Table 2. Haplotype frequencies of TNF-α and IL-10

Gene	Haplotype			Frequency
TNF-α	−1031	−863	−857	
	T	C	C	0.66
	C	**A**	C	0.17
	T	C	**T**	0.16
	C	C	C	0.01
IL-10	−1082	−819	−592	
	A	T	A	0.66
	A	**C**	**C**	0.29
	G	**C**	**C**	0.05

The boldface letters indicate the minor alleles.

Conventional risk factors and atherosclerotic severity

The results obtained using the conventional non-genetic risk factors of atherogenesis are summarized in Table 4. Overall, the conventional risk factors were confirmed to be independent risk factors for the atherosclerosis of systemic arteries, but our results also revealed that their effects differed somewhat from artery to artery. Aging is an independent high-risk factor for all arteries except the coronary, and smoking is an independent high-risk factor for all arteries except the coronary and the intracranial. Females are less susceptible to atherogenesis of the carotid and coronary arteries, while they are more susceptible to atherogenesis of the splenic artery. No significant association was observed between drinking status and atherosclerotic severity in any of the arteries.

Hypertension was found to be an independent risk factor for the atherogenesis of all arteries (OR: 1.72–2.60), while diabetes mellitus and hyperlipidemia affected atherosclerotic severity in seven (OR: 1.42–1.97) and six (OR: 1.45–2.22) specific arteries, respectively.

DISCUSSION

The view that atherosclerosis is a chronic inflammatory disease initiated by the adhesion of immunocompetent cells to the endothelium is verified by many experiments using cholesterol-fed animals, genetic engineering and histological methods. It is now well accepted that pro-inflammatory cytokines promote atherogenesis, and that anti-inflammatory cytokines or agents have the potential to prevent the complications of atherosclerosis (29–31).

In the present study, we investigated the genetic background of atherogenesis, focusing on its inflammatory aspects. We selected nine SNPs of five cytokine genes, all of which have been reported to be associated with altered gene function.

The −1031C allele of TNF-α was found to have significant protective effects on atherogenesis of the intracranial, carotid and femoral arteries. An *in vitro* study has shown that the −863A allele of TNF-α, which is in strong linkage disequilibrium with the variant at position −1031 of TNF-α, reduced the interaction with nuclear protein complexes in electromobility shift assays, and was associated with a 31% decrease in transcriptional activity in chloramphenicol acetyltransferase assays (32). The TNF-α gene is located in the human leukocyte antigen (HLA) class III region in the 6p21.3 band of

Table 3. The results of multiple logistic regression analysis for the third quartile point of the degree of atherosclerosis and each SNP

