($P \leq 0.05$). In three of these loci, the lowest *P*-values were observed for rs3755351 ($P = 1.7 \times 10^{-5}$) in *ADD2*, rs3794260 ($P = 0.0001$) in *KIAA0789* and rs1805762 ($P = 0.0003$) in *M6PR* when case–control comparison was made in the combined data. An SNP (rs3755351) within *ADD2* had the lowest *P*-value and its experiment-wide significance level is 0.13. Thus, these results have nominated several susceptibility genes for hypertension, and independent replication will clarify their etiological relevance.

INTRODUCTION

Essential hypertension (MIM 145500) is a multifactorial trait, in which interactions among genetic, environmental and demographic factors are involved. Substantial contribution of genetic factors to the overall disease etiology has been documented by a number of epidemiological studies. For example, family studies controlling for a common environment indicate that blood pressure heritability is in the range of 15–35% (1–3). Accordingly, considerable efforts have been made in the study of molecular genetics of hypertension, but the inherently complex nature has hampered progress in the elucidation of the genes involved (4). Over the last decade, multiple genome-wide linkage analyses have been conducted by using microsatellite markers to localize genes influencing hypertension status and/or blood pressure levels in a number of populations derived from various ethnic groups. Although no single study has so far yielded definitive evidence for 'principal' hypertension susceptibility gene(s), some of these studies provide consistency of linkage results in a few chromosomal regions (5–7). It is therefore assumed that multiple genes contribute to the etiology of hypertension independently or synergistically, with each gene exerting small effects under a certain environmental condition.

In parallel with family-based linkage analyses across the entire genome, population-based association studies have been performed, particularly focusing on individual candidate genes to search for genetic influences on hypertension. Association studies for mapping disease-related genes have recently gained popularity over traditional family-based linkage analyses mainly because of their far greater statistical power to detect the presence of genes with relatively 'minor' effects (8,9). Some researchers criticize the liability to false-positive or non-replicable claims. Nevertheless, population-based association studies have become an alternative and complementary approach to family-based linkage analyses in practice.

Given the limitation of statistical power that can be achieved by family-based linkage analyses with sample size practically collectable, population-based association studies are now underway in a genome-wide scale for a number of multifactorial diseases (10). Here, we performed a high-density association study of hypertension with a three-tiered genotyping approach in the Japanese population (Fig. 1).

RESULTS

Multi-tiered case–control study

We performed a large-scale case–control association study of hypertension using SNP markers selected from the Japanese SNP (JSNP) database (11,12). These SNP markers were distributed throughout the genome (Table 1). Only male hypertensive individuals were tested in tier 1, and a total of 80 795 SNPs distributed on 22 autosomes were used for the association study. Details of the high-throughput genotyping were same as previously described (13,14), and technical evaluation of our genotyping assay (e.g. overall success rate and accuracy of the genotyping assay) is shown in the supplementary material (Supplementary Explanation). JSNP had been developed as a database for the SNP discovery project with particular focus on common gene variations in the Japanese population. Although SNP marker resources used in the current study showed a certain degree of diversity in terms of the number of typed SNPs per gene locus, this partially reflected the variable size of re-sequenced fragments depending on the individual gene structure (12).

The gene-centered genome-wide exploratory test in tier 1 identified 2676 SNPs with odds ratio (OR) ≥ 1.4 and $P \leq 0.015$ in at least one test comparing allele frequency and/or genotype distribution (dominant or recessive models) between 188 hypertensive patients and 752 population control subjects in either of two panels (see Materials and Methods). In this exploratory test, the SNPs showing inverted tendency of OR between two pairs of case–control comparisons and significant deviations from Hardy–Weinberg equilibrium (HWE) in any panel ($P \leq 0.01$) were excluded. Subsequently, we performed a screening of these 2676 SNPs with 752 hypertensive patients and 752 normotensive controls in tier 2, which constituted the first 'case versus unaffected control' study panel, i.e. comparison between 940 cases and 752 controls, together with the 188 cases in tier 1. On the basis of relatively stringent criteria, we identified 75 SNPs that showed *P*-values of ≤ 0.01 for genotype distribution and *P*-values of ≤ 0.05 for allele frequency in the χ^2-test statistic. To further examine the association signals, we performed a replication study of these 75 SNPs with another panel of 619 hypertensive subjects and 1406 normotensive controls in tier 3. Cases and unaffected controls collected in tiers 2 and 3 were enrolled according to the identical criteria and their baseline characteristics are shown in Table 2. There were some trait differences in cases between tiers 2 and 3, such as blood pressure measurements and percentages of the subjects taking anti-hypertensive medication. This could be largely attributed to differences in sample enrollment settings between tiers 2 and 3; that is, cases in tier 3 were enrolled from either the annual medical checkup of a medical institution or the clinic practices of general practitioners, whereas a major part of cases in tier 2 were from the clinic practices of university hospitals. Among the 75 SNPs showing *P*-values between 0.05 and 4.4×10^{-5} in the first 'case versus unaffected control' study, only nine SNPs showed borderline association (at the level of $P \leq 0.05$) in

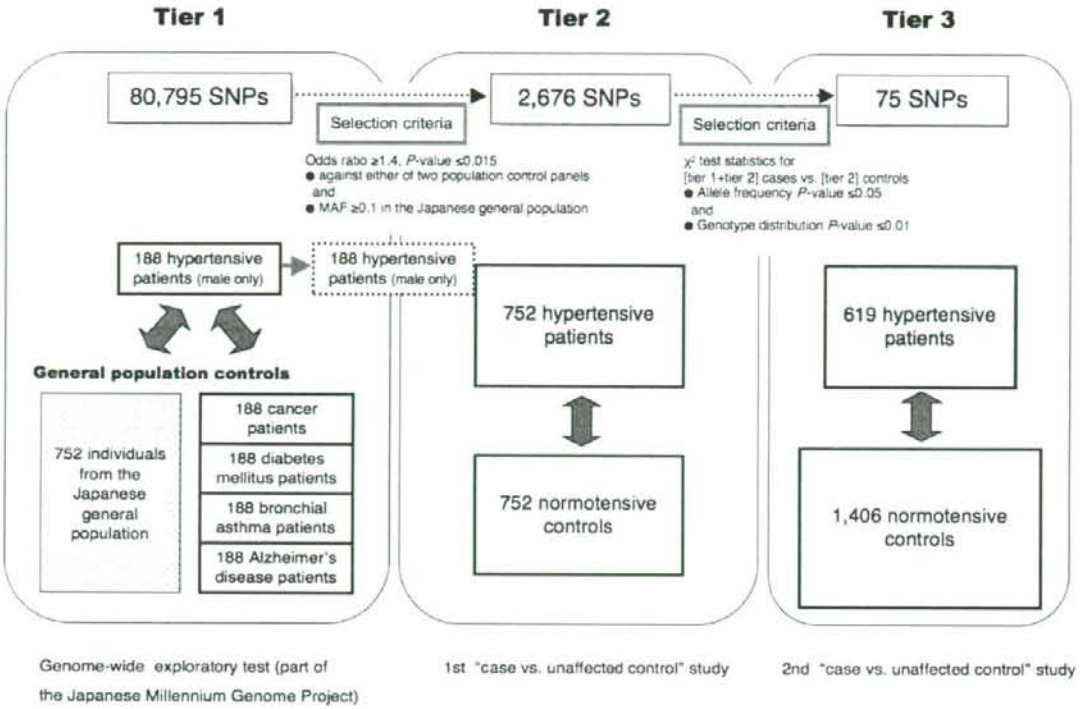

Figure 1. Schematic presentation of a three-tiered screening strategy in the present study. Gene-centered genome-wide exploratory test was performed in tier 1, followed by case–control study of disease-associated SNPs in tier 2 and tier 3 samples. A panel of 188 male hypertensive patients were compared with each of two population control panels in tier 1. Subsequently, 'case versus unaffected control' study was repeated twice to identify the best candidate SNPs. In transitions from tier 1 to tier 2, and from tier 2 to tier 3, the number of SNPs was reduced according to the selection criteria that we arbitrarily defined. See details in the Materials and Methods section.

the second 'case versus unaffected control' study (Fig. 2). Of these, we found six SNPs that showed *P*-values of ≤ 0.05 for both genotype distribution and allele frequency in the χ^2-test statistic (Table 3). rs3755351 and rs3771426 were located within the assumed intron 1 of *ADD2*, rs3787240 and rs3787241 were located in the same intron of *EYA2*, and the remaining two SNPs—rs3794260 and rs1805762—each located in *KIAA0789* and *M6PR*. To adjust for three covariates—age, gender and body mass index (BMI), we also performed logistic regression analysis for the significant SNPs (Supplementary Material, Table S1). With consideration of genetic model consistency, an SNP (rs3755351) showed the strongest association in the identical model (an additive model by logistic regression analysis) among three tiers. Further details of the association results are described in the Discussion.

SNP discovery and further test of association in three selected genes

Because a group of SNPs from three genes, *ADD2*, *KIAA0789* and *M6PR*, were particularly noted for their significant association with hypertension (Table 3), we searched for potentially functional SNPs by re-sequencing the 5′- and 3′-untranslated regions, all exons and exon–intron borders of the individual loci, on the basis of the gene structure deposited in the human genome database (http://www.ncbi.nlm.nih.gov/). We detected a total of 74 SNPs—25 SNPs in *ADD2*, 40 SNPs in *KIAA0789* and 9 SNPs in *M6PR*—and thereby selected 25 tag SNPs for genotyping 2025 subjects in tier 3 (see Supplementary Material, Table S2A, B and C). Apart from four SNPs which had been already included in the JSNP screening marker set, we found four additional SNPs, two in *ADD2* (rs2024453 and rs10084293) and one each in *KIAA0789* (rs9739493) and *M6PR* (rs1805725), to be significantly associated with hypertension (Table 3). Thus, in each gene, we identified at least two SNPs showing modest evidence of association with hypertension ($P \leq 0.05$ level in tier 3) but these SNPs did not necessarily belong to the same linkage disequilibrium (LD) block (Fig. 3 and LD group in Supplementary Material, Table S2A, B and C). The analysis of haplotypes inferable from tag SNPs did not show more significant disease association than the analysis of individual SNPs in any of three genes tested (data not shown).

