molecules in hyperlipidemic apolipoprotein E-deficient mice

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Abstract

Circulating soluble adhesion molecules have been suggested as useful markers to predict several clinical conditions such as atherosclerosis, type 2 diabetes, obesity, and hypertension. To determine genetic factors influencing plasma levels of soluble vascular cell adhesion molecule-1 (VCAM-1) and P-selectin, quantitative trait locus (QTL) analysis was performed on an intercross between C57BL/6J (B6) and C3H/HeJ (C3H) mouse strains deficient in apolipoprotein E-deficient (apoE^{-/-}). Female F₂ mice were fed a western diet for 12 weeks. One significant QTL, named sVcam1 (71 cM, LOD 3.9), on chromosome 9 and three suggestive QTLs on chromosomes 5, 13 and 15 were identified to affect soluble VCAM-1 levels. Soluble P-selectin levels were controlled by one significant QTL, named sSelp1 (8.5 cM, LOD 3.4), on chromosome 16 and two suggestive QTLs on chromosomes 10 and 13. Both adhesion molecules showed significant or an apparent trend of correlations with body weight, total cholesterol, and LDL/VLDL cholesterol levels in the F₂ population. These results indicate that plasma VCAM-1 and P-selectin levels are complex traits regulated by multiple genes, and this regulation is conferred, at least partially, by acting on body weight and lipid metabolism in hyperlipidemic apoE^{-/-} mice.

Keywords

adhesion molecule; soluble; quantitative trait locus; hyperlipidemia; mouse

Introduction

All phases of atherosclerosis involve the recruitment of monocytes from the circulation into the subendothelial space, where they ingest lipids to become foam cells. The transendothelial migration of monocytes is mediated by cellular adhesion molecules expressed on the endothelium. Vascular cell adhesion molecule-1 (VCAM-1) and P-selectin are two adhesion molecules that are expressed by the endothelium, mediating adhesion to and rolling of monocytes along the endothelial border (Blankenberg et al. 2003; Nakashima et al. 1998). Both adhesion molecules are up-regulated on the surface of endothelial cells in lesion-prone sites (Ramos et al. 1999) and are abundantly expressed in atherosclerotic lesions (Johnson-Tidey et al. 1994; O'Brien et al. 1993). Disruptions of VCAM-1 domain 4 or P-selectin inhibit monocyte migration and reduce atherosclerotic lesion size in mice (Cybulsky et al. 2001; Johnson et al. 1997).

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Adhesion molecules are subjected to enzymatic cleavage or to alternative splicing of the messenger RNA, resulting in their release into the circulation (Gearing and Newman 1993). Epidemiological studies have suggested that soluble adhesion molecules can be used as markers to predict risk for several common clinical conditions, such as type 2 diabetes (Koga et al. 1998; Lim et al. 1999; Schmidt et al. 1996; Stehouwer et al. 2002), nondiabetes with insulin resistance (Chen et al. 1999; Hak et al. 2001; Matsumoto et al. 2000; Weyer et al. 2002), obesity (Ferri et al. 1999; Ito et al. 2002; Matsumoto et al. 2002; Strackowski et al. 2002), hypertension (Blann et al. 1994; Buemi et al. 1997; De Caterina et al. 2001; DeSouza et al. 1997), and dyslipidemia (Abe et al. 1998; Hackman et al. 1996). When deficient in apolipoprotein E (apoE^{-/-}), atherosclerosis-susceptible strain C57BL/6 (B6) displays higher plasma soluble VCAM-1 levels than resistant strains C3H/HeJ (C3H) and BALB/c (Pei et al. 2006; Tian et al. 2005). Soluble P-selectin levels are also higher in B6.apoE^{-/-} than in BALB/c.apoE^{-/-} mice (Tian et al. 2005), suggesting a possibility that these circulating adhesion molecules may predict atherosclerosis susceptibility in mice. Thus far, no studies have been carried out to investigate how these inflammatory markers are regulated or whether they are associated with atherosclerotic lesion size. Therefore, in the present study, we performed quantitative trait locus (QTL) analysis to search for chromosomal regions that might influence soluble VCAM-1 and P-selectin levels and evaluated the associations of these adhesion molecules with atherosclerosis and related traits in an intercross between the B6.apoE^{-/-} and C3H.apoE^{-/-} strains.