Gene	TNF-α		IL-1β		IL-10		IL-4	TGF-β1
Position	−863	−857	−511	−1082	−819	−592	−589	+29
Minor allele (frequency)	A (0.17)	T (0.16)	T (0.46)	G (0.05)	C (0.34)	C (0.34)	C (0.32)	T (0.48)
Coronary	0.99 (0.75–1.30)	1.55 (0.66–3.65)	1.12 (0.49–2.54)	1.14 (0.86–1.52)	1.12 (0.72–1.74)	1.12 (0.87–1.44)	1.13 (0.88–1.47)	0.96 (0.74–1.23)	1.21 (0.89–1.64)
Intracranial	**0.70[a] (0.52–0.94)**	1.86 (0.71–4.88)	0.69 (0.32–1.49)	1.06 (0.79–1.42)	0.87 (0.54–1.40)	1.06 (0.81–1.39)	1.05 (0.80–1.38)	1.08 (0.83–1.42)	**1.48[a] (1.05–2.06)**
Carotid	**0.72[a] (0.56–0.92)**	1.75 (0.87–3.54)	1.29 (0.64–2.63)	1.28 (0.99–1.65)	1.30 (0.86–1.96)	1.02 (0.81–1.29)	1.00 (0.80–1.27)	1.03 (0.82–1.30)	0.97 (0.74–1.26)
Subclavian	0.76 (0.58–1.01)	2.13 (0.85–5.35)	1.10 (0.49–2.45)	**1.35[a] (1.01–1.80)**	1.44 (0.94–2.21)	0.93 (0.72–1.20)	0.93 (0.72–1.20)	1.02 (0.79–1.32)	0.82 (0.61–1.10)
Splenic	0.99 (0.75–1.31)	0.76 (0.36–1.59)	0.98 (0.43–2.23)	1.17 (0.88–1.57)	1.20 (0.77–1.85)	1.11 (0.85–1.44)	1.11 (0.85–1.44)	0.94 (0.73–1.22)	0.97 (0.72–1.32)
Superior mesenteric	0.76 (0.57–1.01)	1.14 (0.50–2.58)	1.13 (0.48–2.66)	1.20 (0.89–1.61)	0.79 (0.49–1.28)	0.95 (0.73–1.24)	0.97 (0.75–1.27)	0.87 (0.67–1.13)	0.84 (0.62–1.15)
Aorta	1.09 (0.85–1.40)	0.68 (0.34–1.38)	0.82 (0.40–1.69)	1.18 (0.91–1.53)	1.17 (0.77–1.77)	1.03 (0.81–1.30)	1.01 (0.80–1.28)	1.01 (0.80–1.28)	0.88 (0.67–1.15)
Common iliac	1.03 (0.79–1.34)	0.82 (0.39–1.72)	0.85 (0.40–1.81)	1.20 (0.92–1.57)	1.15 (0.74–1.79)	1.07 (0.84–1.36)	1.04 (0.81–1.32)	1.11 (0.87–1.42)	1.11 (0.83–1.47)
External iliac	1.03 (0.78–1.35)	1.38 (0.61–3.10)	1.14 (0.50–2.56)	1.22 (0.92–1.63)	1.53 (0.996–2.34)	1.29 (0.999–1.67)	1.29 (1.00–1.67)	0.94 (0.73–1.22)	0.91 (0.68–1.22)
Femoral	**0.73[a] (0.54–0.97)**	1.55 (0.64–3.74)	0.86 (0.38–1.93)	1.25 (0.93–1.68)	1.41 (0.91–2.19)	1.27 (0.97–1.66)	1.27 (0.97–1.66)	0.83 (0.64–1.08)	0.90 (0.66–1.23)

All of the results shown are exponented values of the parameter estimates for the logistic regression models: the upper values are the ORs, and the lower values in parentheses are the 95% confidence intervals. The statistically significant values ($P < 0.05$) are shown in boldface. The results are adjusted for age at death, gender, smoking status, drinking status and histories of diabetes mellitus, hypertension and hyperlipidemia.

[a]All of the significant associations will not remain if multiple testing of different loci is taken into account.

Table 4. Results of multiple logistic regression analysis for the third quartile point of the degree of atherosclerosis and the conventional risk factors

