Table 1 Patient characteristics

	Standard therapy (control), $n = 10$	Intensive therapy, $n = 14$	P-value
Age (years), mean ± SD	63 ± 15	61 ± 12	0.7201
Male, *n* (%)	7 (70%)	9 (64%)	0.7697
Risk factors			
Hypertension, *n* (%)	5 (50%)	7 (50%)	1.000
Hyperlipidaemia, *n* (%)	6 (60%)	7 (50%)	0.9448
Smoking, *n* (%)	4 (40%)	5 (36%)	0.8307
Retinopathy, *n* (%)	2 (20%)	2 (14%)	0.7111
Proteinuria, *n* (%)	2 (20%)	4 (29%)	0.6326
CAD, *n* (%)	2 (20%)	3 (21%)	0.9323
Peripheral artery disease, *n* (%)	0 (0%)	2 (14%)	0.6175
Stroke, *n* (%)	1 (10%)	1 (7%)	0.8028
Medications, baseline[a]			
ACE-inhibitor, *n* (%)	4 (40%)	5 (36%)	0.8307
Calcium blocker, *n* (%)	2 (20%)	1 (7%)	0.7543
Beta-blocker, *n* (%)	2 (20%)	1 (7%)	0.7543
Statin, *n* (%)	6 (60%)	5 (36%)	0.4462
Sulphonylurea, *n* (%)	7 (70%)	10 (71%)	0.9395
Biguanide, *n* (%)	1 (10%)	1 (7%)	0.7543
α-Glucosidase inhibitor, *n* (%)	4 (40%)	2 (14%)	0.3380

ACE, angiotensin-converting enzyme.
[a]Medications immediately before additional therapy for dysglycaemia.

(Bond Elut, Varian Inc., CA, USA) were used for three-fold washing with 1 mL serum samples with methanol and distilled water. Thereafter, the samples were eluted with 10% ammonia and dried. The sediment obtained was dissolved in 1 mL of water, the solution was centrifuged, and the supernatant was subjected to high-performance liquid chromatography using ODS columns (Fisher Scientific, St Louis, MO, USA). ADMA concentration was calculated on the basis of the recovery rate of L-monomethyl-arginine (Sigma, St Louis, MO, USA), used as the internal standard. Intra- and inter-assay variabilities were both ~6%, with a detection limit of 0.1 μM/L.

Statistical analyses

Sample size calculations were performed using a primary endpoint variable of FMD. Power calculations indicated that to detect a mean difference in FMD of 4% (SD, 3%), 13 subjects would be needed to complete the study (α statistics, 0.05; power >0.9). All data are expressed as mean ± SD. Two-tailed *t*-tests or the Mann-Whitney *U* test was used to compare the changes in response to treatment. To compare the proportions of patients, Fisher's exact test was used. Linear regression curves and correlations were calculated according to the least-squares method. *P*-values less than 0.05 were considered significant.

Results

The baseline characteristics of 10 control patients who received standard therapy and 14 intensively treated patients are summarized in *Table 1*. All 24 patients completed 3–4-week follow-up measurements.

The control patients were treated by diet alone (three patients) or diet plus oral hypoglycaemic agents (an increased dose of sulphonylurea, six patients and addition of metformin to sulphonylurea, one patient). *Table 2* shows no significant improvements in clinical and biochemical parameters during the observation period of 28 ± 5 days of standard therapy. Neither the fasting blood glucose (from 181 ± 42 to 186 ± 38 mg/dL) nor the response of FMD to reactive hyperaemia (from 3.0 ± 1.3 to 2.6 ± 1.0%) changed.

Table 2 Changes in biochemical and clinical parameters before and after standard treatment of hyperglycaemia in 10 control patients with type-2 DM

	Before	After	P-value
Hb A-1C (%)	9.4 ± 2.2	9.4 ± 2.0	>0.999
Insulin (μU/mL)	4.2 ± 2.0	4.4 ± 2.2	0.834
HOMA-R	1.9 ± 1.2	1.8 ± 1.0	0.842
Total cholesterol (mg/dL)	212 ± 28	210 ± 25	0.868
TG (mg/dL)	128 ± 40	129 ± 45	0.959
HDL cholesterol (mg/dL)	50 ± 19	51 ± 20	0.910
LDL cholesterol (mg/dL)	128 ± 22	127 ± 25	0.925
Systolic BP (mmHg)	139 ± 18	138 ± 20	0.908
Diastolic BP (mmHg)	76 ± 8	78 ± 10	0.627
BMI (kg/m^2)	23.8 ± 2.7	23.4 ± 3.1	0.763

TG, triglyceride; BP, blood pressure. Values are expressed as mean ± SD.

Biochemical and clinical changes after intensive treatment of hyperglycaemia

In the intensive therapy group, the patients were all treated by diet alone (three patients), diet plus oral hypoglycaemic agents (sulphonylurea newly given, one patient; an increased dose of sulphonylurea, one patient; addition of metformin to sulphonylurea, two patients; and addition of α-glucosidase inhibitor to sulphonylurea, one patient), or diet plus insulin (switched from oral hypoglycaemic agents, six patients). The duration of intensive treatment of hyperglycaemia was 34 ± 13 days. Clinical and biochemical parameters at baseline (before treatment) were similar between the standard therapy group and the intensive therapy group (*Tables 2* and *3*). After the intensive

Table 3 Changes in biochemical and clinical parameters before and after intensive treatment of hyperglycaemia in 14 patients with type-2 DM

	Before	After	*P*-value	*P*-value (vs. control after)
Hb A-1C (%)	9.7 ± 1.6	8.6 ± 1.4	0.032	0.287
Insulin (μU/mL)	4.4 ± 2.6	5.3 ± 2.0	0.314	0.233
HOMA-R	2.0 ± 1.1	1.6 ± 0.5	0.226	0.524
Total cholesterol (mg/dL)	202 ± 33	173 ± 28	0.019	0.003
TG (mg/dL)	121 ± 43	105 ± 51	0.378	0.246
HDL cholesterol (mg/dL)	52 ± 21	52 ± 17	>0.999	0.896
LDL cholesterol (mg/dL)	125 ± 25	101 ± 29	0.027	0.032
Systolic BP (mmHg)	134 ± 18	128 ± 14	0.779	0.1626
Diastolic BP (mmHg)	77 ± 7	74 ± 8	0.301	0.2880
BMI (kg/m^2)	23.6 ± 3.6	21.4 ± 3.2	0.049	0.1405

Values are expressed as mean ± SD.