Consideration of study power and multiple testing

We first estimated a type I error probability for the three-tiered screening to be 6.8×10^{-5}: 0.036 for tier 1, 0.0009 for tiers 1

Table 1. Summary of SNPs genotyped in tier-1 screening and genome coverage estimated by HapMap data

Chromosome	From JSNP screening markers		JSNP overlapped with HapMap (overlap)	From HapMap data (Release 21, JPT)			
	Total SNPs in JSNP	Proportion of SNPs unique to JSNP		SNPs in close LD ($r^2 \geq 0.8$) with overlap	(HapMap total − NA) SNPs	Coverage estimate: SNPs in LD ($r^2 \geq 0.8$) (HapMap total − NA) SNPs	Total SNPs in HapMap
1	8378	0.370	5281	26 236	113 362	0.231	139 002
2	7336	0.293	5189	28 763	123 447	0.233	160 546
3	5128	0.358	3290	17 494	91 985	0.190	125 160
4	3172	0.366	2010	11 998	74 080	0.162	114 809
5	4973	0.311	3427	18 432	91 206	0.202	122 243
6	6220	0.272	4527	26 182	110 532	0.237	134 177
7	5813	0.358	3731	18 241	81 727	0.223	99 808
8	2388	0.246	1800	12 644	80 400	0.157	111 953
9	2818	0.218	2203	12 358	70 091	0.176	91 908
10	3159	0.322	2141	13 552	81 103	0.167	100 771
11	3636	0.248	2735	14 953	75 147	0.199	95 905
12	3816	0.223	2964	15 188	73 983	0.205	89 436
13	1291	0.290	917	6717	48 622	0.138	75 956
14	2913	0.219	2275	11 583	50 769	0.228	62 203
15	2311	0.194	1863	9903	46 599	0.213	54 210
16	2677	0.268	1959	8227	43 415	0.189	51 865
17	3246	0.258	2408	10 050	38 550	0.261	41 725
18	1243	0.207	986	6370	41 494	0.154	56 203
19	3392	0.308	2346	7626	25 524	0.299	26 949
20	2588	0.402	1548	8784	41 725	0.211	45 582
21	1761	0.275	1276	5777	23 465	0.246	26 892
22	2536	0.280	1825	7569	24 402	0.310	25 077
Total	80 795	0.298	56 701	298 647	1 451 628	0.206	1 852 380

The numbers of SNPs genotyped in tier-1 screening are demonstrated for each chromosome. Genome coverage was assessed with the HapMap data from JPT ($n = 45$); that is, the proportion of HapMap SNPs showing high r^2 (≥ 0.8) to one of the SNPs genotyped in this study (which are all derived from JSNP) is calculated. Because substantial part of the SNPs have turned out to be unique to JSNP, those overlapping with the HapMap SNPs, in the 'overlap' column, are used to estimate genome coverage. Here, NA represents a category of SNPs which have been mapped to the genome (NCBI B35) but do not have LD information against the HapMap SNPs. In this context, it is appropriate to reduce this NA SNPs from total SNPs deposited in the HapMap data when estimating genome coverage and we therefore use the number of SNPs (HapMap total − NA) as a denominator.

and 2 combined and 0.076 for tier 3 screening. Then, we estimated overall sensitivities (which could represent the statistical power) to be 0.10–0.45, 0.04–0.23 and 0.01–0.08 for a disease-associated SNP of OR = 1.4, 1.3 and 1.2, respectively, assuming the disease allele frequency within 0.1–0.9, the disease prevalence of 0.25 and the multiplicative genotype model. Since we had adopted relatively generous criteria for screening association signals, we evaluated the false discovery rate (FDR) to account for multiple testing (15). FDR for the nine SNPs found as significant was 0.69. A multi-staged screening in the current study could be largely categorized into two steps: tiers 1 and 2 (which constitute the first 'case versus unaffected control' study) and tier 3 (which constitutes the second 'case versus unaffected control' study). We therefore assessed experiment-wise type I errors with particular focus on the last-stage screening in tier 3. By permutation, the chance of observing a *P*-value of 0.0019 (for allele frequency test at rs3755351 in *ADD2*) in tier 3 was estimated to be 0.13.

DISCUSSION

With the recent advent of high-throughput genotyping technologies and high-resolution maps of SNP markers, it is expected that genome-wide association studies allow us to identify systematically the contributions of common genetic variations to human multifactorial diseases (16–18). In this line, our study has attempted to discover common hypertension susceptibility gene variants via a gene-centered genome-wide association design for the first time. Despite the modest genetic impacts assumed for hypertension, e.g. the λ-values (the relative risk for siblings of the affected probands) have been reported to be approximately 4 (19), we have nominated several susceptibility genes for hypertension (Table 3). Among these genes, findings for *ADD2* and *KIAA0789* are particularly noteworthy, because the former has been known to be a physiological candidate gene for hypertension and the latter is a novel gene with as-yet unknown physiological function.

Through a multi-tiered screening, nine SNPs derived from seven distinct gene loci have remained to show some evidence of association out of the 80 795 SNPs initially screened. Although the selection criteria were arbitrarily defined in the present study, a small percentage of the SNPs have passed the criteria in transitions from tier 1 to tier 2 (3.3%) and from tier 2 to tier 3 (2.8%). In the *ADD2* gene, for example, the minor allele frequency (MAF) of rs3755351 is lower in case groups (0.14–0.19) than that in control groups (0.21–0.22) throughout three tiers. A *P*-value of 1.7×10^{-5} and an OR of 1.30 (95% CI 1.15–1.46) are attained for allele frequency comparison of rs3755351 when the subjects studied in different tiers are combined and finally categorized into

Table 2. Clinical characteristics of participants

Variables	Case group Tier 2 panel	Tier 3 panel	Control group Tier 2 panel	Tier 3 panel
Number of subjects (female/male)	752 (353/399)	619 (280/339)	752 (366/386)	1406 (650/756)
Present age, year	62.4 ± 10.3	54.1 ± 8.4[a]	62.0 ± 8.7	58.4 ± 6.6
Age of onset, year	47.3 ± 10.2	43.2 ± 9.9	—	—
Current BMI, kg/m^2	23.9 ± 3.2	25.1 ± 3.6[a]	22.5 ± 2.8	22.4 ± 2.7
Smoking[b]				
None, %	48.6	61.6	66.0	58.6
Previous smoker, %	—	17.0	—	10.2
Current smoker, %	51.4	21.4	34.0	31.2
Blood pressure				
Systolic blood pressure, mmHg	146.4 ± 19.5[a]	150.9 ± 19.3[a]	113.8 ± 9.8	114.4 ± 10.1
Diastolic blood pressure, mmHg	86.4 ± 13.0[a]	91.4 ± 12.2[a]	69.8 ± 7.7	70.3 ± 7.2
Treatment of hypertension, %	92.6	75.4	—	—
Blood chemistry				
Serum creatinine, mg/dl	0.87 ± 0.69[a]	0.75 ± 0.50[a]	0.73 ± 0.18	0.70 ± 0.23
Fasting plasma glucose, mg/dl	105.3 ± 28.7	109.0 ± 31.0[a]	104.0 ± 41.9	99.2 ± 22.7
Serum total cholesterol, mg/dl	204.4 ± 31.1[a]	213.4 ± 33.8	209.2 ± 33.7	215.6 ± 34.1
Serum triglyceride, mg/dl	129.8 ± 82.2[a]	141.3 ± 124.6[a]	108.1 ± 67.1	110.1 ± 71.6
Serum HDL cholesterol, mg/dl	56.2 ± 16.7[a]	61.9 ± 19.5	60.6 ± 16.0	63.5 ± 17.5

Values are means ± SD.
For some variables, subjects with insufficient information are not included in the calculation.
[a]$P < 0.001$, case group versus control group by the unpaired *t*-test in each tier.
[b]Because of differences in the questionnaire, smoking status is categorized into two groups (non-smoker or smoker) in the tier 2 panel.
[c]$P < 0.01$, case group versus control group by the unpaired *t*-test in each tier.

the case (tiers 1–3) and unaffected control (tiers 2 and 3) groups. None of our results appears to be significant with the use of a strict Bonferroni correction, a very conservative evaluation of significance, and further replication in an independent population is indispensable.

The candidacy of *ADD2* as a hypertension susceptibility gene has been supported by several physiological and biochemical findings (20–22), together with some evidence from the studies of molecular genetics (23–27). Adducin is a ubiquitously expressed membrane-skeleton heteromeric protein composed of different subunits, α-, β- and γ-subunits. It is known to play a substantial role in the regulation of membrane ion transport. Point mutations of the α- and β-adducins account for up to 50% of the blood pressure difference between Milan hypertensive and normotensive rat strains, probably via the modulation of the Na^+–K^+ ATPase activity (one of major Na^+-channels) in the kidney (23,24). In this line, of note is the fact that β-adducin-deficient mice show significant increases in systolic and diastolic blood pressures and pulse pressure (21). The human homolog of β-adducin spans over 100 kb on chromosome 2p13 and comprises 17 exons. It has been reported that a common SNP (rs4984) identified at position 1797 in exon15 is associated with an increased risk of hypertension under certain pathological conditions in European populations (25–27), whereas this SNP itself is not polymorphic in Asian populations (http://www.ncbi.nlm.nih.gov/SNP/). Also, it has to be noted that one previous study (28) showed significant evidence for hypertension linkage in the 2p13 region (a peak of 2.84 LOD at 93 cM), where the *ADD2* locus is exactly located among several positional candidate genes. Despite our investigation in the *ADD2* locus, we could not find either a clear LD block-like structure or potentially functional SNPs in the vicinity of three disease-associated SNPs (rs2024453, rs3755351 and rs3771426), which are located in the putative promoter region and intron 1, apart from rs10084293 located within an LD block of *ADD2* (Fig. 3). We have assessed the independence of multiple associated SNPs in *ADD2* by logistic regression analysis and have found that the observed association in this gene could be explained principally by the most significant SNP (rs3755351) (see Supplementary Explanation). Once these associations are validated in an independent study panel, further extensive searches of functional SNPs in the *ADD2* locus are warranted.