Material and methods

B X H F₂ cross

The construction of a F₂ cross between B6.apoE^{-/-} and C3H.apoE^{-/-} mice was as reported (Su et al. 2006b). Briefly, female B6.apoE^{-/-} mice were crossed with male C3H.apoE^{-/-} mice to generate F₁ hybrids, which were subsequently intercrossed by brother-sister mating to generate 234 female F₂ progeny. At 6 weeks of age, they were switched onto a Western diet containing 42% fat, 0.15% cholesterol, and 19.5% casein without sodium cholate (TD 88137, Teklad, Madison, WI) and maintained on the diet for 12 weeks. All procedures were carried out in accordance with current National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee.

Measurements of plasma lipids, P-selectin, and VCAM-1

At the end of Western diet feeding, mice were fasted overnight before blood was collected by retro-orbital venous plexus puncture with the animals under isoflurane anesthesia. Plasma total cholesterol, HDL cholesterol, and triglyceride levels were determined by using the Thermo DMA (Louisville, CO) cholesterol and triglyceride kits, as we previously reported (Tian et al. 2005). Plasma P-selectin and VCAM-1 were measured with ELISA kits from the R&D Systems (Minneapolis, MN, USA) by following the manufacturer's instructions.

Aortic lesion analysis

Methods for quantification of atherosclerotic lesions in aortic root were as previously reported (Su et al. 2006b).

Genotyping

Genomic DNA was isolated from the tail of mice by using the standard phenol/chloroform extraction and ethanol precipitation method. F₂ mice were genotyped with microsatellite markers distinguishing strain B6 from strain C3H and covering all chromosomes at an average interval of 12 cM by PCR. Parental and F₁ DNA served as controls for each marker.

Gene expression analysis

The expression level of Thpo in endothelial cells was determined by quantitative RT-PCR. Endothelial cells were isolated and cultured as we previously described (Shi et al. 2000). Briefly, under sterile conditions, the thoracic aorta of mice was harvested and cut into rings ~3 mm long. The aortic segments were placed on Matrigel and incubated in DMEM supplemented with 15% FBS, 1% penicillin/streptomycin, 90 µg/ml heparin, 60 µg/ml endothelial cell growth supplements, and 100 U/ml Fungizone. The vessel segments were removed once cell outgrowth was observed. The cells were passaged with Dispase and then plated into gelatin-coated culture dishes. The subsequent passages were performed with 0.25% trypsin-EDTA. Immunostaining for the von Willebrand factor and the DiI-labeled AcLDL uptake experiment confirmed the reliability of this method in yielding pure endothelial cells. Total RNA was extracted with trizol reagent (Invitrogen) and reverse transcribed into cDNA with ThermoScript™ RT-PCR System (Invitrogen). The cDNA product was amplified by PCR for 27 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 45 seconds at 72°C. The PCR products were separated in 2.5% agarose gels. GAPDH was amplified simultaneously in a separate set of tubes under the same conditions.

Statistical analysis

Phenotype and genotype data of F₂ mice were analyzed with the R/qtl software (<http://www.jax.org/staff/churchill/labsite/software/Jqtl/index.html>). LOD scores were generated to define the significance of the associations of genetic markers with each individual trait. One thousand permutations of the trait values were used to define the genome-wide LOD score threshold required to be significant or suggestive for each specific trait. Loci that exceeded the 95th percentile of the permutation distribution were defined as significant ($P < 0.05$) and those exceeding the 37th percentile were suggestive ($P < 0.63$) according to the criteria recommended by the genetics community in 2003 (Abiola et al. 2003). The confidence interval (CI) for each QTL was determined by the posterior probability test. ANOVA was used for determining whether the mean phenotype values of progeny with different genotypes at a specific marker were significantly different. Triglyceride and lesion size were log-transformed to achieve normal distributions before linear regression analysis was performed. All other traits data exhibited a normal distribution and were not transformed.