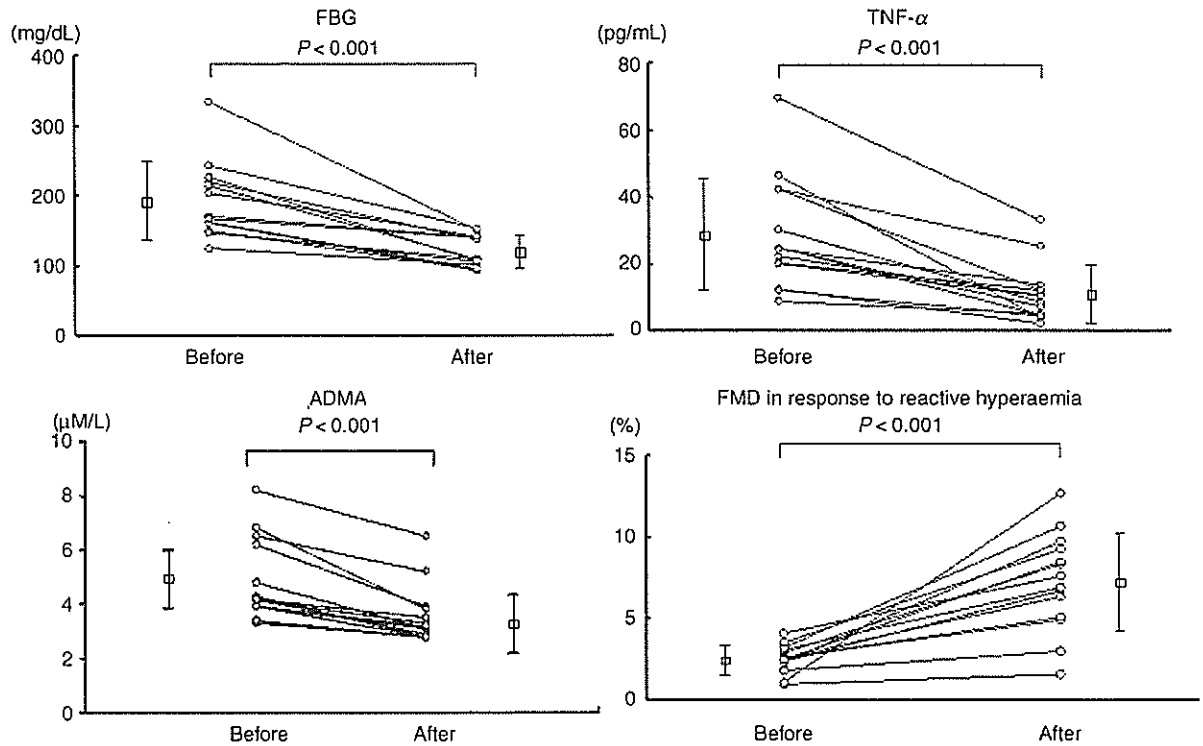

Figure 1 Individual measurements of fasting blood glucose (FBG), TNF-α and ADMA levels, and FMD in response to reactive hyperaemia before and after intensive treatment of hyperglycaemia in 14 patients with type-2 DM.

treatment, the fasting glucose level significantly decreased from 190 ± 55 to 117 ± 21 mg/dL ($P < 0.001$), as shown in *Figure 1*. Significant decreases in Hb A-1C, total cholesterol, and LDL cholesterol levels and BMI were observed, whereas no changes in HOMA-R index; insulin, triglyceride, or HDL cholesterol levels; and systolic and diastolic blood pressures were observed (*Table 3*). Two of three patients with coronary artery disease were taking statins at the time of the study.

The levels of plasma TNF-α (from 29 ± 16 to 11 ± 9 pg/dL, $P < 0.001$) and ADMA (from 4.8 ± 1.5 to 3.5 ± 1.1 μM/L, $P < 0.001$) significantly decreased after the intensive control of glucose level (*Figure 1*). However, HGF level did not significantly change throughout the study (from 0.19 ± 0.05 to 0.20 ± 0.08 ng/mL).

Brachial artery reactivity after intensive treatment of hyperglycaemia

Before treatment under hyperglycaemic condition, the baseline brachial arterial diameter was 4.5 ± 0.3 mm, and FMD in response to reactive hyperaemia was 2.4 ± 0.9%. After the intensive control of glucose level, FMD significantly ($P < 0.001$) increased to 7.2 ± 3.1% (*Figure 1*), whereas the baseline diameter (4.5 ± 0.2 mm) did not change. There was a similar increase in blood flow during reactive

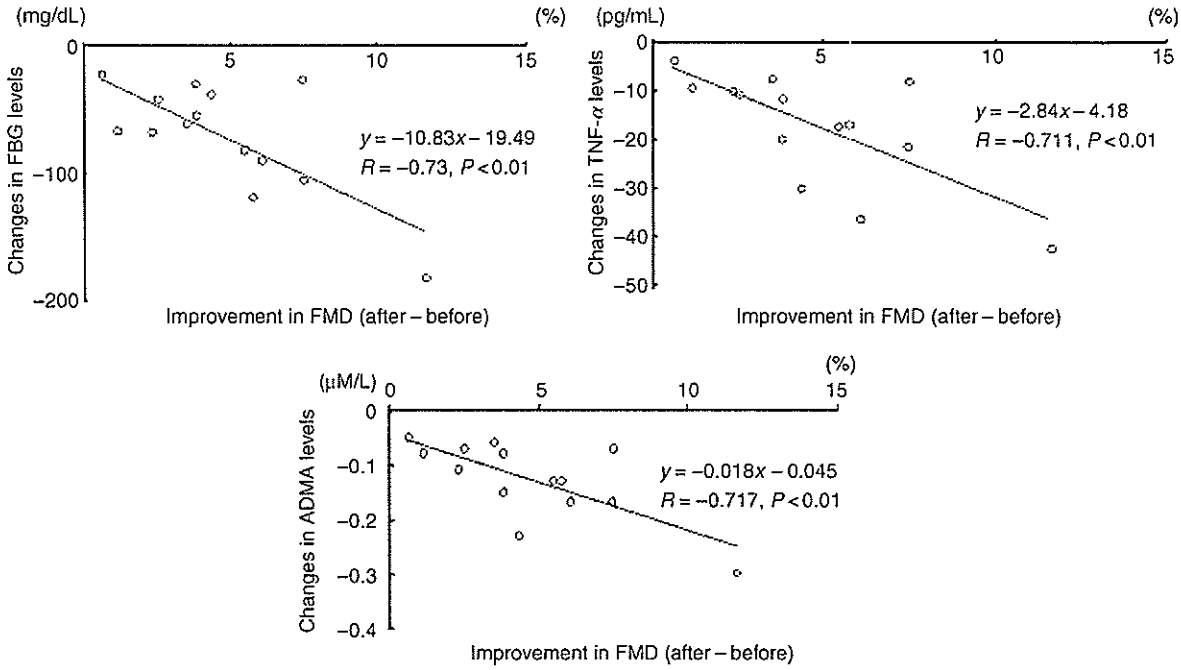

Figure 2 Correlation of improvement of FMD after treatment with decreases in levels of FBG, TNF-α, and ADMA.

hyperaemia (293 ± 16 vs. 296 ± 20%) and a similar baseline heart rate (67 ± 7 vs. 65 ± 8 bpm) before and after the treatment.