	Sex	Age at death (years)			Smoking	Drinking	Hypertension	Diabetes Mellitus	Hyperlipidemia
		60 versus 90	70 versus 90	80 versus 90					
Coronary	**0.68* (0.50–0.93)**	0.64 (0.38–1.08)	0.64 (0.43–0.94)	0.76 (0.53–1.11)	1.31 (0.97–1.77)	0.82 (0.61–1.11)	**2.26† (1.74–2.94)**	**1.97† (1.42–2.72)**	**2.22† (1.54–3.19)**
Intracranial	1.18 (0.85–1.63)	**0.19† (0.10–0.37)**	**0.36† (0.24–0.53)**	**0.69† (0.48–0.98)**	0.97 (0.71–1.33)	1.03 (0.74–1.43)	**1.83† (1.38–2.41)**	1.05 (0.72–1.52)	1.21 (0.81–1.81)
Carotid	**0.74* (0.56–0.99)**	**0.14† (0.08–0.23)**	**0.31† (0.22–0.45)**	**0.49† (0.34–0.70)**	**2.15† (1.63–2.83)**	0.89 (0.67–1.17)	**2.55† (1.97–3.30)**	**1.60* (1.15–2.24)**	**2.13† (1.46–3.13)**
Subclavian	0.81 (0.59–1.11)	**0.17† (0.09–0.30)**	**0.31† (0.21–0.46)**	**0.57† (0.40–0.81)**	**2.44† (1.79–3.33)**	1.02 (0.76–1.38)	**2.60† (1.99–3.39)**	0.98 (0.69–1.40)	**1.92† (1.31–2.81)**
Splenic	**1.63* (1.18–2.25)**	**0.11† (0.05–0.23)**	**0.36† (0.25–0.52)**	**0.54† (0.38–0.76)**	**1.72† (1.26–2.35)**	1.01 (0.73–1.39)	**1.72† (1.31–2.26)**	**1.42* (1.00–2.02)**	1.14 (0.77–1.69)
Superior mesenteric	1.25 (0.90–1.73)	**0.15† (0.08–0.30)**	**0.37† (0.25–0.54)**	**0.60† (0.42–0.86)**	**1.89† (1.37–2.60)**	0.88 (0.64–1.20)	**1.97† (1.50–2.59)**	**1.72* (1.22–2.43)**	1.10 (0.74–1.65)
Aorta	0.89 (0.66–1.18)	**0.07† (0.04–0.11)**	**0.17† (0.12–0.25)**	**0.37† (0.26–0.54)**	**2.42† (1.82–3.22)**	0.77 (0.58–1.02)	**2.18† (1.68–2.82)**	**1.51* (1.08–2.11)**	**1.92† (1.32–2.81)**
Common iliac	1.09 (0.80–1.47)	**0.04† (0.02–0.07)**	**0.14† (0.09–0.21)**	**0.36† (0.23–0.55)**	**3.55† (2.62–4.81)**	0.77 (0.57–1.04)	**1.87† (1.42–2.47)**	1.34 (0.94–1.91)	**1.73* (1.15–2.59)**
External iliac	0.79 (0.58–1.08)	**0.13† (0.07–0.23)**	**0.23† (0.16–0.34)**	**0.37† (0.26–0.54)**	**3.52† (2.56–4.82)**	1.09 (0.81–1.46)	**1.91† (1.46–2.49)**	**1.47* (1.04–2.07)**	**1.63* (1.11–2.39)**
Femoral	1.13 (0.81–1.56)	**0.07† (0.04–0.15)**	**0.18† (0.12–0.26)**	**0.31 (0.22–0.45)**	**1.99† (1.44–2.75)**	1.14 (0.83–1.56)	**2.22† (1.69–2.92)**	**1.83† (1.29–2.60)**	1.45 (0.98–2.16)

The results of the multiple logistic regression analysis are presented as OR estimates with the corresponding 95% confidence intervals. The upper values are the ORs, and the lower values in parentheses are the 95% confidence limits. Statistically significant values ($P < 0.05$) are shown in boldface. *$P < 0.05$; †$P < 0.001$.

the short arm of chromosome 6, and the −1031C/−863A allele has been reported to be in significant linkage disequilibrium with HLA-B61, -B39 and -DRB1*0901 (33). Although it is plausible that TNF-α gene variants are associated with inflammatory disease, we cannot exclude the possibility that these associations might be due to the polymorphisms in the adjacent HLA genes. Since our results show that a functional genetic variation that lowers the transcriptional activity of TNF-α has a significantly protective effect, we prefer to think that this might be the causative polymorphism.

The −511T allele of IL-1β is associated with increased IL-1β protein production, according to the results of an *ex vivo* blood stimulation assay (34). This supports our finding that the −511T allele is a risk factor for atherogenesis in the subclavian artery.

It has been reported that the homozygote for C in the +29 allele of TGF-β1 is associated with higher mRNA expression, quantified by means of TGF-β1 gene-specific competitor constructs in competitive PCR (35), and also by means of enzyme-linked immunosorbent assay (ELISA) with higher serum concentrations of TGF-β1 (18). Although TGF-β1 has also been reported to have pro-atherogenic properties through its role in vascular remodeling (36,37), our results reveal that the +29T allele is a risk factor for atherogenesis of the intracranial arteries. This might be due to the fact that the anti-inflammatory activity of TGF-β1 is more preponderant than the pro-atherogenic property of TGF-β1 in the formation of atherosclerosis in intracranial arteries.