Results

Plasma soluble VCAM-1 and P-selectin levels of B6.apoE^{-/-}, C3H.apoE^{-/-}, F₁, and F₂ mice

Plasma VCAM-1 and P-selectin levels of female B6.apoE^{-/-}, C3H.apoE^{-/-}, F₁, and F₂ mice were measured after being fed the Western diet for 12 weeks. As shown in Figure 1, B6.apoE^{-/-} mice had significantly higher plasma levels of soluble VCAM-1 than C3H.apoE^{-/-} mice (723 ± 61 vs. 386 ± 76 ng/ml; $P < 0.0001$; $n = 6$). F₁s had a plasma VCAM-1 (540 ± 69 ng/ml; $n = 6$) level intermediate between the two parental strains. Plasma P-selectin levels of the two parental strains were not statistically significant although B6.apoE^{-/-} mice had a higher level (144 ± 37 vs. 122 ± 7 ng/ml; $n = 4\sim 8$; $P = 0.32$). F₁ mice had P-selectin levels (122 ± 10 ng/ml; $n = 9$) that were comparable to their C3H parent. There was a wide range of variations in soluble VCAM-1 and P-selectin levels among F₂s, suggesting complex inheritance of the traits. The trait values of soluble VCAM-1 and P-selectin of F₂ mice were approximately normally distributed (Figure 2).

Loci for soluble VCAM-1 and P-selectin

234 female F₂ mice derived from B6.apoE^{-/-} and C3H.apoE^{-/-} mice were analyzed to search for loci contributing to variations in plasma soluble VCAM-1 and P-selectin levels (Figure 3). Genome-wide scans of the F₂ mice revealed one significant locus on chromosome 9, and three

suggestive loci on chromosomes 5, 13 and 15, respectively, that affected plasma VCAM-1 levels. Details of the QTLs detected, including peak position, nearest marker locus, LOD score, and confidence interval (CI) are presented in Table 1. The chromosome 9 locus peaked at 71 cM and had a significant LOD score of 3.9 (Figure 4A). We designated this locus as sVcam1 to represent the first QTL identified that affects plasma VCAM-1 levels in the mouse. This locus exhibited a dominant inheritance pattern from the B6 allele in that F₂ mice with the heterozygous BC genotype near the peak marker D9Mit18 had a plasma VCAM-1 level comparable to those with the BB genotype but lower than those with the CC genotype (Figure 5A). The B6 allele was associated with decreased plasma VCAM-1 levels in the F₂ cross. The chromosome 5 locus peaked at 52 cM and had a suggestive LOD score of 2.2 (Figure 4B). The C3H allele was related to a decreased VCAM-1 level in a dominant fashion (Figure 5B). The other two loci on chromosomes 13 and 15 had a suggestive LOD score of 2.1 (Table 1) and the F₂ mice with the BC genotype had either significantly lower or higher VCAM-1 level than those with the BB or CC genotype (It is called heterosis when the heterozygote has a trait value significantly larger or smaller than both homozygotes) (Figure 5C and D).

One significant locus on chromosome 16 and two suggestive loci on chromosomes 10 and 13 were identified to affect plasma P-selectin levels (Figure 4B). All three loci influenced P-selectin levels in a heterosis manner because the heterozygote did not match the phenotype of either homozygote (Figure 5E, F and G). The chromosome 16 locus peaked at 8.5 cM and had a significant LOD score of 3.4 (Table 1). This locus was designated as sSelp1. Both chromosomes 10 and 13 QTLs had a suggestive LOD score of 2.5 and the former locus peaked at 45 cM and the latter at 41 cM (Figure 4B).