Our high-density association study has also highlighted the *KIAA0789* gene located on chromosome 12q23.3. This gene encodes a hypothetical protein, LOC9671, which is expressed principally in the central nervous system and modestly in the pancreas (unpublished data). The predicted gene structure of *KIAA0789* involves 9 exons, spanning ~120 kb. There is a clear LD block in the 5′ region of the putative exon 1 (~3.8 kb in size), whereas we have found two other LD block-like structures within the *KIAA0789* gene (Fig. 3). Two disease-associated SNPs (rs3794260 and rs9739493) have turned out to reside in different LD blocks, and the construction of their haplotypes does not seem to provide much additional information on disease association. Although the precise gene structure and gene function remain unknown, *KIAA0789* appears to contain a carboxy-binding WSC domain, and its homologs are likely to exist in mice and rats according to the database information (http://www.ncbi.nlm.nih.gov/). Again, detailed investigation including independent

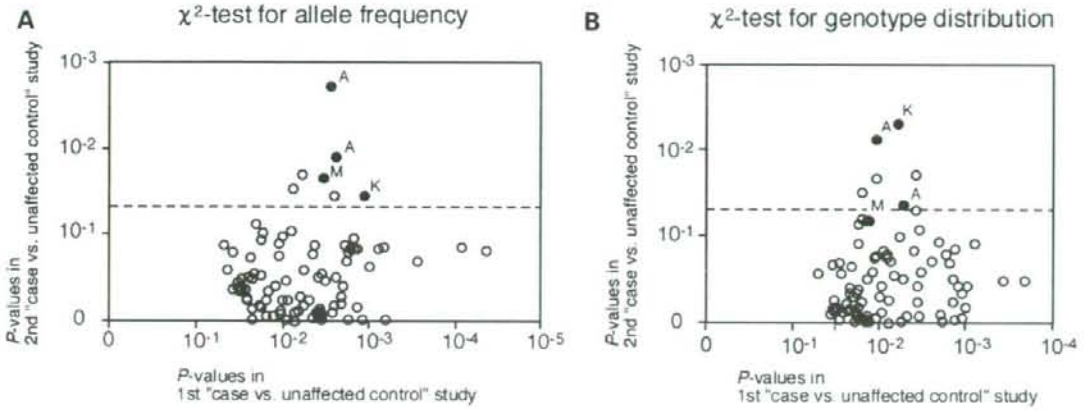

Figure 2. Statistical significance of χ^2-test of disease association in the two-staged 'case versus unaffected control' study. $-\mathrm{Log}_{10}$ *P*-values were used instead of raw *P*-values in each scatter plot. The dashed lines indicate $P = 0.05$. (**A**) As for SNPs genotyped in the second 'case versus unaffected control' panel, *P*-values for allele frequency in the second 'case versus unaffected control' panel were plotted against those in the first 'case versus unaffected control' panel, where SNPs located in three gene loci—*ADD2*, *KIAA0789* and *M6PR*—are depicted with solid circles to which the following symbols are attached: A, *ADD2*; K, *KIAA0789*; M, *M6PR*. (**B**) As for SNPs genotyped in the second 'case versus unaffected control' panel, *P*-values for genotype distribution ([2 × 3] contingency table) in the second 'case versus unaffected control' panel were plotted against those in the first 'case versus unaffected control' panel, where SNPs located in three gene loci—*ADD2*, *KIAA0789* and *M6PR*—are depicted with solid circles as mentioned earlier.

replication of disease association will lead us to clarify the etiological relevance of *KIAA0789* to hypertension.

Another, potential disease association, though modest statistical significance, has been found for *M6PR*. The *M6PR* gene encodes a cation-dependent receptor for mannose-6-phosphate groups on lysosomal enzymes and plays a critical role in the segregation and targeting of lysosomal enzymes to lysosomes. Thus far, no functional relation between *M6PR* and hypertension has been reported. Similar to *KIAA0789*, this gene could also allow us to identify a novel, as-yet unnoticed blood pressure regulatory mechanism.

We should bear in mind several limitations inherent in the present study. First, the level of genome coverage is an issue of heated debate (10,18). Some people may argue that our *a priori* marker selection strategy is gene-centric without utilizing LD information and hence it is not sufficient to pick up as many modest associations as possible in genome-wide searches of hypertension susceptibility genes. A comprehensive framework of common variations throughout the human genome has been made available by the recent completion of the International HapMap Project (29). On the basis of our assessment, the JSNP screening markers in this study cover 20.6% of the HapMap SNPs, whereas a substantial proportion (~30%) of SNPs appear to be unique to JSNP (Table 1 and Supplementary Material, Fig. S1). Under these circumstances, an ideal set of SNPs for our study would encompass deliberately selected tag SNPs (principally common genetic variants) and additional 'singleton' SNPs (sometimes rare genetic variants). Besides this argument of tag SNPs, there are two points of weakness regarding genome coverage as follows: (i) sex chromosome markers have been excluded from the analysis because of the pre-determined policy of multi-disease collaborative study in the Japanese Millennium Genome Project, and (ii) a substantial part of the expressed human genes is not covered by the JSNP database (11), in which the fundamental SNP data were almost fixed in the middle of 2003. Second, the statistical power attainable by our study panel needs to be taken into consideration. For the last few years, genotype costs have fallen dramatically, yet present economic and experimental conditions make it necessary, in practice, to reduce the number of genotyped samples down to a moderately sized case group (188 subjects in our study) at the initial screening with approximately 80 000 SNPs. We arbitrarily set the selection criteria of OR $\geq$ 1.4 and $P \leq 0.015$ in transition from tier 1 to tier 2, where the overall statistical power is estimated to be 10–45% for a disease-associated SNP of OR = 1.4 and 1–8% for that of OR = 1.2, assuming the disease allele frequency within 0.1–0.9 and the disease prevalence of 0.25. Thus, it is likely that our study design allows for capturing less than half of the true disease associations particularly with regard to modest genetic susceptibility. Third, ethnic diversity has not been tested within the scope of the present study. Instead of using commercially available SNP sets aimed at full genomic coverage, we have attempted to focus on potentially functional variants and also relatively common SNPs (MAF $\geq$ 0.1) in the Japanese population. Accordingly, some of disease-associated SNPs listed in Table 3 may be rare or not polymorphic in the other ethnic groups. To clarify allele frequency representation of individual loci and etiological impacts attributable to them, further examination is required in the context of ethnic diversity.

During our preparation of this report, two genome-wide association studies for hypertension and/or blood pressure have been performed in Caucasians (30,31). When our results are compared with public data sets for these association statistics, a few SNPs in the regions of interest appear to show a tendency of association with hypertension or blood pressure;

Table 3. Summary of genomic SNPs associated with hypertension status in two-staged 'case versus unaffected control' study

dbSNP number	Gene symbol	Major minor allele	Orientation	Minor allele frequency: Case			Control			Association analysis: *P*-value in the first 'case (tier 1 and 2) versus unaffected control (tier 2)' study				*P*-value in the second 'case (tier 3) versus unaffected control (tier 3)' study				Total subjects: case (tiers 1–3) versus unaffected control (tiers 2 and 3)				
				Tier 1	Tier 2	Tier 3	Tier 1 (+Ref. 2)[a]	Tier 2	Tier 3	[2 × 3] Contingency	Dominant	Recessive	Allele	[2 × 3] Contingency	Dominant	Recessive	Allele	[2 × 3] Contingency *P*-value	Dominant *P*-value	Recessive *P*-value	Allele frequency model *P*-value	OR (95% CI)[b]
rs3753353	ADD2	C/A	Ind	0.14	0.19	0.17	0.22 (0.21)	0.23	0.21	0.011	0.041	0.007	0.003	0.007	0.071	0.001	0.0102	0.000091	0.001	0.00006	0.00062	1.30 (1.15–1.46)
rs3731426	ADD2	T/C	Ind	0.13	0.16	0.16	0.19 (NA)	0.2	0.19	0.005	0.006	0.016	0.003	0.043	0.093	0.025	0.012	0.0003	0.003	0.0007	0.00005	1.28 (1.13–1.45)
rs2024453	ADD2	T/C	ns	—	—	0.29	—	—	0.33	—	—	—	—	0.034	0.013	0.132	0.023	—	—	—	—	—
rs10084293	ADD2	G/A	ns	—	—	0.44	—	—	0.45	—	—	—	—	0.049	0.063	0.063	0.027	—	—	—	—	—
rs3794260	KIAA0709	G/A	Ind	0.14	0.17	0.17	0.21 (0.18)	0.21	0.20	0.006	0.161	0.002	0.001	0.005	0.001	0.256	0.035	0.0008	0.006	0.001	0.0001	1.26 (1.12–1.42)
rs6734193	KIAA0709	T/C	Ind	—	—	0.41	—	—	0.44	—	—	—	—	0.026	0.008	0.641	0.041	—	—	—	—	—
rs1805076	MOPR	C/G	ns	0.21	0.22	0.22	0.25 (0.24)	0.26	0.25	0.015	0.045	0.009	0.003	0.061	0.332	0.010	0.022	0.001	0.023	0.0001	0.0005	1.23 (1.06–1.37)
rs1805725	MOPR	T/G	Ind	—	—	0.54	—	—	0.49	—	—	—	—	0.017	0.102	0.125	0.005	—	—	—	—	—
rs3747240	EVA2	C/T	Ind	0.23	0.21	0.22	0.19 (0.18)	0.18	0.19	0.003	0.001	0.059	0.008	0.053	0.227	0.934	0.028	0.001	0.002	0.001	0.0010	0.82 (0.73–0.92)
rs3747241	EVA2	G/A	Ind	0.24	0.21	0.22	0.19 (0.19)	0.18	0.19	0.008	0.003	0.080	0.014	0.164	0.267	0.042	0.037	0.002	0.003	0.01	0.0017	0.83 (0.74–0.93)
rs3761987	—	T/A	Ind	0.43	0.37	0.37	0.36 (0.36)	0.33	0.34	0.004	0.001	0.101	0.004	0.019	0.566	0.015	0.134	0.0007	0.031	0.004	0.0016	0.86 (0.78–0.94)
rs3741691	THAP2	A/C	Ind	0.26	0.22	0.22	0.21 (0.20)	0.19	0.21	0.011	0.043	0.007	0.002	0.021	0.139	0.066	0.317	0.007	0.727	0.003	0.0064	0.85 (0.76–0.96)
rs1298463	CCDC131	A/G	Ind	0.26	0.22	0.22	0.21 (0.19)	0.19	0.21	0.016	0.052	0.010	0.004	0.031	0.182	0.073	0.112	0.012	0.736	0.0011	0.0096	0.86 (0.77–0.96)

Two SNPs of EVA2, rs3747240 and rs3747241, are located closely (only 296 bp apart) and in complete LD ($r^2 = 1.00$) to each other.