Therefore, overall, we detected some SNPs that are related to atherogenesis. In general, pro-inflammatory alleles confer risk and anti-inflammatory alleles are protective, although their influence is selective and limited to specific arteries. The reason why inflammation-related SNPs confer site-specific atherogenic risks is difficult to explain in a straightforward manner. However, our pathological observation revealed that individual differences in atherosclerotic severity at each artery have a wide range of variety. For example, one subject suffered from atherosclerosis only at the aortic and femoral arteries, while another's was confined to the coronary arteries. The anatomy of the vessels, blood flow and shear stress regulate the expression of specific genes in the endothelium, especially those encoding cell-adhesion and inflammation-related molecules (38,39), and they are thought to contribute to such differences. Along the same lines, a previous report from the Honolulu heart study has shown that risk factors for the coronary arteries and aorta have different components (40). Recently, Vanderlaan *et al.* (41) have proposed that regional differences in the hemodynamic profile prime the endothelial phenotype to respond distinctly to systemic risk factors, such as hypercholesterolemia, genetics, immune status, gender and oxidative stress. We speculate that cytokine polymorphisms interact with these artery-specific atherogenic factors and enhance atherosclerosis. If this hypothesis does not hold, then an unknown mechanism may underlie the involvement of cytokines in the inflammation of the arteries in a site-specific manner. It seems worthwhile to investigate how local differences influence site-specific atherogenesis. In particular, our results raise the possibility that the development of atherosclerosis in the coronary arteries is more affected by aging, smoking at the early stage of the disease, and being female

than the development of atherosclerosis in other arteries. In fact, the coronary arteries have been shown to be the first sites of atherosclerotic involvement among the systemic arteries (42). Therefore, further investigations are needed to evaluate the factors that affect the progression of coronary atherosclerosis in younger populations.

We also evaluated the association between the conventional risk factors of atherosclerosis and atherosclerotic severity in each artery. Advanced age and smoking were associated with atherosclerotic severity in all arteries, except the coronary and the coronary and intracranial, respectively. Hypertension was associated with atherosclerotic severity in all arteries, while diabetes mellitus and hyperlipidemia affected it in most, if not all, of the arteries, which is consistent with the fact that these are *bona fide* risk factors. Generally, the conventional risk factors showed higher ORs than the genetic variants, and their influence tended to be systemic (Table 4).

In the present study, we demonstrated that functional SNPs in TNF-α (−1031T/C), IL-1β (−511C/T) and TGF-β1 (C29T) play a role in atherogenesis, although their influence was less than that of systemic diseases such as hypertension and diabetes, as well as smoking and aging, and limited to specific arteries. Clearly, the subjects of our study are elderly persons who escaped from fatal cardiovascular diseases, and environmental factors would be more influential. Because the observed ORs of the genetic variants were close to 1, it is possible that they were mere chance observations. Nevertheless, even after excluding the risk-allele-bearing subjects, we could detect associations between genetic variants and atherosclerosis. We believe that our results point out the importance of inflammation-related genetic diversity in atherosclerosis. However, factors other than those found in this study must also be involved. Since progress in understanding the genetic factors of atherogenesis will enable us to develop preventive and therapeutic strategies for atherosclerosis, we intend to expand our investigation of the relationship between genes and atherosclerosis, especially with respect to the inflammatory aspects, by increasing the number of subjects and polymorphisms in future studies.