Nitroglycerin-mediated vasodilation was 9.8 ± 1.0% before treatment; however, in contrast to FMD, it did not change after treatment (10.0 ± 1.6%).

Correlation with FMD improvement

As shown in *Figure 2*, the improvement of FMD after treatment correlated inversely with the changes in fasting glucose ($R = -0.730$, $P < 0.01$), TNF-α ($R = -0.711$, $P < 0.01$), and ADMA ($R = -0.717$, $P < 0.01$) levels. However, the improvement of FMD did not correlate significantly with the changes in Hb A-1C level ($R = 0.408$, $P = 0.148$), total cholesterol level ($R = 0.325$, $P = 0.256$), or BMI ($R = 0.270$, $P = 0.351$).

Six-to-12-month follow-up

A follow-up study was performed 6–12 months after the discharge. In eight of 14 patients with an Hb A-1C level of <8.0% at this follow-up period, fasting blood glucose level and FMD remained at 127 ± 26 mg/dL and 8.4 ± 1.0%, respectively. In contrast, in the remaining six patients with an Hb A-1C level of ≥8.0%, fasting blood glucose level and FMD worsened to be 178 ± 30 mg/dL and 3.1 ± 1.1%, respectively. There were inverse correlations of FMD with fasting blood glucose ($R = -0.577$) and Hb A-1C levels ($R = -0.860$).

Discussion

The major finding in the present study is that the intensive treatment of hyperglycaemia is associated with the improvement of endothelial function, coupled with the decrease in plasma TNF-α and ADMA (an endogenous inhibitor of NO synthase) levels in patients with type-2 DM.

Previous studies revealed that an acute increase in blood glucose level impairs endothelium-dependent vasodilation in healthy subjects[5,6] and further inhibits it in patients with type-2 DM.[6] DM is a state of chronic hyperglycaemia, and glycaemic control is one of the major goals of diabetes management.[18] As shown in *Figure 1*, endothelial dysfunction improves after a 5-week intervention targeting hyperglycaemia in type-2 diabetes patients, accompanied by a relatively small but significant decrease in Hb A-1C level. In contrast, either hyperglycaemia or endothelial function did not change in control outpatients who received routine treatment. These findings suggest that hyperglycaemia may be a fundamental abnormality underlying the mechanism that causes endothelial dysfunction in DM. However, we must acknowledge a potential limitation that an appropriate control group should have included patients who were admitted to the hospital, but did not receive intensive treatment. In addition, the number of statistical tests performed and relatively small sample size of the study population may potentially infiltrate type-I error.

In patients with type-2 DM, TNF-α levels were elevated in both blood and tissue.[19-21] Taken together with results from knockout mice deficient in TNF-α or its receptors,[11] it is suggested that TNF-α is a factor contributing to the pathogenesis of type-2 DM. Hyperglycaemia is an important stimulus for TNF-α synthesis in human peripheral monocytes *in vitro*.[22] A previous *in vivo* study demonstrated that the administration of TNF-α impairs endothelial-dependent vasodilation in rats.[23] In the present study, as shown in *Figure 1*, plasma TNF-α level decreased after the intensive treatment of hyperglycaemia. This finding indicates the therapeutic potential of a strict glycaemic control against inflammatory cytokines that play a prominent role in atherogenesis.[7]

TNF-α and hyperglycaemia could impair dimethylarginine dimethylaminohydrolase and cause the accumulation of ADMA, an endogenous, competitive inhibitor of NO synthase, contributing to the derangements of NO pathways in the vessel.[10,24] The intra-arterial infusions of ADMA significantly impair endothelium-dependent flow responses in the human forearm.[25] In the present study, we found that the ADMA level increased in patients with type-2 DM (*Figure 1*), and its decrease after the strict glycaemic control correlated significantly with the improvement of FMD (*Figure 2*). Not only ADMA, but also TNF-α itself downregulates NO synthase by decreasing mRNA's half-life.[26] Moreover, both inflammatory cytokines and high glucose levels enhance the production of oxygen-derived free radicals,[27,28] which rapidly inactivate NO.[29] In patients with type-2 DM, the extent of urinary excretion of the isoprostanes (8-iso-prostaglandin $F_{2\alpha}$) significantly decreased ~4 weeks after an intensive therapy for hyperglycaemia, an intervention similar to that used in the present study.[30] Taking together a recent report that lowering serum TNF-α level alone (without glycaemic control) does not improve endothelial function,[31] these findings suggest that the hyperglycaemia-induced oxidative stress could be a key factor in the pathophysiology of diabetes.

HGF is characterized to be one of the most potent mitogens among the growth factors for vascular endothelial cells and contributes to vascular protection or repair.[13] Because its production is suppressed by glucose in a dose-dependent manner *in vitro*,[13] we hypothesized that endothelial dysfunction might be associated with the decreased production of HGF in diabetic patients. However, this was not the case. The level of HGF did not change throughout this study. Moreover, as shown in *Table 3*, insulin sensitivity, as assessed using HOMA-R index,[14] did not change significantly. Insulin resistance contributes, in part, to the pathogenesis of type-2 DM and may be potentially linked with endothelial dysfunction and ADMA.[32] To address this important issue, we need to further assess insulin sensitivity with a more specific method such as steady-state plasma glucose measurement.