Also, rs3741691 and rs1298463 are located closely (4430 apart) and have turned out to be in strong LD ($r^2 > 0.99$–1.00) to each other.

[a]For tier 1 control subjects, the figures in parentheses are minor allele frequencies (MAFs) calculated separately in the Ref. 1 panel—the other disease patients who can be regarded as ambient general controls. Ref. 2 panel—752 individuals from the Japanese general population (see Materials and Methods).

[b]The OR was calculated as the ratio of the odds of disease in chromosomes with major alleles relative to those without them.

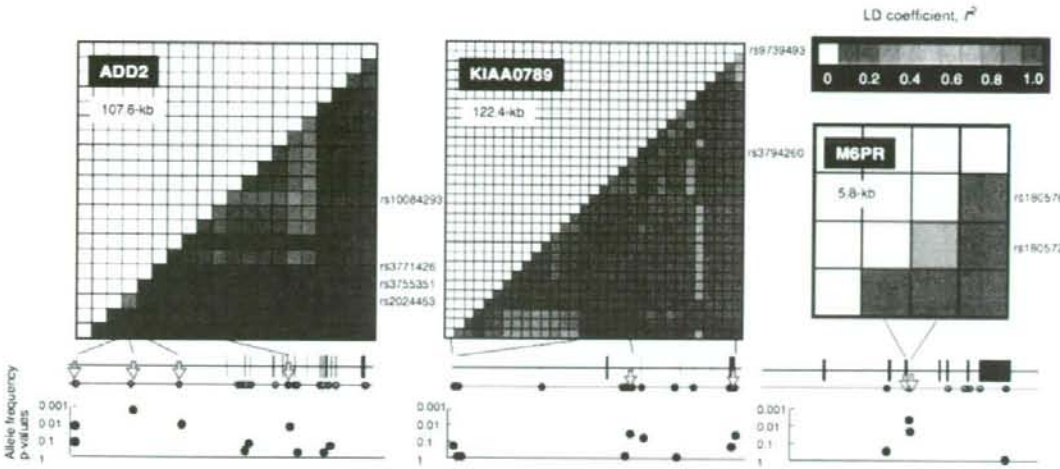

Figure 3. LD relations between SNPs in the *ADD2*, *KIAA0789* and *M6PR* genes (top) and disease association of markers from the corresponding genomic regions (bottom). In the top, the LD between a pair of markers is indicated by the color of the block above and to the left of the intersection of the markers. For the sake of readability, only the names of SNPs showing significant association are shown to the right of the vertical axis of the LD plot. The rest of the SNP information is described in Supplementary Material, Table S2A, B and C. In the upper bottom, the location of genetic markers studied in the corresponding genomic region is shown with relation to gene structure. Here, green and red circles indicate the SNPs with low (MAF < 0.05) and high (MAF ≥ 0.05) allele frequencies in the Japanese, respectively. In the lower bottom, $-\log_{10}$ P-values of the differences in allele frequencies between case and control subjects in tier 3 (i.e. the second 'case versus unaffected control' study) were plotted against the location of individual genetic markers genotyped.

for example, an SNP (rs17006246) in *ADD2*, which is in strong LD ($r^2 = 0.806$ and $D' = 1$ in the HapMap JPT population) with rs3755351, the most significant SNP in our study, is modestly associated with hypertension status ($P = 0.029$) in the Diabetes Genetics Initiative (DGI) study but the direction of effect is opposite between rs3755351 typed in this study and rs17006246 typed in the DGI study. On the other hand, rs1805740, in strong LD with an SNP (rs1805762) in *M6PR*, is modestly associated with hypertension status ($P = 0.036$) in the Wellcome Trust Case Control Consortium study with the same direction of effect as in this study (see Supplementary Material, Table S3).

In summary, our high-density association study provides a list of gene loci potentially predisposing people to hypertension, which awaits replication across populations. With the available samples, we have observed an association of SNPs including three SNPs clusters (or gene loci) in the Japanese populations. In face of the complex nature of disease etiology, it seems to be a formidable task but worth challenging that we eventually apply the SNPs information to improved prevention, diagnosis and treatment of hypertension.

MATERIALS AND METHODS

Study design

We performed a large-scale association study for genes susceptible to hypertension by using a three-tiered genotyping approach (tiers 1, 2 and 3) as depicted in Figure 1. All methods of the study were approved by the review committees of the individual institutions involved in the present study. All subjects provided written informed consent for participation.

In the gene-centered genome-wide exploratory test in tier 1, we carried out genotyping of 83 802 SNPs (3007 of which were excluded from the analysis because they are on sex chromosomes or in the unknown locations) using genomic DNAs from 188 Japanese male hypertensive patients and 752 unrelated Japanese individuals (referred to as general population controls) and another panel of 752 Japanese subjects (referred to as arbitrarily defined controls) who were affected with any of the other four common diseases including gastric cancer, diabetes mellitus, bronchial asthma and Alzheimer's disease; each of these was investigated as the 'Japanese Millennium Genome Project' (Fig. 1). The theoretical basis of adopting this exploratory test scheme was previously reported elsewhere (32). Cases were enrolled from the clinical practice or the annual medical checkup of university hospitals and medical institutions according to the uniformly defined criteria. These included (i) systolic blood pressure ≥ 160 mmHg, diastolic blood pressure ≥ 95 mmHg, or both on two consecutive visits for untreated subjects; (ii) patients receiving long-term antihypertensive treatments; (iii) no secondary form of hypertension as evaluated by an extensive workup; (iv) family history of hypertension, i.e. at least one hypertensive subjects detectable among parents and siblings of the participants; (v) an age of onset known to be between 30 and 59 years. Moreover, only male subjects with BMI < 25 kg/m^2 were selected in tier 1. We compared allele frequencies and/or genotype distributions in hypertensive patients and two population control panels and evaluated deviation from HWE at each of the genotyped loci. For the subsequent screening

in tier 2, we selected SNPs (i) with OR ≥ 1.4 and $P \leq 0.015$ against either of two population control panels and with concordant OR tendency against two control panels; (ii) with MAF ≥ 0.1 and (iii) not showing significant deviations ($P = 0.01$ level) from Hardy–Weinberg expectations in the patient or control panels.

In tier 2 (which comprised 752 hypertensive patients and 752 normotensive controls), we further tested the SNPs thus screened in tier 1, which effectively constituted the first 'case (tiers 1 and 2) versus unaffected control (tier 2)' study. Here, cases in tier 2 were selected according to the criteria (i)–(v) mentioned earlier for tier 1. Normotensive controls, on the other hand, were defined as follows: (i) systolic blood pressure ≤ 130 mmHg and diastolic blood pressure ≤ 85 mmHg without receiving antihypertensive treatments; (ii) age ≥ 50 years and (iii) no family history of hypertension. Both males and females were included in tier 2 without reference to BMI. We selected SNPs (i) with P-value ≤ 0.05 when comparing allele frequency; and (ii) with P-value ≤ 0.01 when comparing genotype distribution between (tiers 1 and 2) cases and (tier 2) controls by χ^2 test statistics.

In tier 3 (which comprised 619 hypertensive patients and 1406 normotensive controls), we performed the second 'case versus unaffected control' study to examine significant associations observed in tiers 1 and 2. The diagnostic criteria in tier 3 were identical to those in tier 2. For the assessment of assumptions when using statistical models in the present study, quantile–quantile plots of P-values were depicted for each stage of association test described in Supplementary Explanation.

No significant population stratification was observed for samples in tier 1 when it was assessed with the methods reported by Patterson *et al.* (33). However, the presence of population stratification was indicated for samples in the first stage 'case (tiers 1 and 2) versus unaffected control' study. We observed moderate bias in genotype frequency of some SNPs between the two tiers, which may have resulted from technical/experimental artifacts between genotyping of cases in tiers 1 and 2. Therefore, the trend test statistic at this analytical stage was corrected according to the significant eigenvector (see Supplementary Explanation). Stratification in tier 3 was not detected but could not be ruled out because of the relatively small number of SNPs ($n = 75$) genotyped in tier 3. As for the nine SNPs that showed significant disease association after multi-stage screening, they were not correlated with the significant eigenvector detected in tiers 1 and 2 cases and tier 2 controls. The P-values for nine SNPs were similar between the nominal and the EIGENSTRAT-corrected ones; for example, the nominal P-value was 0.0029 and the EIGENSTRAT-corrected P-value was 0.0069 at rs3755351 in *ADD2*.

SNP marker resource and genotyping

Most of the SNP markers used in the present study were same as the markers used in the previous reports (14) and derived from the JSNP database. The samples in tiers 1 and 2 were genotyped by PCR amplification of multiple genomic fragments with 20 ng of genomic DNA followed by characterization with the invader assay. Genotyping of the samples in tier 3 was undertaken using the TaqMan® SNP Genotyping Assays (Applied Biosystems). To secure the accuracy and completeness of genotyping, which is critical for large-scale studies (34), we attached a set of 'flags' to individual SNP data mainly dependent on the data completeness, after two independent investigators had checked the raw data robustness by looking at the scatter plot of the assay.

SNP discovery in the selected genes

Approximately 38 kb of genomic sequence spanning the exons and the 5′- and 3′-untranslated regions of three genes, *ADD2*, *KIAA0789* and *M6PR*, was re-sequenced in 48 Japanese control individuals to identify potentially functional SNPs. Since *KIAA0789* had not been fully annotated, the arbitrary positions of translation initiation sites were estimated according to the human genome database. From the SNPs thus identified, tag SNPs were selected for the three genes with the algorithm that we previously reported (35). These tag SNPs were then used for the case–control analysis in tier 3 to further examine association signals seen throughout the multi-staged screening. We deposited the identified SNP information in the NCBI's SNP database and also in our own database, JMDBase (Japan Metabolic Disease Database).