Limitation of the study

The subjects of this study were autopsy cases of patients in a community-based geriatric hospital. Therefore, our approach had two limitations. The first is the selection bias from chance of admission, cause of death and autopsy practice. The rate of autopsy in this hospital was ~40%. Those who died suddenly outside the hospital and were medicolegal cases were not autopsied for this study. According to the 2001 population survey report of Japan (http://www.dbtk.mhlw.go.jp/toukei/data/010/2001/toukeihyou/0003845/t0066001/c080_001.html), more than 81% of elderly persons (70–89 years) die in a hospital, whereas 12% die at home. Although the rate of in-hospital death for the elderly is high, this might be a minor confounding factor for the data. We therefore compared the direct causes of death between our subjects and the census data in 'Abridged Life Tables For Japan 2003' by the Ministry of Health, Labour and Welfare of Japan (http://www.mhlw.go.jp/english/database/db-hw/lifetb03/index.html). Although almost all death rates for major diseases per 100 000 between 80 and 84 years of age in Tokyo, Japan were consistent with our autopsy data, those for malignancy and cerebrovascular disease were ~15% lower and 10% higher, respectively, than our autopsy data. Hasuo *et al.* compared the death certificates issued without knowledge of the autopsy findings with pathological reports of autopsy cases originating from the Hisayama study (43). They indicated that cerebral stroke and cardiac disease tended to be overdiagnosed, whereas malignant neoplasms were underdiagnosed. Thus, the subjects in our study did not differ greatly from the standard elderly residents of Tokyo, Japan. Second, since post-mortem blood samples were not collected, we cannot check the inflammatory markers at death, but had to rely on patients' clinical charts retrospectively to obtain available clinical information.

Table 5. The characteristics of the subjects ($N = 1503$)

Age (at death)	mean ± SD	80.3 ± 8.9
Sex	Male/female	696/807 (0.86:1)
Smoking status[a]	Yes/No	717/677 (1.06:1)
Drinking status[a]	Yes/No	499/887 (0.56:1)
Major clinical diagnoses		
	Hypertension	440 (29.3%)
	Cerebrovascular disease[b]	438 (29.1%)
	Ischemic heart disease[c]	248 (16.5%)
	Diabetes mellitus	221 (14.7%)
	Hyperlipidemia	165 (11.0%)
	Arteriosclerosis obliterans	59 (3.9%)
Direct causes of death		
	Malignancy	498 (33.1%)
	Coronary heart disease	181 (12.0%)
	Pneumonia	201 (13.4%)
	Cerebrovascular disease	83 (5.5%)
	Miscellaneous	540 (35.9%)

[a]The information on the smoking and drinking statuses was taken from the patients' medical charts.
[b]The cerebrovascular diseases encountered included transient ischemic attack, reversible ischemic neurological deficit, subarachnoid hemorrhage, cerebral hemorrhage and cerebral infarction.
[c]The ischemic heart diseases encountered included angina pectoris and myocardial infarction.

SUBJECTS AND METHODS

Subjects

The subjects were 1503 consecutive autopsy cases of elderly patients who had suffered in-hospital death. The autopsies were performed at the Tokyo Metropolitan Geriatric Hospital in Tokyo, Japan from 1995 to 2004 (men: 696; women: 807; mean age at death: 80.3 ± 8.9 years) (Table 5). The subjects were registered in the Internet-based database of Japanese SNPs for geriatric research (the JG-SNP) (http://www.tmgh.metro.tokyo.jp/jg-snp/english/E_top.html) (44). The major clinical diagnoses and direct cause of death in this population are summarized in Table 5 (see the JG-SNP for detailed information). The direct cause of death was determined based on the descriptions given in the death certificate issued after the autopsy. The details of the underlying disease, history of smoking and drinking, serum lipid data and presence of atherosclerotic complications were obtained from the clinical records of the patients.