Impaired endothelium is a key factor for diabetic macroangiopathy.[7] Thus, restoring endothelial function has important clinical implications for reducing the risk of cardiovascular diseases in diabetic patients. The present results, although obtained in a short period, suggest that a long-term maintenance of strict glycaemic control is important. If hyperglycaemia continues, then the expression level of NO synthase and the generation of NO may be chronically reduced, leading to a persistent dysfunction of the vascular endothelium and the consequent atherogenesis. In the UKPDS conducted for more than 15 years,[4] the difference in Hb A-1C level between the conventionally and intensively treated groups was significant throughout the study. However, Hb A-1C level progressively increased in both groups. The median Hb A-1C level was 6.6% in the first 5 years, but increased to 8.1% in the last 5 years, even in the intensively treated group. A difficulty in maintaining a good glycaemic control may explain, in part, the borderline decrease in the extent of myocardial infarction ($P = 0.05$) induced by the intensive treatment. Taking the multifactorial aetiology of macrovascular disease into account, the results of the UKPDS also suggest that the optimum treatment of patients with type-2 DM would include the control of blood pressure and correction of lipid abnormalities in addition to the control of glucose level. For the assessment of the effectiveness of therapeutic/dietary interventions and for the early detection of vascular dysfunction, plasma ADMA may be useful as a potential biochemical marker.[9,33] Metformin,[34] angiotensin-converting enzyme inhibitors/angiotensin II receptor blocker,[35] and statins[36] could decrease ADMA level. Although these drugs were not newly given in the present patients, it is possible that an increased utilization of and compliance with medications and an improved diet during hospitalization may contribute, at least in part, to endothelial function improvement. Insulin-sensitizing rosiglitazone also decreases ADMA level.[37] A recent study has suggested that obese and insulin resistance are not strongly associated with the development of type-2 DM in Japanese patients with a BMI of ~23 kg/m^2 (from the Japan Diabetes Complications Study), unlike in European patients with a BMI of ~29 kg/m^2 (from the UKPDS).[38]

In conclusion, in patients with type-2 DM, the intensive treatment of hyperglycaemia is associated with the improvement of endothelial dysfunction, coupled with decreases in TNF-α and ADMA levels. A strict glycaemic control may exert anti-cytokine and anti-atherogenic effects and may therefore be pathophysiologically important.

Acknowledgement

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Conflict of interest: none declared.

References

1. Haffner SM, Lehto S, Ronnemaa T, Pyorala K, Laakso M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* 1998;339:229-234.
2. Capes SE, Hunt D, Malmberg K, Gerstein HC. Stress hyperglycaemia and increased risk of death after myocardial infarction in patients with and without diabetes: a systematic overview. *Lancet* 2000;355:773-778.
3. Grundy SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, Mitch W, Smith SC Jr, Sowers JR. Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association. *Circulation* 1999;100:1134-1146.
4. UK Prospective Diabetes Study (UKPDS) Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 1998;352:837 853.
5. Williams SB, Goldfine AB, Timimi FK, Ting HH, Roddy MA, Simonson DC, Creager MA. Acute hyperglycemia attenuates endothelium-dependent vasodilation in humans *in vivo*. *Circulation* 1998;97:1695-1701.
6. Kawano H, Motoyama T, Hirashima O, Hirai N, Miyao Y, Sakamoto T, Kugiyama K, Ogawa H, Yasue H. Hyperglycemia rapidly suppresses flow-mediated endothelium-dependent vasodilation of brachial artery. *J Am Coll Cardiol* 1999;34:146 154.
7. Ross R. Atherosclerosis-an inflammatory disease. *N Engl J Med* 1999; 340:115-126.
8. Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. *J Clin Invest* 1997;100:2153-2157.
9. Cooke JP. Does ADMA cause endothelial dysfunction? *Arterioscler Thromb Vasc Biol* 2000;20:2032-2037.

10. Ito A, Tsao PS, Adimoolam S, Kimoto M, Ogawa T, Cooke JP. Novel mechanism for endothelial dysfunction: dysregulation of dimethylarginine dimethylaminohydrolase. *Circulation* 1999;99:3092-3095.
11. Moller DE. Potential role of TNF-alpha in the pathogenesis of insulin resistance and type 2 diabetes. *Trends Endocrinol Metab* 2000;11: 212-217.
12. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 1997;20:1183-1197.
13. Morishita R, Nakamura S, Nakamura Y, Aoki M, Moriguchi A, Kida I, Yo Y, Matsumoto K, Nakamura T, Higaki J, Ogihara T. Potential role of an endothelium-specific growth factor, hepatocyte growth factor, on endothelial damage in diabetes. *Diabetes* 1997;46:138-142.
14. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;28:412-419.
15. Shinozaki K, Suzuki M, Ikebuchi M, Takaki H, Hara Y, Tsushima M, Harano Y. Insulin resistance associated with compensatory hyperinsulinemia as an independent risk factor for vasospastic angina. *Circulation* 1995;92:1749-1757.
16. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972;18:499-502.
17. Pettersson A, Uggla L, Backman V. Determination of dimethylated arginines in human plasma by high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 1997;692:257-262.
18. Keen H, Clark C, Laakso M. Reducing the burden of diabetes: managing cardiovascular disease. *Diabetes Metab Res Rev* 1999;15:186-196.
19. Winkler G, Salamon F, Harmos G, Salamon D, Speer G, Szekeres O, Hajos P, Kovacs M, Simon K, Cseh K. Elevated serum tumor necrosis factor-alpha concentrations and bioactivity in type 2 diabetics and patients with android type obesity. *Diabetes Res Clin Pract* 1998;42(Suppl.):169-174.
20. Zinman B, Hanley AJ, Harris SB, Kwan J, Fantus IG. Circulating tumor necrosis factor-alpha concentrations in a native Canadian population with high rates of type 2 diabetes mellitus. *J Clin Endocrinol Metab* 1999;84:272-278.
21. Clausell N, Kalil P, Biolo A, Molossi S, Azevedo M. Increased expression of tumor necrosis factor-alpha in diabetic macrovasculopathy. *Cardiovasc Pathol* 1999;8:145-151.
22. Morohoshi M, Fujisawa K, Uchimura I, Numano F. Glucose-dependent interleukin 6 and tumor necrosis factor production by human peripheral blood monocytes *in vitro*. *Diabetes* 1996;45:954-959.
23. Wang P, Ba ZF, Chaudry IH. Administration of tumor necrosis factor-alpha *in vivo* depresses endothelium-dependent relaxation. *Am J Physiol* 1994;266:H2535-H2541.
24. Lin KY, Ito A, Asagami T, Tsao PS, Adimoolam S, Kimoto M, Tsuji H, Reaven GM, Cooke JP. Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase. *Circulation* 2002;106:987-992.
25. Calver A, Collier J, Leone A, Moncada S, Vallance P. Effect of local intra-arterial asymmetric dimethylarginine (ADMA) on the forearm arteriolar bed of healthy volunteers. *J Hum Hypertens* 1993;7:193-194.
26. Yoshizumi M, Perrella MA, Burnett JC Jr, Lee ME. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. *Circ Res* 1993;73:205-209.
27. Matsubara T, Ziff M. Increased superoxide anion release from human endothelial cells in response to cytokines. *J Immunol* 1986;137: 3295-3298.
28. Marfella R, Quagliaro L, Nappo F, Ceriello A, Giugliano D. Acute hyperglycemia induces an oxidative stress in healthy subjects. *J Clin Invest* 2001;108:635-636.
29. Gryglewski RJ, Palmer RM, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* 1986;320:454-456.
30. Davi G, Ciabattoni G, Consoli A, Mezzetti A, Falco A, Santarone S, Pennese E, Vitacolonna E, Bucciarelli T, Costantini F, Capani F, Patrono C. *In vivo* formation of 8-iso-prostaglandin f2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation. *Circulation* 1999;99:224-229.
31. Bilsborough W, O'Driscoll G, Stanton K, Weerasooriya R, Dembo L, Taylor R, Green D. Effect of lowering tumour necrosis factor-alpha on vascular endothelial function in type II diabetes. *Clin Sci* 2002;103:163-169.
32. Nash DT. Insulin resistance, ADMA levels, and cardiovascular disease. *JAMA* 2002;287:1451-1452.
33. Fard A, Tuck CH, Donis JA, Sciacca R, Di Tullio MR, Wu HD, Bryant TA, Chen NT, Torres-Tamayo M, Ramasamy R, Berglund L, Ginsberg HN, Homma S, Cannon PJ. Acute elevations of plasma asymmetric dimethylarginine and impaired endothelial function in response to a high-fat meal in patients with type 2 diabetes. *Arterioscler Thromb Vasc Biol* 2000;20:2039-2044.
34. Asagami T, Abbasi F, Stuelinger M, Lamendola C, McLaughlin T, Cooke JP, Reaven GM, Tsao PS. Metformin treatment lowers asymmetric dimethylarginine concentrations in patients with type 2 diabetes. *Metabolism* 2002;51:843-846.
35. Delles C, Schneider MP, John S, Gekle M, Schmieder RE. Angiotensin converting enzyme inhibition and angiotensin II AT1-receptor blockade reduce the levels of asymmetrical N(G), N(G)-dimethylarginine in human essential hypertension. *Am J Hypertens* 2002;15:590-593.
36. Lu TM, Ding YA, Leu HB, Yin WH, Sheu WH, Chu KM. Effect of rosuvastatin on plasma levels of asymmetric dimethylarginine in patients with hypercholesterolemia. *Am J Cardiol* 2004;94:157-161.
37. Stuhlinger MC, Abbasi F, Chu JW, Lamendola C, McLaughlin TL, Cooke JP, Reaven GM, Tsao PS. Relationship between insulin resistance and an endogenous nitric oxide synthase inhibitor. *JAMA* 2002;287:1420-1426.
38. Sone H, Ito H, Ohashi Y, Akanuma Y, Yamada N; Japan Diabetes Complication Study Group. Obesity and type 2 diabetes in Japanese patients. *Lancet* 2003;361:85.