Statistical analysis

The SNPs were tested individually for the statistical significance of disease association with the χ^2-test statistic, which evaluated three inheritance models—[2×3] contingency table, dominant and recessive models—for genotype distributions and independence on [2×2] contingency table for allele frequencies. Here, the most significant P-values among three inheritance models were adopted for genotype distributions when we selected SNPs for screening in tier 3. The criteria for declaring suggestive evidence of disease association were arbitrarily set at each analytical stage as summarized in Figure 1, and they are described in the Results section. SNPs' genotype departures from HWE were tested using the χ^2-test with 1 degree of freedom.

In the three genes showing significant association signals, the extent of LD was measured in terms of an LD coefficient r^2 before the analysis of haplotype structure. Within each LD block, haplotypes were inferred from genotype data by the SNPHAP software for the case and control groups, respectively.

We randomly permutated the genotype of individuals across different panels, 100 times per SNP, and counted the ratio of permutations that fulfill the screening criteria. This ratio indicates the specificity of the study. According to the P-value distribution of the permutations, we evaluated the probability of observing an SNP with P-value no larger than the actual minimum. This probability indicates the experiment-wise P-value. For the specific prevalence and penetrance, we calculated genotype frequency and randomly generated genotypes according to their frequency. We generated genotypes for 1000 simulations of each panel and computed the ratio of simulations that could pass the screening. This ratio is considered the sensitivity of the study.

Values were expressed as means ± SD unless otherwise indicated.

Uniform resource locators

The JSNP database is available at http://snp.ims.u-tokyo.ac.jp/index.html. The National Center for Biotechnology Information's SNP database is available at http://www.ncbi.nlm.nih.gov/SNP/. The JMDBase is available at http://www.jmdbase.jp. SNPHAP is available at http://www.gene.cimr.cam.ac.uk/clayton/software/

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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Which Factors Predict the Recovery of Natural Heart Function After Insertion of a Left Ventricular Assist System?

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Background: Recent reports have demonstrated that use of a left ventricular assist system (LVAS) can initiate recovery of cardiac function, and subsequent weaning from the LVAS has attracted considerable interest. In this study we investigated reliable predictors of LVAS weaning.

Methods: Eighty-two patients underwent LVAS implantation between April 1994 and July 2006 at our institution. Cardiac function was restored in 8 patients, who were weaned from LVAS after a mean of 5 months (Group R). Thirty-three patients remained on LVAS support for >1 year (Group N) because natural heart function did not show adequate improvement. We retrospectively evaluated the differences between these two groups. Group R was younger, and had a shorter duration of heart failure than Group N (23.4 vs 36.7 years and 13.3 vs 56.1 months, $p < 0.01$, respectively). Pathologic findings showed that the interstitial fibrosis score was lower in Group R ($p < 0.01$). Three months after LVAS insertion, B-type natriuretic peptide (BNP) and fractional shortening (FS) were more favorable (66.6 ± 46 vs 264.5 ± 170 pg/ml, $p < 0.01$, and 23 ± 17.1 vs 12 ± 9.1%, $p < 0.05$, respectively) in Group R. Furthermore, Group R received a higher dose of β-blocker (15.4 ± 8.4 vs 5.8 ± 3.9 mg, $p < 0.05$).

Conclusions: Younger age, shorter history of heart failure, and less interstitial fibrosis were effective predictors of weaning from LVAS. Restoration of natural heart function was more rapid and more persistent in candidates for LVAS explantation, and presence of β-blocker played a prominent role in improving cardiac function after LVAS implantation. J Heart Lung Transplant 2008;27:869-74. Copyright © 2008 by the International Society for Heart and Lung Transplantation.

The left ventricular assist system (LVAS) is a powerful tool for saving patients with end-stage heart failure. The primary objective of this device is to provide sufficient circulation, to help patients recover from secondary organ dysfunction, and to stabilize them until their own heart function recovers or suitable donor organs are found. However, relatively few patients receive the benefit of heart transplantation, especially in Japan, due to a shortage of donor organs. In a previous study, we have described the possibility of natural heart recovery after profound heart failure using long-term LVAS support.[1] Several recent reports have demonstrated the restoration of native cardiac function during LVAS support, and weaning from LVAS is recognized as a desirable option. Several factors are associated with improvement of natural heart function after LVAS implantation. Levin et al reported reverse remodeling with a decreased LV mass in LVAS-supported patients.[2] Reduced cellular edema,[3] improved myocardial metabolism,[4] reversal of neurohumoral stimulation[5] and decreased apoptosis[6] have also been suggested. Assessment of myocardial recovery during LVAS support is also an area of interest.[7] However, it remains unclear which patients are appropriate candidates for LVAS explantation. In this study we investigated the factors that could predict weaning from LVAS.

METHODS

Patient Population

Between April 1994 and July 2006, 82 patients except post-cardiotomy cases underwent LVAS implantation for end-stage heart failure at our institution. All patients had New York Heart Association Class IV status and were supported by intravenous inotropic agents and/or percutaneous mechanical support. Among these patients, natural heart function was restored and general condition was sufficiently stable in 8 patients (ages 17

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to 38 years, 7 males and 1 female; 7 with dilated cardiomyopathy [DCM], 1 with myocarditis) and they were weaned from LVAS after 89 to 310 days (recovery group: Group R). Thirty-three patients were supported by LVAS for >1 year. They remained generally stable, but they could not be weaned from LVAS because of poor native heart function (non-recovery group: Group N). This group comprised 22 males and 11 females, ages 16 to 55 years, and whose etiologies were as follows: 27 had DCM; 3 were in the dilated phase of hypertrophic cardiomyopathy (dHCM); and 3 had secondary cardiomyopathy (sarcoidosis, myopathy and drugs). Of these, 15 patients underwent heart transplantation, 13 died (6 cerebral hemorrhages, 1 cerebral infarction, 6 infections), and 5 remain on the waiting list. Another 3 patients were weaned from LVAS due to cerebral events despite insufficient natural heart recovery. LVAS support was discontinued within 1 year in the other 35 patients because of transplantation or death.

In Group R, 3 patients were given a Toyobo LA LVAS, 4 a Toyobo LV LVAS and 1 a Novacor device. In Group N, 30 patients were given a Toyobo LV LVAS and 3 a HeartMate VE device. We retrospectively evaluated the differences between Group R and Group N. To assess natural heart function, we followed-up echocardiographic parameters and the brain natriuretic peptide (BNP) levels at 1 and 3 months after LVAS implantation. Medical therapy regimens were also evaluated. The investigations complied with the principles outlined in the Declaration of Helsinki. The study was approved by the institutional review board of the National Cardiovascular Center, and all patients provided written informed consent.

Management After LVAS Implantation

After general stabilization, we re-administered a β-blocker (carvedilol), an angiotensin-converting enzyme inhibitor (ACE-I, enarapril) and an aldosterone antagonist (spironolactone).

The maximum titrated doses were 20, 5 and 25 mg, respectively. The criteria by which we introduced or increased these drugs were as follows: systolic blood pressure >80 mm Hg; heart rate >60 beats/min; and no sign of deterioration of heart failure. Adequate rehabilitation was also combined with medical treatments. Nutritional states were assessed and the patients received nutritional intervention if necessary. The pump rate was gradually reduced to 60/min when cardiac function showed no deterioration.

Weaning Protocol

Device explantation was considered if the patients met the following criteria: left ventricular diameter in diastole (LVDd) <55 mm; fractional shortening (FS) >20%; and BNP < 100 pg/ml under minimal LVAS support (60 pumps/min). Candidates for LVAS explantation then underwent dobutamine stress testing. Dobutamine was titrated from 5 to 40 μg/kg/min, and hemodynamic and echocardiographic data were evaluated at each dose level. The test outcome was classified as favorable if the patients showed an increase in cardiac output and FS with an increase in dobutamine, without an increase in pulmonary capillary wedge pressure (PCWP), LVDd and symptoms of heart failure. Those who responded appropriately to dobutamine stress testing were considered candidates for LVAS explantation.

Statistical Analysis

We used Student's unpaired *t*-test to compare continuous variables (all data expressed as mean ± SD) and the chi-square test to compare categoric variables. In time-course analysis (Figure 1), data were analyzed by 2-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $p < 0.05$ was considered statistically significant. All analyses were performed using SPSS software (version 14-J).

RESULTS

Before LVAS Implantation

Table 1 summarizes the demographics and baseline characteristics of Groups R and N. Group R was significantly younger and had a shorter duration of heart failure than Group N ($p < 0.01$, respectively). Group R had less myocardial fibrosis than Group N ($p < 0.01$). Myocardial hypertrophy tended to be milder in Group R, but the difference did not reach statistical significance. The ratio (%) of patients with dilated cardiomyopathy was similar in both groups. Hemodynamic parameters, echocardiographic parameters, dose of intravenous inotropic agents, ratio (%) of patients supported by percutaneous mechanical assist devices, BNP levels, and degree of other organ dysfunction or anemia did not significantly differ between the two groups. The regimens of medical treatment did not significantly differ between the two groups (Table 2), but the percentage of patients who were given an ACE-I, a β-blocker, a spironolactone or an amiodarone tended to be higher in Group N.

One Month After LVAS Implantation

Echocardiographic parameters (Dd and FS) and BNP levels were more favorable in Group R, but the differences were not statistically significant (Table 3). The ratio (%) of patients who tolerated treatment with a β-blocker was significantly higher in Group R ($p < 0.05$) (Table 4).