Associations of soluble VCAM-1 and P-selectin with plasma lipids, body weight, and atherosclerotic lesions

The associations of soluble VCAM-1 and P-selectin with plasma lipids, body weight, and atherosclerotic lesion size were determined in the F₂ population. As shown in Table 2, both soluble VCAM-1 and P-selectin levels were significantly correlated with body weight ($R = 0.161$ and $P = 0.015$ for VCAM-1; $R = 0.233$ and $P = 0.00044$ for P-selectin). Soluble P-selectin levels were significantly correlated with total cholesterol ($R = 0.184$; $P = 0.0057$) and LDL/VLDL cholesterol levels ($R = 0.184$; $P = 0.0056$). Soluble VCAM-1 levels showed an apparent trend of associations with total cholesterol ($R = 0.122$; $P = 0.065$) and LDL/VLDL cholesterol levels ($R = 0.119$; $P = 0.073$). Neither adhesion molecule exhibited a correlation with HDL cholesterol levels ($R = 0.047$ and $P = 0.483$ for VCAM-1; $R = 0.025$ and $P = 0.708$ for P-selectin). Neither soluble P-selectin ($R = 0.10$ and $P = 0.14$) nor VCAM-1 ($R = 0.01$ and $P = 0.85$) showed a significant association with plasma triglyceride levels. Unexpectedly, circulating VCAM-1 levels showed a significant but inverse association with atherosclerotic lesion size ($R = -0.15$; $P = 0.03$) in the F₂ mice. There was no correlation between soluble P-selectin and lesion size ($R = 0.03$; $P = 0.68$).

Positional candidate gene

Thpo (thrombopoietin) and Adipoq (adiponectin, C1Q and collagen domain containing) are two positional candidate gene underlying the sSelp1 QTL. Their expression in endothelial cells from the two parental strains was evaluated by RT-PCR. As shown in Figure 6, the expression level of Thpo was significantly higher in B6 than in C3H mice. In contrast, the expression levels of Adipoq or Gapdh were comparable between the two strains.

Discussion

In the present study, we examined the genetic basis of variation in circulating soluble adhesion molecules using an intercross between B6.apoE^{-/-} and C3H.apoE^{-/-} mice and identified one

significant QTL and three suggestive QTLs for soluble VCAM-1 and one significant QTL and two suggestive QTLs for soluble P-selectin. We also observed significant or an apparent trend of correlations of soluble VCAM-1 and P-selectin with body weight, total cholesterol, and LDL/VLDL cholesterol levels and an inverse correlation of soluble VCAM-1 with atherosclerotic lesion size in the F₂ population.

We previously demonstrated significant variations in circulating VCAM-1 and P-selectin levels among mouse strains when deficient in apoE (Pei et al. 2006; Tian et al. 2005). To the best of our knowledge, genetic factors that influence circulating adhesion molecule levels in the mouse remain undefined. Our present study indicates that plasma soluble adhesion molecule levels are complex traits influenced by multiple genes. One significant QTL and three suggestive QTLs were identified to affect circulating VCAM-1 levels in the intercross derived from B6.apoE^{-/-} and C3H.apoE^{-/-} mice. The locus on chromosome 9 had a significant LOD score of 3.9 and we have designated this QTL as sVcam1 to represent the first locus identified for soluble VCAM-1 in the mouse. Candidate genes in the region include acetyl-Coenzyme A acyltransferase 1A (71 cM), cytochrome P450, family 8, subfamily b, polypeptide 1 (71 cM), and sterol carrier protein 2-pseudogene 2 (70.5 cM). All three genes are involved in cholesterol metabolism (Nicholls et al. 2006; Norlin and Wikvall 2007). An elevation in plasma cholesterol levels is accompanied by an increase in circulating adhesion molecules when apoE^{-/-} mice are fed a high fat/cholesterol diet (Tian et al. 2005). Hyperlipidemia results in deposition of apoB-containing lipoproteins such as LDL and VLDL in the subendothelium of the arterial wall. The accumulated LDL undergoes oxidative modification by arterial wall cells to become oxidized LDL. The majority of circulating VCAM-1 originates from the endothelium, where it can be induced by native LDL (Allen et al. 1998) and oxidized LDL (Liao et al. 1997). We also identified three suggestive loci on chromosomes 5, 13 and 15 influencing circulating VCAM-1 levels. No QTL for soluble VCAM-1 was found on chromosome 3, where the Vcam1 gene is located, suggesting that the structural gene had no influence on circulating VCAM-1 levels.