Assessment of Genetic Effects of Polymorphisms in the MCP-1 Gene on Serum MCP-1 Levels and Myocardial Infarction in Japanese

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Background Recently, the Framingham Heart Study reported that genetic variations in *CCL2* influence serum levels of monocyte chemoattractant protein-1 (MCP-1) and the incidence of myocardial infarction (MI). The purpose of the present study was to investigate the possible involvement of *CCL2* in the pathogenesis of atherosclerosis and MI in Japanese.

Methods and Results Multiple regression analysis indicated that the MCP-1 levels were significantly influenced by various factors including age, body mass index, smoking, alcohol intake, high density lipoprotein-cholesterol, and systolic blood pressure. Moreover, the serum MCP-1 level was significantly correlated with intima–media thickness ($p<0.0001$). However, this association disappeared when other clinical confounding factors were included in the analyses. Comprehensive analysis of common polymorphisms of *CCL2* in a large community-based population and in subjects with MI found that the A(–2138)T polymorphism affected the serum MCP-1 level in a subgroup of subjects 65 years and older. However, no significant differences in the frequencies of any of the polymorphisms or haplotypes were found between subjects with and without MI. None of the polymorphisms in *CCL2* affected carotid atherosclerosis.

Conclusions The serum MCP-1 level was a good surrogate marker of atherosclerosis in the present study population. Although genetic variations in *CCL2* may have some influence on MCP-1 production, their influence does not seem to contribute appreciably to atherosclerosis in Japanese. The present results did not support the recently published findings from the Framingham Heart Study. The discrepancy between the 2 studies may be related to differences in confounding factors that contribute to MCP-1 levels and in the haplotype structure of the 2 populations. (*Circ J* 2006; **70:** 805–809)

Key Words: Atherosclerosis; Epidemiology; Monocyte chemoattractant protein-1; Myocardial infarction; Polymorphisms

Monocyte chemoattractant protein-1 (MCP-1; gene name *CCL2*) has been suggested to play an important role in the initiation of atherosclerosis by recruiting monocytes to sites of injured endothelium. MCP-1 promotes monocyte differentiation to lipid-laden macrophages, and also contributes to the proliferation of arterial smooth muscle cells![1–4]

In various murine models of atherosclerosis, deletion of *CCL2* has resulted in large reductions in atherosclerotic plaque size[5] but conversely, overexpression of MCP-1 in the leukocytes of susceptible mice resulted in increased plaque size[6]

Several human epidemiological studies have also suggested links between MCP-1 levels and atherosclerotic disease![7–10] Higher MCP-1 levels have been associated with increased risks of myocardial infarction (MI), sudden death, coronary angioplasty, and stent restenosis. Very recently, the Framingham Heart Study reported that *CCL2* polymor-

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Table 1 Characteristics of the Study Population

	Suita	*MI*	*p value*
n	2,266	342	
M (%)	46.0	87.1	<0.0001
Age	65.2 (11.0)	57.9 (9.9)	<0.0001
BMI	22.8 (3.1)	23.9 (2.9)	<0.0001
HTN (%)	38.7	53.4	<0.0001
DM (%)	9.4	40.4	<0.0001
TG	107 (71)	125 (69)*	0.0007
TC	209 (33)	197 (37)*	<0.0001
HDL-C	60 (16)	43 (13)*	<0.0001
Smoking	16.3	61.1	<0.0001
MCP-1	243 (958)**	–	
log (MCP-1)	5.23 (0.42)**	–	
IMT	0.79 (0.13)***	–	
MI	34 (1.5%)	342 (100%)	

Values are expressed as mean (SD).
**n=235, **n=2,180, ***n=2,035.*
MI, myocardial infarction; M, male subjects; BMI, body mass index (kg/m²); HTN, hypertensive subjects; DM, diabetes mellitus; TG, triglycerides (mg/dl); TC, total cholesterol (mg/dl); HDL-C, high density lipoprotein cholesterol (mg/dl); Smoking, current smokers; MCP-1, serum MCP-1 level (ng/ml); log (MCP-1), logarithmic transformation of MCP-1 level; IMT, intima media thickness (mm).