Three Months After LVAS Implantation

FS was significantly higher, and BNP levels was significantly lower ($p < 0.05$ and $p < 0.01$, respectively) in Group R than in Group N (Table 5). Furthermore, the

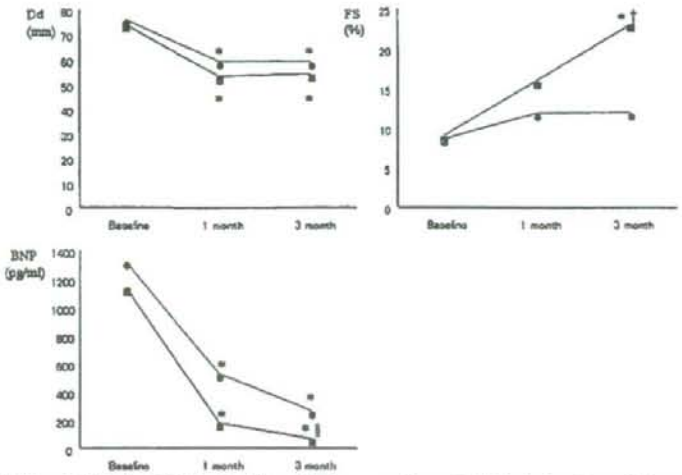

Figure 1. Changes in Dd, FS and BNP after LVAS implantation. Filled squares: Group R; filled circles: Group N. $^{*}p < 0.05$ vs baseline, $^{\dagger}p < 0.05$ vs Group N and $^{\S}p < 0.01$ vs Group N. LVDd, left ventricular end-diastolic dimension; FS, fractional shortening; BNP, brain natriuretic peptide.

increasing rate of FS and the decreasing rate of BNP (3 months after vs before LVAS implantation) were significantly higher in Group R ($p < 0.05$, respectively, data not shown). The dose of β-blocker was higher in Group R ($p < 0.05$) (Table 6). More patients tolerated treatment with an ACE-I or a β-blocker, and Dd tended to be smaller in Group R, but statistical significance was not demonstrated.

Time Course After LVAS Implantation

Figure 1 shows changes in Dd, FS and BNP after LVAS implantation. Improvement of Dd was almost complete

Table 1. Demographics and Baseline Characteristics of Study Population

	Group R ($n = 8$)	Group N ($n = 33$)	p-value
Age (years)	23.4 ± 7.1	36.7 ± 12.4	<0.01*
Gender (% female)	12.5	35.3	0.21
Etiology (% dilated cardiomyopathy)	87.5	79.4	0.6
Duration of heart failure (month)	13.3 ± 22	56.1 ± 52	<0.01*
Myocardial fibrosis (score)	1.4 ± 0.5	2.5 ± 0.6	<0.01*
Myocardial hypertrophy (score)	1.7 ± 0.5	2.2 ± 0.8	0.1
Dose of inotropic agents (DOA + DOB)	9.7 ± 5.6	10.2 ± 4.8	0.83
Use of mechanical support (% IABP and/or PCPS)	62.5	67.6	0.78
Systolic blood pressure (mm Hg)	93 ± 9.0	86 ± 12	0.16
Heart rate (bpm)	116 ± 13	103 ± 25	0.19
Cardiac output (liters/min)	3.21 ± 1.0	3.36 ± 1.0	0.77
Pulmonary capillary wedge pressure (mm Hg)	27.2 ± 4.3	27.2 ± 8.5	0.1
Right atrial pressure (mm Hg)	14.2 ± 5.8	10.2 ± 6.1	0.17
Left ventricular diastolic dimension (mm)	74.1 ± 8.9	75.9 ± 11	0.66
Fractional shortening (%)	9.0 ± 3.7	8.6 ± 4.6	0.84
Wall thickness (mm)	7.6 ± 0.4	7.4 ± 1.4	0.7
B-type natriuretic peptide (pg/ml)	1,140 ± 660	1,282 ± 1,074	0.76
Total bilirubin (mg/dl)	2.6 ± 1.0	1.8 ± 1.0	0.06
Creatine (mg/dl)	1.1 ± 0.5	1.4 ± 1.1	0.52
Hemoglobin (g/dl)	11.4 ± 2.5	10.5 ± 1.8	0.31

Myocardial fibrosis or hypertrophy was classified as mild, moderate or severe and scored as follows: 1 = mild; 2 = moderate; 3 = severe. Dose of inotropic agents is shown as the sum of dopamine (DOA) + dobutamine (DOB). Wall thickness is shown as the mean of the septum and posterior wall. IABP, intra-aortic balloon pump; PCPS, percutaneous cardiopulmonary support.

*Statistically significant.

Table 2. Medical Regimens Before LVAS Implantation

	Group R	Group N	*p*-value
ACE-I (%)	37.5	55.9	0.35
β-blocker (%)	12.5	47.1	0.07
Furosemide (%)	100	82.4	0.2
Spironolactone (%)	25	55.9	0.12
hANP (%)	37.5	23.5	0.42
Amiodarone (%)	12.5	50	0.05
Digitalis (%)	37.5	29.4	0.66

Ratio (%) represents drug induction rate. LVAS, left ventricular assist system; ACE-I, angiotensin-converting enzyme inhibitor.

within 1 month in both groups. Augmentation of FS continued during the follow-up period in Group R, but was complete at about 1 month in Group N. BNP levels decreased during the first month and continued to decrease thereafter in both groups.

Prognosis of Patients After LVAS Explantation

Table 7 shows prognosis of patients after LVAS explantation. Three of 8 patients have continued to maintain normal ventricular function during follow-up periods ranging from 8 months to 8 years. Four patients developed recurrent but mild heart failure, and were treated in the outpatient clinic for up to 10.5 years. All are being given an ACE-I (enarapril, mean dose 3.75 mg) and a β-blocker (carvedilol, mean dose 16 mg). The other patient did well up to 8 to 9 years after LVAS removal, but then had episodes of heart failure that required re-LVAS implantation 12 years after explantation. He is now on the waiting list.

DISCUSSION

This study has demonstrated that: (1) young patients with a short history of heart failure and less myocardial fibrosis are candidates for LVAS removal; (2) patients who can be weaned from LVAS show rapid and persistent improvement of natural heart function; and (3) a β-blocker is a potent agent that can induce LVAS removal.

Several mechanisms about restoration of the natural heart by LVAS have been reported. Wohlschlaeger et al showed that ventricular pressure and volume unloading by LVAS reduces harmful neurohumoral and cytokine stimulation (systemic and local), and decreases myocardial apoptosis.[8] Heerdt et al suggested that LVAS support increases the gene and protein levels of SERCA 2a, normalizes Ca^{2+} handling[9] and improves myocardial contraction. Brodde et al demonstrated an up-regulation of a β-receptor after LVAS support.[10] The regression of myocyte hypertrophy and interstitial fibrosis has been also suggested.[11,12] These effects, which occur as a result of maximal ventricular unloading, lead to functional recovery of the native heart.

Basal cardiac states, however, might influence the process of functional improvement. Histologic analysis has demonstrated that less myocardial fibrosis is one of the predictors of LVAS weaning.[13] This finding was also demonstrated in our study. Furthermore, in the present study, myocardial hypertrophy tends to be less common in patients who could be weaned from the device, but a significant difference was not detected. Our study found that younger patients with a shorter duration of heart failure before LVAS implantation were suitable candidates for LVAS explantation. These features indicate less pre-operative myocardial degeneration. The timing of LVAS implantation is very important. LVAS implantation in necessary before myocardial damage becomes irreversible for restoration of natural heart after LVAS implantation. Cardiac function and dysfunctional severity of other organs before LVAS implantation were not statistically different between Groups R and N.

The process of natural heart improvement might reach completion within 4 to 5 months after device implantation.[14] Continued ventricular unloading be-

Table 3. Echocardiographic Parameters and BNP Levels 1 Month After LVAS Implantation

	Group R	Group N	*p*-value
Left ventricular diastolic diameter (mm)	53.7 ± 12.4	59.5 ± 17.6	0.42
Fractional shortening (%)	16.1 ± 12.7	11.9 ± 7.7	0.43
BNP (pg/ml)	176.8 ± 151.6	526.2 ± 483.8	0.09

BNP, B-type natriuretic peptide; LVAS, left ventricular assist system.

Table 4. Medical Regimens at 1 Month After LVAS Implantation

	Group R	Group N	*p*-value
ACE-I (%)	71.4	41.2	0.14
β-blocker (%)	71.4	26.5	<0.05[a]
Furosemide (%)	85.7	88.2	0.85
Spironolactone (%)	57.1	70.6	0.49
Amiodarone (%)	0	20.6	0.19
Digitalis (%)	57.1	26.5	0.11

Ratio (%) represents drug induction rate. LVAS, left ventricular assist system.
[a]Statistically significant.

Table 5. Echocardiographic Parameters and BNP Levels 3 Months After LVAS Implantation

	Group R	Group N	*p*-value
Left ventricular diastolic diameter (mm)	54.7 ± 11.7	58.9 ± 15.4	0.49
Fractional shortening (%)	23.0 ± 17.1	12.0 ± 9.0	<0.05[a]
BNP (pg/ml)	66.6 ± 46.1	264.6 ± 170.1	<0.01[a]

BNP, B-type natriuretic peptide; LVAS, left ventricular assist system.
[a]Statistically significant.

Table 6. Medical Regimens at 3 Months After LVAS Implantation

	Group R	Group N	*p*-value
ACE-I (%)	85.7	55.9	0.14
β-blocker (%)	85.7	55.9	0.14
β-blocker (mg)	15.4 ± 8.4	5.8 ± 3.9	<0.05*
Furosemide (%)	57.1	85.3	0.09
Spironolactone (%)	57.1	70.6	0.49
Amiodarone (%)	57.1	32.4	0.22
Digitalis (%)	57.1	29.4	0.16

Ratio (%) represents drug induction rate. LVAS, left ventricular assist system; ACE-I, angiotensin converting enzyme inhibitor.
*Statistically significant.

yond this time frame may induce myocardial atrophy and fibrosis. Farrar et al reported that waiting 50 days would capture half of the patients who would ultimately recover ventricular function followed by successful device removal, and waiting up to 90 days could capture 80% of them.[5] We evaluated several parameters at 1 and 3 months after LVAS implantation. Natural heart function was restored more rapidly and the improvement persisted for longer in the weaned patients (Group R). They recovered completely, essentially within 3 months, and were weaned from LVAS after a mean of 5 months of support. BNP was the first representative indicator of native cardiac recovery, which was followed by echocardiographic improvement. None of the patients in whom restoration of the native heart was not indicated for these periods could be weaned from LVAS. This timing is compatible with the findings of Farrar et al.