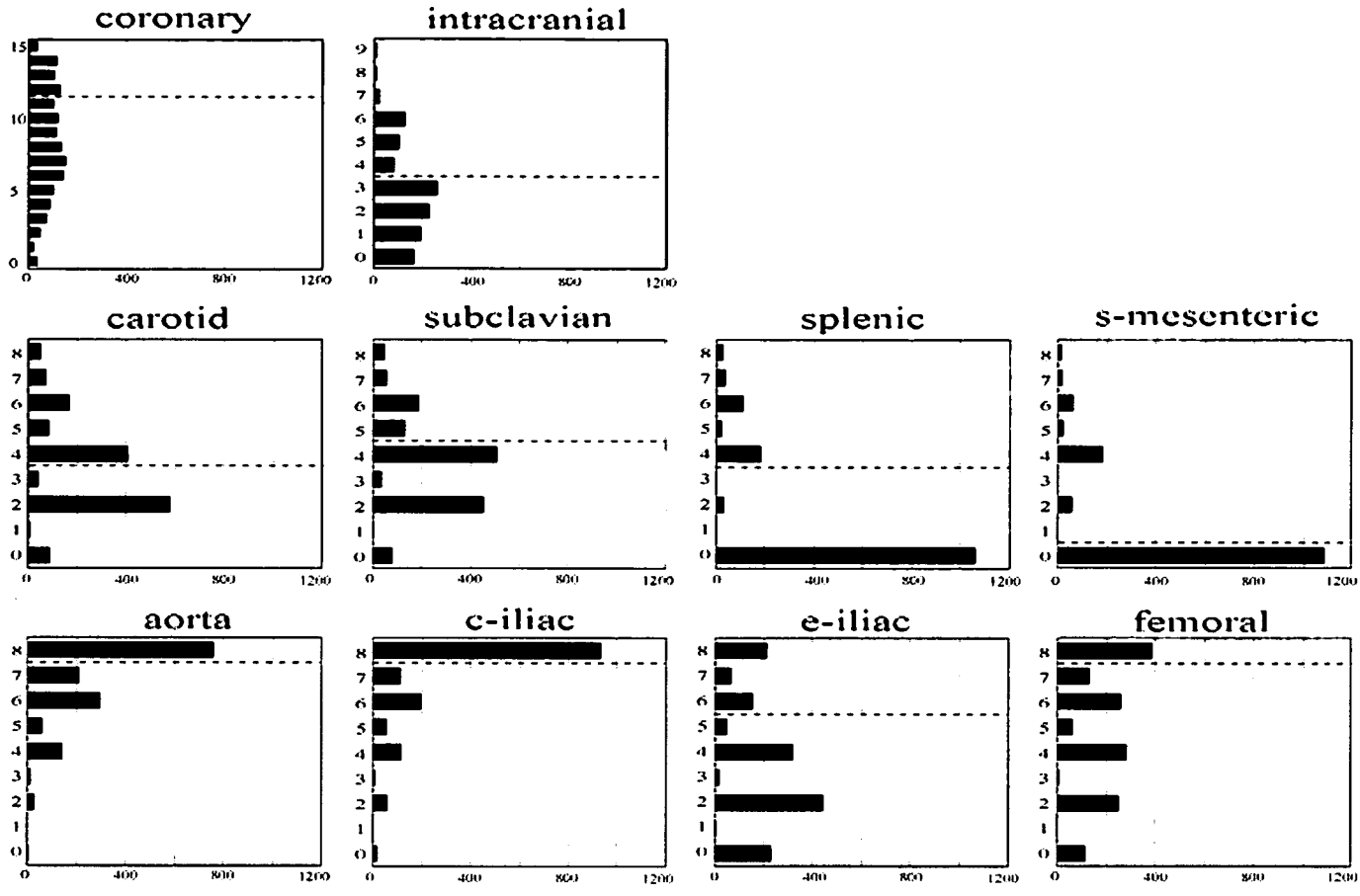

Figure 1. Distributions of atherosclerotic severity in individual arteries. These graphs show the distributions of atherosclerotic severity in the individual arteries. The *Y*-axis is the atherosclerotic severity and the *X*-axis is the number of subjects. The dashed line indicates the 75% point of each distribution. These graphs show that the atherosclerotic severity differs greatly from artery to artery.

Written informed consent was obtained from the bereaved families of the patients at the time of autopsy. This study was approved by the ethical committees of the Tokyo Metropolitan Geriatric Hospital and the Tokyo Medical and Dental University.

Pathological assessment of atherosclerosis

Macroscopic evaluation of the degree of atherosclerosis was performed for the following large- and medium-sized arteries: the carotid, subclavian, splenic, superior mesenteric, common iliac, external iliac, femoral, coronary and intracranial, as well as the aorta.