Table 2 Probes and Primers in TaqMan

Polymorphisms	Probe		Primer	
	VIC	FAM	Forward	Reverse
G(−2581)A	acagctGtcacttc	agacagctAtcacttc	ttccactcactctctcacgc	gacttggccttgcatatatcaga
A(−2138)T	ctctctctaatcTgttagtgcat	ctctctctaatcAgttagtgcat	ccgaagcatgactggattat	cctaggccatctcatctcatct
A(−1811)G	aaatggccActccatag	aatggccGctccata	cuaagcagggctcgagtg	cctgggactagacttgatgtctca
C(−972)G	cttagctgtCtgcccat	ttagctgtGtgcccatt	gcctcctaactcataatgacttagcc	ctctgtcctcagcatcttccaa
G(−928)C	aagcaGgcaactagt	ccaagcacgCaacta	tggaagatgctgaggacagaga	ggaaacgtgtacaagtcctccaa
C(7320112)G	atgagctcttCtcttct	tgagctcttGtcttct	tgaggtataggcagaagcactgg	aatgcaaaaggcaggcagga

Table 3 Summary of *CCL2* Polymorphisms

Polymorphism	Sequence	Region		Mi-AF
G(−2581)A	GACAGCT[G/A]TCACTTT	Promoter		0.332
G(−2411)C	CAAAGCT[G/C]GGAAGTT	Promoter		0.082
A(−2138)T	CACTAAC[T/A]GATTAGA	Promoter		0.049
A(−1811)T	AATGGCC[A/T]CTCCATA	Promoter		0.082
C(−972)G	TAGCTGT[C/G]TGCCCAT	Promoter		0.005
G(−928)C	CCAAGCA[G/C]GCAACTA	Promoter		0.049
C(−362)G	CGCTTCA[C/G]AGAAAGC	Promoter		0.332
C(7320112)G	GCTCTTT[C/G]TCTTCTC	Intron1		0.086
T(7320249)C	CCTGCTG[T/C]TATAACT	Exon2	Cys−>Cys	0.044
C(7320891)T	AGACACC[C/T]TGTTTTA	Exon3	3′−UTR	0.332

Mi-AF (minor allele frequency) was calculated based on the sequencing data of 93 subjects.

phisms are associated with serum MCP-1 levels and MI.[11] In genetic association studies, validation in other study populations is very important to confirm that the observed effects are not statistical errors, so the purpose of the present study was to assess the genetic effects of *CCL2* polymorphisms on serum MCP-1 levels and atherosclerosis in Japanese subjects.

Methods

Study Population

The selection criteria and design of the Suita study have been described previously.[12–14] The genotypes were determined in 2,266 subjects (including 34 MI subjects) recruited from the Suita study between September 2003 and March 2005. Serum MCP-1 levels were measured in 2,180 subjects. The MI group consisted of 342 randomly selected inpatients and outpatients with documented MI who were enrolled in the Division of Cardiology at the National Cardiovascular Center between May 2001 and April 2003.[15,16] All the subjects enrolled in the present study gave written informed consent. The present study was approved by the Ethics Committee of the National Cardiovascular Center and by the Committee on Genetic Analysis and Gene Therapy of the National Cardiovascular Center. The characteristics of the study population are shown in Table 1. Subjects with systolic blood pressure (SBP) ≥140 mmHg, diastolic blood pressure ≥90 mmHg, and/or who were taking antihypertensive medication were categorized as having hypertension. Subject with fasting blood glucose ≥126 mg/dl, hemoglobin A1c ≥6.5%, and/or who were being treated for diabetes mellitus was categorized as having the disease.

Fasting serum samples were collected and stored at −80°C. MCP-1 levels were measured in duplicate with a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The inter- and intra-assay variabilities were 6.3% and 6.2%, respectively. Because the distribution of serum MCP-1 levels was skewed, the values were logarithmically transformed in the statistical analysis.

The details of the method used for the carotid ultrasonic examination have been reported previously.[14] We used a high-resolution B-mode ultrasonic machine with 7.5-MHz transducers, which gave an axial resolution of 0.2 mm. The regions between 30 mm proximal from the beginning of the dilation of the bifurcation bulb and 15 mm distal from the flow divider of both common carotid arteries (CCAs) were scanned. All measurements were made at the time of scanning with the instrument's electronic caliper and were recorded as photocopies. The intima–media thickness (IMT) was measured on a longitudinal scan of the CCA at a point 10 mm proximal from the beginning of the dilation of the bulb.

DNA Study

The promoter (up to −2.8 kb) and exons 1, 2, and 3 (including 3'UTR) regions were sequenced in 93 subjects, which included the top 12 subjects with high serum MCP-1 levels and the bottom 12 subjects with low serum MCP-1 levels. The sequence primers will be provided on request. The genotypes were determined by the TaqMan method (Table 2). The success rate of genotyping was greater than 96%.

Statistical Analysis

Values are expressed as mean ± standard deviation (SD). All statistical analyses were performed with the JMP statistical package (SAS Institute Inc, Cary, NC, USA). Multiple regression analysis was performed to obtain predictors of the serum MCP-1 level and to assess the contribution of polymorphisms of *CCL2* to the serum MCP-1 level. Multiple logistic analysis was performed to obtain predictors for MI. Residuals of the serum MCP-1 level and IMT were calculated by adjusting for appropriate confounding factors. R-square values between polymorphisms and haplotype frequencies in the control and MI groups were analyzed using the SNPAlyze Pro statistical package (version 3.2, Dynacom Inc). A statistical power calculation was per-

Table 4 Linkage Disequilibrium Among the Polymorphisms of *CCL2*

	G(−2581)A	A(−2138)T	A(−1811)T	C(−972)G	G(−928)C	C(7320112)G
G(−2581)A						
A(−2138)T	0.12356					
A(−1811)T	0.16035	0.00565				
C(−972)G	0.02467	0.00086	0.00034			
G(−928)C	0.12411	0.97084	0.00582	0.00089		
C(7320112)G	0.15605	0.00546	0.00716	0.00108	0.00562	

Linkage disequilibrium (LD) among the polymorphisms of CCL2 was calculated from the TaqMan data of the Suita subjects. R-square values between polymorphisms are shown. Tight LD was observed between the A(−2581)T and G(−928)C polymorphisms.

Table 5 Predictors of Serum MCP-1 Level

Predictor	t-ratio	p value
Age	7.9	<0.0001
BMI	−3.21	0.0014
SBP	2.42	0.0155
Alcohol	2.71	0.0067
Smoking	3.36	0.0008
HDL-C	−2.59	0.0096

Predictors of serum MCP-1 levels were identified by multiple regression analysis (n=2,180). Alcohol, ethanol consumption per day (g/day); Smoking, number of cigarettes per day X years.
SBP, systolic blood pressure. See Table 1 for other abbreviations.