Recently, the β-blocker has been recognized as being highly beneficial for patients with chronic heart failure, and is becoming the first-line drug treatment for heart failure.[15-17] However, the effect of a β-blocker in patients with LVAS is unclear. We found here that the ratio (%) of patients who tolerated treatment with a β-blocker at 1 month after LVAS insertion and the dose of a β-blocker at 3 months after device implantation were significantly higher in weaned than in non-weaned patients. This result indicates that a β-blocker is useful in patients with LVAS. Several mechanisms underlying the favorable effects of β-receptor blockage have been suggested. A β-blocker restores the function of the calcium-release channel and improves cardiac muscle performance.[18] It also improves myocardial energetics, attenuates myocardial apoptosis, and abrogates induction of the fetal gene program.[19] These effects ultimately help to prevent and reverse ventricular remodeling. Also, these mechanisms might strengthen restoration of the natural heart induced by LVAS. Our findings directly show the importance of β-blocker treatment in patients with first-time LVAS. The percentage of patients who tolerated treatment with an ACE-I after LVAS implantation was also higher in the weaned group, but the values did not reach statistical significance. Conversely, more patients were given a β-blocker, ACE-I, spironolactone and amiodarone before LVAS implantation in the non-weaned group. This may be dependent on the longer duration of heart failure in those patients.

Study Limitations

The present study has several limitations. First, the population size in this investigation was relatively small because the percentage of patients able to be weaned from LVAS is small. Second, the etiologies of patients are various due to the same reason (we could not focus specifically on DCM patients). Third, we demonstrated the effect of a β-blocker. However, we could not standardize the medical regimens after LVAS implantation. Further examinations on larger numbers of patients with uniform etiology and medical treatments are necessary.

In conclusion, weaning from LVAS might be feasible in selected patients. Adjunctive treatments as well as adequate unloading are important in those who

Table 7. Prognosis After Explanation of the Left Ventricular Assist System

Patient no.	Age (years)	Gender	Left ventricular diastolic dimension (mm)	Fractional shortening (%)	B-type natriuretic peptide (pg/ml)	New York Heart Association class	Current status	Duration after explantation
1	29	M	69	5	124	I	Re-LVAS implantation, in hospital, on waiting list	12 years
2	31	M	66	17	103	II	Well, at home	10 years 5 months
3	33	M	50	28	12	I	Well, at home	8 years
4	44	F	53	36	21	I	Well, at home	5 years 7 months
5	25	M	69	10	548	I	Well, at home	5 years 5 months
6	30	M	72	8	275	II	Well, at home	4 years 1 month
7	19	M	91	12	848	II	Well, at home	3 years
8	26	M	51	31	26	I	Well, at home	8 months

have the capability of natural heart restoration. Further studies on LVAS weaning are desirable.

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ORIGINAL ARTICLE

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Acute myocardial infarction as a systemic prothrombotic condition evidenced by increased von Willebrand factor protein over ADAMTS13 activity in coronary and systemic circulation

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Abstract The aim of the present study is to clarify the roles of circulating ADAMTS13 and von Willebrand factor (VWF) in the formation of coronary artery thrombi in acute myocardial infarction (AMI). Twenty-six AMI patients, 37 age-matched healthy controls, and 20 young controls were studied. Plasma ADAMTS13 activity and levels of VWF antigen (VWF:Ag) and unusually large VWF multimer (UL-VWFM) were measured in the femoral vein (FV), aortic root (Ao), and coronary sinus (Cs) immediately before percutaneous coronary intervention (PCI) during the acute phase of AMI, as well as 6 months later. During the acute phase of AMI, plasma levels of VWF:Ag were similar in FV, Ao, and Cs, and were higher than those of age-matched control. In contrast, ADAMTS13 activity in three sampling points in AMI patients was similar to that of age-matched controls. Thus, the ratio of VWF:Ag to ADAMTS13 activity in the acute phase of AMI was significantly higher in all three sampled sites than that of age-matched controls. In the chronic phase, plasma levels of VWF:Ag, ADAMTS13 activity, and the ratio of VWF:Ag to ADAMTS13 activity were similar to those of age-matched controls. UL-VWFM was detected in the acute phase of AMI but not in the chronic phase. The present study showed that the plasma VWF:Ag levels are increased and ADAMTS13 activity is relatively decreased in both systemic and coronary circulation during the acute phase of AMI, suggesting that an imbalance between the enzyme and its substrate may play a role in the formation of occlusive thrombi in a coronary artery.

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Introduction

The rapid closure of the coronary artery by acutely formed arterial thrombi, which are composed of platelets, fibrin, and inflammatory cells, is the major cause of acute myocardial infarction (AMI).[1,2] Although the exact mechanism of coronary thrombus formation is not fully understood, the binding of von Willebrand factor (VWF) to glycoproteins Ibα and IIb/IIIa on the surface of platelets is known to lead to platelet activation and subsequent aggregation, which is an initial step toward formation of coronary thrombi.[3,4] Earlier reports have shown that circulating levels of VWF antigen (VWF:Ag) is elevated in patients during the acute phase of AMI,[5,6] and increased levels of plasma VWF:Ag can predict primary and secondary coronary events.[7–9] Thus, VWF appears to be involved in the formation of coronary thrombi as a cause of AMI, although blocking of VWF function has not yet been clinically proven to prevent the onset of AMI. It is not clear, however where and how VWF is produced during AMI.

Von Willebrand factor is synthesized in vascular endothelial cells and then released into the plasma as unusually large VWF multimer (UL-VWFM),[4] which has most potent biological activities interacted with platelet, and is rapidly degraded into smaller VWF multimers by ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13),[4,9] a metalloproteinase that specifically cleaves multimeric VWF between Tyr1605 and Met1606 within the VWF A2 domain.[9] Loss-of-function mutation of ADAMTS13 leads to Upshaw–Schulman syndrome, a form of congenital thrombotic thrombocytopenic purpura. Reduction of ADAMTS13 activity keeps circulating UL-VWFM levels high, which leads to platelet clumping and formation of platelet-rich thrombi. Recently, Sakai et al.[6] reported that UL-VWFM was detected in plasma drawn from peripheral veins in patients with AMI. To understand

the mechanism for the formation of coronary arterial thrombi in AMI, we measured plasma ADAMTS13 activity together with circulating levels of its substrate, VWF:Ag, in three sites: the aorta (Ao) near the ostium of the infarction-related coronary artery, the coronary sinus (Cs), and the femoral vein (FV). Samples were taken immediately before the percutaneous transluminal coronary intervention (PCI) during the acute phase of AMI and compared with those taken during the chronic phase.

Materials and methods

Patients

We studied 26 Japanese patients with AMI (5 women and 21 men; mean age 67.8 ± 11.6 years; range 38–89 years) admitted to the Nara Medical University Hospital between August 2004 and February 2005. The diagnosis of AMI was based on sustained chest pain of typical character and location, electrocardiographic ST-T elevation in two or more leads, disrupted regional wall motion on echocardiograms, and plasma levels of cardiac enzymes, including creatine phosphokinase (CK) and its MB fraction, that were greater than twice the normal upper limit. Of the 26 patients, 18 had hypertension, 21 had dyslipidemia, 13 had diabetes mellitus, 5 were obese, and 19 smoked. All of the patients received emergency coronary angiography and PCI within 24 h from the onset of AMI (the first symptoms). Clinical characteristics and drugs used are summarized in Table 1. The culprit lesions were in the right coronary artery in 6 patients, the left anterior descending coronary artery in 18, and the left circumflex coronary artery in 2. The peak CK level in AMI patients averaged 2960 IU/l and ranged from 344 to 12930 IU/l. All of the patients received intracoronary stents, implanted at the culprit lesions, and were subsequently given aspirin (81 mg/day, per os) and ticlopidine (200 mg/day, per os) or cilostazol (200 mg/day, per os) as antiplatelet therapy. An angiotensin-converting enzyme inhibitor and/or angiotensin-II receptor blocker were also administered to all patients. In addition, 10 patients received a β-blocker, 6 a calcium channel blocker, 7 a diuretic, and 15 a statin. Six months after the first onset of AMI, coronary angiography was again carried out in all of the patients. Written informed consent was obtained from all patients and control subjects participating in the study. The protocol was approved by the institutional review board of Nara Medical University (#2002-009).

Young and age-matched healthy control subjects

Study participants included both young and age-matched healthy control subjects. Young healthy subjects consisted of 30 volunteers (15 women and 15 men) aged from 20 to 39 years with a mean age of 30 ± 12.0 years, and age-matched healthy subjects consisted of 37 healthy volunteers (19 women and 18 men) aged from 39 to 93 years with a mean age of 64.2 ± 14.0 years. Both groups had no history of angina, myocardial infarction, coronary artery bypass graft surgery, PCI, or any electrocardiographic abnormalities. Blood samples were collected from the antecubital vein early in the morning, before breakfast. Nine of the age-matched controls (4 women and 5 men, mean age 48.1 ± 4.8 years, range 41–52 years) were also studied to evaluate the circadian variation of VWF:Ag and ADAMTS13 activity in plasma. In those subjects, blood samples were collected from the antecubital vein in the morning (09:30) and in the evening (20:00).

Table 1. Characteristics of patients with acute myocardial infarction

	Patient	Age-matched control subjects	*P* value
Age (years)	67.8 (38–89)	64.2 (39–93)	0.29
Sex (female/male)	5/21	19/18	<0.01
Coronary risk factor (yes/no)			
Hypertension	8/18	0/37	<0.01
Dyslipidemia	21/5	2/35	<0.01
Diabetes mellitus	13/13	3/34	<0.01
Obesity	5/21	7/30	0.41
Smoking	19/7	7/30	<0.01
Peak CK (IU/l) (mean)	2960 (344–12930)		
Location of AMI			
RCA/LAD/LCx	6/18/2		
Medication (yes/no)			
Aspirin	26/0		
Ticlopidine or Cilostazol	26/0		
ACE-I or ARB	26/0		
β-Blocker	10/16		
Calcium-antagonist	6/20		
Diurea	7/19		
Statin	15/11		

Values in parentheses indicate range
CK, creatine phosphokinase; AMI, acute myocardial infarction; RCA, right coronary artery; LAD, left anterior descending artery; LCx, left circumflex artery; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin-II receptor blocker

Blood sampling

In the AMI patients, emergency cardiac catheterization was performed within 90 min of their arrival in our hospital. Blood samples were collected using a 7-F sheath inserted into the patient's femoral vein (FV), a 6-F Cs catheter placed in the Cs through an FV sheath, and a 4-F Judkins catheter placed at the Ao. Unfractionated heparin and contrast medium were not used before pre-PCI blood sampling. Blood was sampled at the femoral vein (FV), the aortic root near the ostium of the infarction-related coronary artery (Ao), and the coronary sinus vein (Cs) immediately before and after emergency PCI. Six months after the onset of AMI, all 26 patients underwent a second round of coronary angiography, at which time blood was again collected from the same three areas. In young healthy and age-matched control subjects, blood samples were drawn from the antecubital vein. Preliminary experiments showed that there was no difference in plasma levels of VWF:Ag and ADAMTS13 activity among the antecubital vein, the FV, and the right atrium.