We adopted the method of pathological assessment of atherosclerosis reported previously by us (45–47). For eight arteries, i.e. the carotid, subclavian, splenic, superior mesenteric, common iliac, external iliac, femoral and the aorta, the atherosclerotic severity is scored according to the ratio of the occupying atheroma to the entire intimal area, while for the coronary and intracranial arteries, it is the sum of stenotic scores of their respective branches. At a medium-size artery, such as the coronary or intracranial, the stenotic degree is clinically valuable, and basically parallels the atherosclerotic severity as the ratio of the occupying atheroma. On the other hand, at a large-size artery such as the aorta, the stenotic degree is not useful, and is evaluated conventionally based on the ratio of the occupying lesion. Although we evaluated atherosclerosis by means of two pathological methods, together they both indicate the degree of atherosclerosis.

Selected SNPs and genotyping using melting curve analysis

We selected nine SNPs in five genes that are considered to be relevant to atherosclerosis, especially with respect to the inflammatory aspect, i.e. −1031T/C, −863C/A and −857C/T in the promoter region of the TNF-α gene, −511C/T in the promoter of the IL-1β gene, −1082A/G, −819T/C and −592A/C in the promoter of the IL-10 gene, −589T/C in the promoter of the IL-4 gene, and C29T (with a proline to leucine amino-acid change in codon 10) in the TGF-β1 gene.

DNA samples were extracted from the renal cortex by the phenol/chloroform method, and 10-ng DNA aliquots were used for the genotyping reaction. The PCR primer pairs used in the study are summarized in Table 1. The PCR reactions and melting curve analyses carried out for the purpose of detecting polymorphisms in TNF-α (−1031T/C, −863C/A and −857C/T), IL-1β (−511C/T), IL-10 (−1082A/G, −819T/C and −592A/C), IL-4 (−589T/C) and TGF-β1

(C29T) were performed using the LightTyper (Roche Diagnostics) according to the protocols previously reported by us (48). The rates of genotyping error were 0.3–3.5%. The pathological assessment and genotyping were done at different facilities in double-blind fashion.

Statistical analysis

The Hardy–Weinberg equilibrium of the alleles at individual loci was assessed using Fisher's exact test. Lewontin's D prime (D') and correlation coefficient (r^2) were calculated as two measures of linkage disequilibrium (LD) between SNPs.

As shown in Figure 1, the distributions of the atherosclerotic severity differed greatly from artery to artery: some were continuous, while others were discrete. For the sake of unity of the analysis methods, we considered all arteriosclerosis degrees as discrete variables by dividing them into upper and lower than each 75% point (shown by dashed lines at each artery). For the arteries whose distributions seemed to be continuous, we also analyzed them as continuous variables by means of multiple regression analysis, but the results were the same as those of the analysis using discrete variables (data not shown). To identify the SNPs that affect atherogenesis, we determined their relationships according to the genotypes in a dominant model for each minor allele. All of the variables of atherosclerotic severity, minor allele and haplotype distributions were categorized on a scale of 0 or 1, depending on whether they were lower or higher than each 75% point, and whether or not each minor allele or haplotype was absent or present, respectively. The conventional risk factors of age (at death), gender, drinking status and smoking status, as well as histories of hypertension, diabetes mellitus and hyperlipidemia, were also categorized as follows: gender (male versus female), years of age at death (60 versus 90, 70 versus 90, 80 versus 90), and histories of smoking, drinking, hypertension, diabetes mellitus and hyperlipidemia (absent versus present) as 0 versus 1, respectively.

Multiple logistic regression analyses were performed to assess the associations of individual minor SNPs and haplotypes with the risk of atherosclerosis, with adjustment for the conventional risk factors. Using the EM algorithm, we estimated the frequencies in this population of the four and three most common (>1% frequency) haplotypes of TNF-α and IL-10, respectively, and imputed the subject-specific expected haplotypes.

The statistical analyses were performed using the SAS 9.1.3 statistical software. The subject-specific haplotypes were estimated using the existing procedure in SAS/GENETICS version 9.1.3. In general, association studies involving many genes have the problem of multiple statistical comparisons. This study, however, was a hypothesis-generative search, and the groups with severe atherosclerosis differed greatly from artery to artery (Fig. 1), so we considered two-tailed P-values <0.05 to be statistically significant with no correction.

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Conflict of Interest statement. The authors hereby declare that there is no financial conflict of interest.

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