Table 6 Predictors of Intima-Media Thickness

Predictor	t-ratio	p value
log (MCP-1)	0.13	0.7191
Age	353.82	<0.0001
SBP	29.67	<0.0001
Sex	33.21	<0.0001
BMI	33.45	<0.0001

n=2,034, F=128.197, p<0.0001.
The serum MCP-1 levels were assessed in 2,034 of the 2,035 subjects assessed by carotid sonography.
See Tables 1,5 for abbreviations.

Table 7 Influence of the Polymorphisms of *CCL2* on Serum MCP-1 Level

	AA	Aa	aa	p value
G(−2581)A	0.002 (0.399)	0.004 (0.418)	−0.020 (0.387)	0.692
n	936	961	270	
A(−2138)T	−0.006 (0.402)	0.049 (0.436)	−0.054 (0.211)	0.122 (0.052)
n	1,909	253	7	
A(−1811)T	0.006 (0.407)	−0.031 (0.406)	−0.079 (0.292)	0.268 (0.117)
n	1,839	313	13	
C(−972)G	−0.001 (0.406)	0.045 (0.404)	–	0.409
n	2,111	54		
G(−928)C	−0.006 (0.403)	0.048 (0.430)	−0.054 (0.211)	0.123 (0.052)
n	1,896	262	7	
C(7320112)G	0.004 (0.401)	−0.013 (0.023)	−0.163 (0.108)	0.259 (0.349)
	1,840	311	14	
A(−2138)T	−0.012 (0.351)	0.081 (0.462)	−0.051 (0.153)	0.0126 (0.0041)
Age ≥65 years	1,041	154	4	
G(−928)C	−0.012 (0.351)	0.081 (0.500)	−0.051 (0.153)	0.0124 (0.0040)
Age ≥65 years	1,035	156	4	

Residuals of log (MCP-1) were calculated by adjusting for Age, BMI, SBP, alcohol, smoking, and HDL-C. Values are expressed as mean (SD). p values calculated by grouping AA/Aa + aa are shown in parentheses. The effects of the A(−2138)T and G(−928)C polymorphisms on the MCP-1 level were more significant in subjects aged 65 years and older.
See Tables 1,5 for abbreviations.

formed with the statistical package SamplePower (version 2.0, SPSS, Chicago, IL, USA).

Results

Sequence Analysis of CCL2

Sequence analyses in 93 subjects revealed the existence of 10 polymorphisms (Table 3) of *CCL2*. The G(−2581)A was in almost complete linkage disequilibrium (LD) with the C(−362)G and C(7320891)T polymorphisms. The A(−1811)G polymorphism was in almost complete LD with the G(−2411)C polymorphism. Thus, the genotypes of the C(−362)G, C(7320891)T, and G(−2411)C polymorphisms were not determined in the present study. Because the polymorphism in exon 2 [T(7320249)C] was synonymous (Cys−>Cys), this polymorphism was also not determined in the present study. The genotypes of the remaining 6 polymorphisms were determined by the TaqMan method in a total of 2,570 subjects. The LD values calculated from R-square values among these SNPs are shown in Table 4.

Clinical Correlates of Serum MCP-1 Level

Multiple regression analysis indicated that the MCP-1 level was significantly influenced by various factors (p< 0.0001, R-square=0.054) including age (p<0.0001), body mass index (BMI; p=0.0014), smoking (p=0.0008), alcohol intake (p=0.0067), high-density lipoprotein cholesterol (p=0.0096), and SBP (p=0.0155) (Table 5).

Many studies have reported that the serum MCP-1 level is an excellent indicator of atherosclerosis and in our study population the serum MCP-1 level significantly correlated with IMT (p<0.0001, R-square=0.009). However, this association disappeared when other clinical confounding factors were included in the multiple regression analyses (Table 6).

Table 8 *CCL2* Polymorphisms and Incidence of MI

	MI (−)			MI			p value
	AA	Aa	aa	AA	Aa	aa	
G(−2581)A (%)	946 (43.35)	966 (44.13)	274 (12.52)	149 (40.93)	176 (48.53)	39 (10.71)	0.2857
A(−2138)T (%)	1,931 (88.25)	250 (11.43)	7 (0.32)	218 (87.36)	45 (12.36)	1 (0.27)	0.8686 [0.6289]
A(−1811)T (%)	1,861 (85.02)	314 (14.34)	14 (0.64)	304 (83.29)	56 (15.34)	5 (1.37)	0.3337 [0.3999]
C(−972)G (%)	2,130 (97.53)	54 (2.47)		357 (98.08)	7 (1.92)		0.5548
G(−928)C (%)	1,918 (87.82)	259 (11.86)	7 (0.32)	319 (87.40)	45 (12.33)	1 (0.27)	0.9578 [0.8200]
C(7320112)G (%)	1,855 (84.94)	315 (14.42)	14 (0.64)	302 (82.74)	61 (16.71)	2 (0.55)	0.5229 [0.2880]

Genotype frequencies between subjects with and without MI are shown. p values calculated by grouping AA/Aa + aa are shown in square parentheses.
See Table 1 for abbreviation.

Table 9 Influence of *CCL2* Polymorphisms on IMT

	AA	Aa	aa	p value
G(−2581)A	−0.003 (0.104)	0.001 (0.105)	0.006 (0.115)	0.421
n	865	908	255	
A(−2138)T	0.000 (0.106)	−0.001 (0.103)	0.065 (0.118)	0.319
n	1,784	237	6	(0.958)
A(−1811)T	0.000 (0.105)	0.003 (0.112)	−0.049 (0.068)	0.227
n	1,717	294	12	(0.752)
C(−972)G	0.000 (0.106)	0.000 (0.113)	–	0.964
n	1,970	53		
G(−928)C	0.000 (0.106)	−0.003 (0.103)	0.065 (0.118)	0.291
n	1,771	246	6	(0.802)
C(7320112)G	−0.001 (0.106)	0.005 (0.101)	0.039 (0.159)	0.275 (0.278)

Residuals of IMT were calculated by adjusting for sex, age, BMI, and SBP. Values are expressed as mean (SD). p values calculated by grouping AA/Aa + aa are shown in parentheses.
See Tables 1,5 for abbreviations.