In this study, we detected one significant QTL, named sSelp1, on chromosome 16 and two suggestive QTLs on chromosomes 10 and 13 affecting soluble P-selectin levels in the cross. All three QTLs influenced soluble P-selectin in a heterosis manner. One likely candidate gene for sSelp1 is Thpo (13.8 cM), encoding thrombopoietin, a humoral growth factor that induces P-selectin expression and primes platelet activation in response to several agonists (Lupia et al. 2006; Schattner and Lazzari 2002). In the present study, we found that Thpo was differentially expressed in endothelial cells of the two strains. Adipoq (16 cM), encoding adiponectin, is another candidate gene underlying sSelp1. Adiponectin is involved in regulation of body weight and lipid metabolism. The present observation on the association of soluble P-selectin levels with body weight and LDL/VLDL cholesterol levels in the F₂ mice suggests a genetic link between adiponectin and P-selectin.

In this study, we found that both soluble VCAM-1 and P-selectin were positively correlated with body weight in the F₂ population. This finding is in agreement with the observations made in human populations (Shai et al. 2006; Ziccardi et al. 2002). The significant correlation between the two traits was probably partially mediated through the effect of lipoprotein-lipids. In the same cross, we previously observed significant correlations of body weight with plasma levels of LDL/VLDL cholesterol ($R = 0.39$, $P = 4.9 \times 10^{-9}$) and triglyceride ($R = 0.44$, $P = 3.56 \times 10^{-10}$) (Su et al. 2006a). As discussed above, native LDL can directly stimulate the endothelium to express VCAM-1 and probably other adhesion molecules. Thus, F₂ mice with larger body weight tended to have higher levels of LDL/VLDL and triglyceride, which would stimulate the endothelium to express more adhesion molecules. On the other hand, F₂ mice with smaller body weight tended to have lower LDL/VLDL and triglyceride levels and would be expected to express less adhesion molecules on endothelial cells. Interestingly, we did observe a significant correlation of LDL/VLDL with soluble P-selectin and an apparent trend

of correlation with soluble VCAM-1 in the cross. We also observed an apparent trend of associations of soluble P-selectin with plasma triglyceride but not with HDL levels in the F₂ mice. This is in line with the findings of De Pergola et al (De Pergola et al. 2008), showing that soluble P-selectin concentrations were positively correlated with plasma triglyceride but not HDL levels in 50 non-diabetic women, 17 with normal weight and 33 overweight or obese.

In the F₂ population on the Western diet, plasma levels of soluble VCAM-1 were negatively correlated with atherosclerotic lesion size. Plasma VCAM-1 levels were also inversely correlated with lesion size when these mice were fed the chow diet (before initiation of the Western diet) (data not shown). The reasons for the negative correlation between the two traits are unknown. However, it is known that plasma soluble VCAM-1 levels and atherosclerotic lesion size were each controlled by a QTL on chromosome 9 in the F₂ intercross (Su et al. 2006b). Thus, there was a possibility that the two traits were regulated by the same gene or two closely linked genes that exerted an opposite effect on the traits. An inverse correlation may simply reflect the fact that atherosclerosis development is complex. Previous studies on the relationship of soluble adhesion molecules with atherosclerosis in humans are not consistent. Some studies have shown that soluble VCAM-1 is significantly correlated with carotid intima-media thickness, an index of early atherosclerosis (Kohara et al. 2002; Peter et al. 1997; Rohde et al. 1998). However, in a further study evaluating the respective contribution of soluble VCAM-1 in peripheral arterial disease, the effect of VCAM-1 lost significance when adjusted for smoking (Blann et al. 1998).

No correlation was detected between plasma levels of soluble P-selectin and atherosclerotic lesion size in our F₂ mice. Only few data are available on soluble P-selectin levels and the extent of atherosclerosis. There is some evidence for increased levels of sP-selectin in patients with plaques (Chironi et al. 2006; Tan et al. 2005), but these results were derived from small populations.