Blood was collected into plastic tubes with 1/10th volume of 3.8% sodium citrate. Platelet-poor plasma was prepared by centrifugation at 3000× *g* at 4°C for 15 min and stored in aliquots at –80°C until analysis.

Assays of ADAMTS13 activity, VWF:Ag, and UL-VWFM

Plasma ADAMTS13 activity was determined using a highly sensitive enzyme-linked immunosorbent assay (ELISA) recently developed by our laboratory.[10] The assay system includes a recombinant GST-VWF73-His polypeptide as a substrate and a murine monoclonal antibody that specifically recognizes the Tyr1605 residue in the VWF-A2 domain exposed by ADAMTS13 cleavage; it does not recognize the uncleaved form of the peptide. Plasma VWF:Ag was measured by a sandwich enzyme immunoassay using rabbit anti-human VWF polyclonal antibody (Dako, Kyoto, Japan). Plasma ADAMTS13 activity and VWF:Ag levels were expressed as percentages of those of reference peripheral plasma obtained from 20 healthy volunteers aged 20–40 years. The lower detection limit of the ELISA for ADAMTS13 activity was 0.5% of the reference peripheral plasma activity. Plasma UL-VWFM was analyzed by sodium dodecyl sulfate – 0.9% agarose gel electrophoresis using 1 µl samples, after which VWF multimers were visualized by Western blotting and luminography, as described previously.[11]

Statistical analysis

The data are expressed as mean ± SD. Comparison between acute and chronic data was performed using the paired

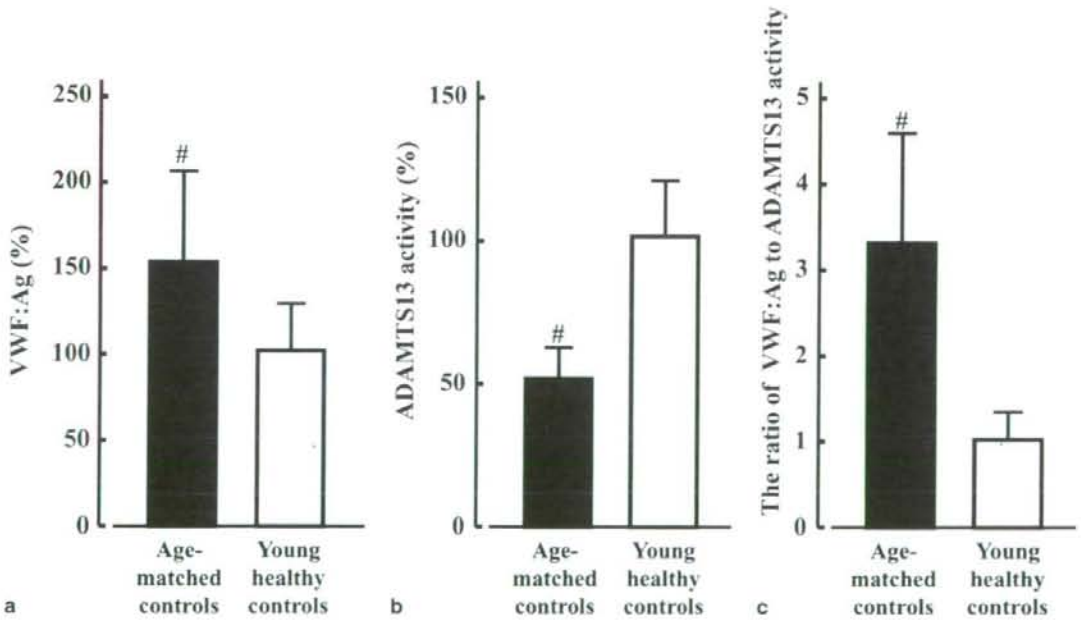

Fig. 1a–c. Comparison of plasma von Willebrand factor antigen (*VWF:Ag*) levels and ADAMTS13 activity between healthy young subjects and age-matched controls. **a** Plasma VWF:Ag levels. **b** Plasma ADAMTS13 activity. **c** Ratios of VWF:Ag to ADAMTS13 activity. Shown are mean ± SD; $^{\#}P < 0.001$ vs young subjects

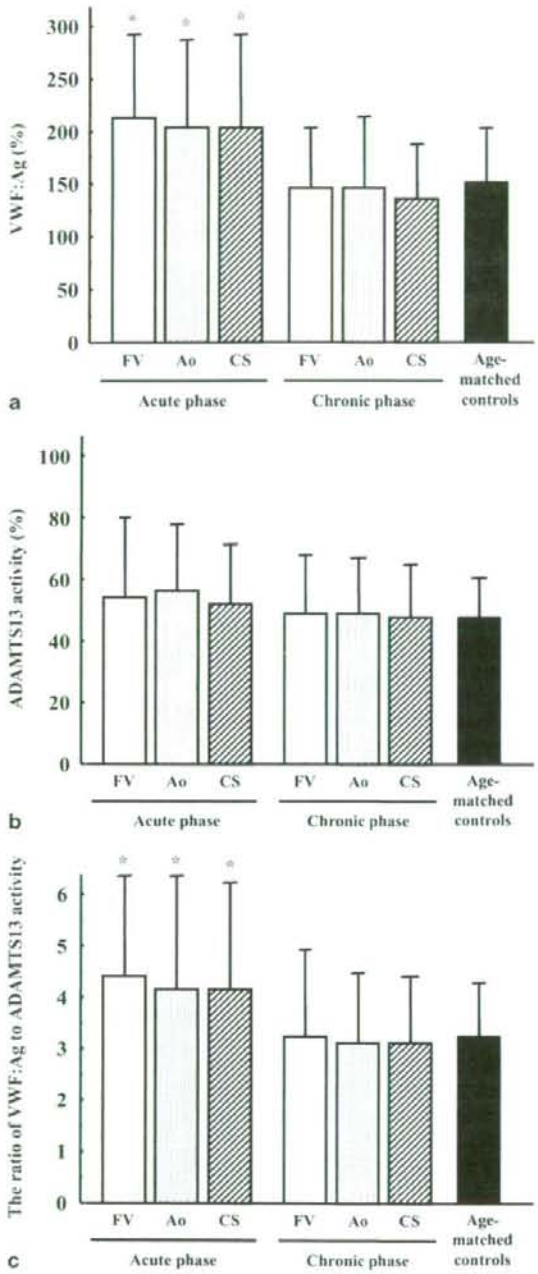

Fig. 2a–c. Plasma von Willebrand factor antigen (*VWF:Ag*) levels and ADAMTS13 activity and the ratio of VWF:Ag to plasma ADAMTS13 activity during the acute and chronic phases of AMI. **a** VWF:Ag levels, **b** ADAMTS13 activity, and **c** ratios of VWF:Ag to ADAMTS13 activity before percutaneous coronary intervention (PCI) during the acute phase and chronic phase of acute myocardial infarction (AMI). Measurements were made using plasma samples collected from the femoral vein (*FV*), aortic root (*Ao*), and coronary sinus (*Cs*) of the AMI patients and peripheral blood samples collected from control subjects. Shown are means ± SD; $^*P < 0.05$ vs age-matched controls

Student's *t*-test or Wilcoxon signed-rank test, when appropriate. Comparison among the three groups of subjects was performed by analysis of variance. The analyses were carried out using the statistical software Statview (version 5.0; SAS Institute, Cary, NC, USA). A P value of less than 0.05 was considered statistically significant.

Results

Differences between healthy young and age-matched controls

Plasma levels of VWF:Ag were significantly higher in healthy age-matched controls than in the young subjects (151% ± 58% vs 102% ± 33%, $P < 0.001$) (Fig. 1). Conversely, the plasma ADAMTS13 activity was lower in the age-matched controls than in the young subjects (51% ± 15% vs 104% ± 22%, $P < 0.001$), resulting in a three-fold higher ratio of VWF:Ag to ADAMTS13 activity in the age-matched controls than in young healthy controls (3.3 ± 1.4 vs 1.0 ± 0.3, $P < 0.001$) (Fig. 1).

VWF:Ag levels

During the acute phase of AMI before PCI, plasma VWF:Ag levels were significantly higher ($P < 0.01$) at the FV (211% ± 75%), Ao (204% ± 78%), and Cs (205% ± 90%) than in peripheral blood samples from the age-matched controls (151% ± 58%) (Fig. 2a). During the chronic phase, these values ($P < 0.05$) fell to levels similar to those seen in the age-matched controls (FV, 149% ± 69%; Ao, 148% ± 73%; and Cs, 133% ± 52%). There also were no differences in VWF:Ag levels among sampling sites (Fig. 2a).

ADAMTS13 activity

Plasma ADAMTS13 activity did not differ among blood samples collected from the FV, Ao, and Cs before PCI during the acute phase of AMI (FV, 55% ± 22%; Ao, 57% ± 22%; Cs, 54% ± 19%), or during the chronic phase of AMI (FV, 51% ± 19%; Ao, 52% ± 17%; Cs, 51% ± 22%). In fact, all of these values were similar to ADAMTS13 activity in peripheral blood from the age-matched controls (51% ± 15%) (Fig. 2b). Moreover, ADAMTS13 activity in the acute phase was similar to that in the chronic phase at each sampling point. There was no significant inverse correlation between ADAMTS13 activity and plasma level of VWF:Ag in the acute phase of AMI.

The ratio of VWF:Ag to ADAMTS13 activity

During the acute phase of AMI, the ratio of VWF:Ag to ADAMTS13 activity before PCI was significantly higher ($P < 0.05$) in the FV (4.5 ± 2.4), Ao (4.2 ± 2.5), and Cs (4.2 ± 2.4) than in the peripheral blood samples from age-matched