In summary, this study has demonstrated a genetic control of circulating soluble VCAM-1 and P-selectin levels in hyperlipidemic apoE-deficient mice. We have also demonstrated associations of soluble VCAM-1 and P-selectin with body weight and LDL/VLDL cholesterol levels in the F₂ cross, suggesting a role for adhesion molecules in obesity development and lipid metabolism.

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Figure 1. Plasma soluble VCAM-1 (A) and P-selectin (B) levels in Western diet-fed female B6.apoE^{-/-} (B6), C3H.apoE^{-/-} (C3H), F₁, and F₂ mice. Each dot represents an individual value of one mouse. Mean $\pm$ SD of each group is shown on the figure. The horizontal lines denote means.

Figure 2. Distributions of plasma soluble VCAM-1 and P-selectin levels in female F₂ mice derived from B6.apoE^{-/-} and C3H.apoE^{-/-} mice. Mice were fed a Western diet for 12 weeks.

Figure 3.

Genomewide scans for main effect loci affecting plasma levels of soluble VCAM-1 (A) and P-selectin (B) in F_2 mice. Chromosomes 1 through X are represented numerically on the X-axis. The Y-axis represents the LOD score. The horizontal broken lines represents the suggestive ($P = 0.63$) and significant ($P = 0.05$) levels as determined by permutation tests using 1,000 permutations.

Figure 4.

Detailed LOD score plots of significant and suggestive QTLs for soluble VCAM-1 and P-selectin. The X-axis depicts the plot positions in cM for each chromosome and Y-axis depicts the LOD score. A: Soluble VCAM-1 QTLs on chromosomes 9, 5, 13, and 15; B: Soluble P-Selectin QTLs on chromosomes 16, 10, and 13.

Figure 5.

The allele effects at different QTLs in the F₂ offspring on soluble VCAM-1 (A-D) and P-Selectin levels (E-G). Chromosome number and the QTL position in cM are given for each QTL. Homozygosity for B6 alleles is represented by BB, homozygosity for C3H alleles is represented by CC, and heterozygote at a locus is represented by BC.

Figure 6. mRNA levels of Thpo, Adipoq, and Gapdh in endothelial cells from B6 and C3H mice analyzed by RT-PCR. Each lane represents an individual mouse.

Table 1

Significant and suggestive QTLs for soluble VCAM-1 and P-selectin identified in the B6.apoE^{-/-} and C3H.apoE^{-/-} intercross fed a western diet.

Trait	Nearest Marker	Peak (cM)	LOD ^a	CI (cM) ^b
sVcam-1	D9Mit18	71	3.9	56-71
sVcam-1	D5Mit155	52	2.2	22-82
sVcam-1	D13Mit63	29	2.1	15-39
sVcam-1	D15Mit92	29.4	2.1	15.4-47.9
sP-selectin	D10Mit42	45	2.5	35-65
sP-selectin	D13Mit250	41	2.5	33-63
sP-selectin	D16Mit165	8.5	3.4	5.4-17.4

^a Suggestive and significant LOD scores are 2.0/3.4 for soluble VCAM-1 and 2.0/3.3 for soluble P-selectin, as defined by 1000 permutation tests. Significant LOD scores are bolded.

^b CI, confidence interval, is calculated by the posterior probability test.

Table 2

Associations of soluble VCAM-1 or P-selectin with plasma lipids, body weight, and atherosclerotic lesion size in the B6.apoE^{-/-} and C3H.apoE^{-/-} intercross fed a western diet.

	VCAM-1		P-selectin	
	R value	P value	R value	P value
Body weight	0.161	0.015	0.233	0.00044
Total cholesterol	0.122	0.065	0.184	0.0057
LDL/VLDL cholesterol	0.119	0.073	0.184	0.0056
HDL cholesterol	0.047	0.483	0.025	0.708
Triglyceride	0.01	0.85	0.10	0.14
Lesion size	-0.15	0.03	0.03	0.68

R, correlation coefficient; P, statistical significance. Significant correlations are denoted in bold. Plasma triglyceride levels and lesion size were log-transformed, and all other trait values exhibited normal distributions and thus were not transformed before linear regression was performed.