Table 10 Haplotype Analysis of the 2 Study Populations

Suita	Framingham	G(−2581)A	A(2138)T	A(−1811)G	G(−928)C	C7320112G	MI (−)	MI	Framingham
Haplo1	H1	G	A	A	G	C	65.2	65.0	27.0
Haplo2	H4 + H5	A	A	A	G	C	13.2	10.9	26.9
Haplo3	H6	A	A	G	G	C	7.8	9.0	4.2
Haplo4	–	A	A	A	G	G	7.5	8.7	–
Haplo5	–	A	T	A	C	C	6.1	6.5	–
–	H2	A	T	A	G	G	<0.01	<0.01	20.3
–	H3	A	A	A	C	C	<0.01	<0.01	18.6

Haplotype frequencies in the MI (−) and MI groups were calculated. Haplotype frequencies reported in the Framingham study are also shown for reference.
See Table 1 for abbreviation.

Thus, the serum MCP-1 level was only a surrogate marker of atherosclerosis in the present study population.

Influence of Polymorphisms on Serum MCP-1 Level

Next, we examined the influence of polymorphisms of *CCL2* on residuals of the MCP-1 level after adjusting for the above-mentioned confounding factors (Adj-MCP1) (Table 7). Two polymorphisms, A(−2138)T and G(−928)C, tended to affect Adj-MCP1. The A(−2138)T and G(−928)C polymorphisms were in tight LD (R-square=0.97084) in this study population (Table 4). Interestingly, the influence of these polymorphisms on Adj-MCP1 seemed to be exaggerated in subjects 65 years and older whose MCP-1 levels were significantly higher than those of younger subjects.

Association Study Between CCL2 Polymorphisms and MI

No significant difference was found in the frequencies of any of the polymorphisms between the cases and controls (Table 8). Multiple logistic analyses including age and BMI indicated that none of the polymorphisms contributed to MI. Moreover, none of them affected IMT after adjusting for sex, age, SBP, and BMI (Table 9).

Haplotype Analysis

We constructed haplotypes based on the G(−2581)A, A(−2138)T, A(−1811)T, G(−928)C, and C7320112G polymorphisms and identified 5 common haplotypes that accounted for 99.7% of all haplotypes. The C(−972)G polymorphism was not included because of its low frequency. No significant difference was observed in haplotype fre-

quencies between subjects with and without MI (Table 8).

The haplotype frequencies reported in the Framingham study[11] were significantly different from those in the present study population (Table 10). Although H2 and H3, which accounted for 20.3% and 18.6%, respectively, in the Framingham study, were very rare in this study population, Haplo4 and 5, which were rare in the Framingham study, were common.

Discussion

This report describes a comprehensive analysis of the common polymorphisms of *CCL2* in both a large community-based population and subjects with MI. No significant differences in the frequencies of any of the polymorphisms were found between cases and controls. Moreover, none of the polymorphisms of *CCL2* affected carotid atherosclerosis as assessed by IMT. However, the A(−2136)T and G(−928)C polymorphisms tended to affect the serum MCP-1 level. Although genetic variations in *CCL2* may have some influence on MCP-1 production, their does not seem to contribute appreciably to atherosclerosis in Japanese subjects. Thus, our findings do not support the recently published result from the Framingham Heart Study[11] that genetic variations in *CCL2* significantly influence serum MCP-1 levels and the incidence of MI.

There may be several reasons for this discrepancy. The MCP-1 levels in the Framingham Heart Study were approximately 1.4-fold higher than those in the present study population. Genetic variation might well have an influence under a stimulated state. MCP-1 levels are influenced by various factors, as described in Table 5. It is conceivable that subjects in the Framingham Heart Study may have had higher MCP-1 levels because of stimulation by atherogenic factors that may be more prevalent in Caucasians. Indeed, the influence of genetic variations was more evident in the present study population when the analysis was limited to older subjects who had higher MCP-1 levels (Table 7).

In the Framingham Heart Study, the haplotype H2 was reported to contribute to higher MCP-1 levels, and the frequency of this haplotype was 20.3%[11] It is defined by the (−2138)T and (77320112)G genotypes, and although the A(−2138)T and G(7320112)C polymorphisms were observed in the present study population, the H2 haplotype was not ($p<0.01\%$). This difference in the haplotype structure between Caucasians and Japanese might also contribute to the discrepancy between the 2 studies.

The reported positive association between the A(−2581)T polymorphism and MI in the Framingham Heart Study was based on 1,797 study subjects, including just 107 MI subjects[11] which was insufficient statistical power ($p<0.50$) to conclude that there was a positive association between the genotype and MI. Moreover, although the H2 haplotype was reported to be associated with the serum MCP-1 level, the H1 haplotype but not the H2 haplotype, was reported to be associated with MI. This inconsistency might also indicate that the Framingham study had insufficient statistical power.

Although the serum MCP-1 level is an excellent indicator of atherosclerosis[7–10] MCP-1 itself appears to make only a slight contribution to atherosclerosis (Table 6). Thus, it is unlikely that genetic polymorphisms that may only slightly influence the serum MCP-1 level will contribute significantly to the occurrence of MI and atherosclerosis. Our present findings suggest that, although genetic variations in *CCL2* may have some influence on MCP-1 production, their influence on the incidence of MI is not appreciable in Japanese. The present study also indicates the importance of clarifying the haplotype structure for comparing genetic association studies involving different ethnic backgrounds.

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References

1. Yla-Herttuala S, Lipton BA, Rosenfeld ME, Sarkioja T, Yoshimura T, Leonard EJ, et al. Expression of monocyte chemoattractant protein-1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc Natl Acad Sci USA* 1991; **88:** 5252–5256.
2. Yu X, Dluz S, Graves DT, Zhang L, Antoniades HN, Hollander W, et al. Elevated expression of monocyte chemoattractant protein-1 by vascular smooth muscle cells in hypercholesterolemic primates. *Proc Natl Acad Sci USA* 1992; **89:** 6953–6957.
3. Nelken NA, Coughlin SR, Gordon D, Wilcox JN. Monocyte chemoattractant protein-1 in human atheromatous plaques. *J Clin Invest* 1991; **88:** 1121–1127.
4. Viedt C, Vogel J, Athanasiou T, Shen W, Orth SR, Kubler W, et al. Monocyte chemoattractant protein-1 induces proliferation and interleukin-6 production in human smooth muscle cells by differential activation of nuclear factor-*k*B and activator protein-1. *Arterioscler Thromb Vasc Biol* 2002; **22:** 914–920.
5. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, et al. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* 1998; **2:** 275–281.
6. Aiello RJ, Bourassa PA, Lindsey S, Weng W, Natoli E, Rollins BJ, et al. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 1999; **19:** 1518–1525.
7. de Lemos JA, Morrow DA, Sabatine MS, Murphy SA, Gibson CM, Antman EM, et al. Association between plasma levels of monocyte chemoattractant protein-1 and long-term clinical outcomes in patients with acute coronary syndromes. *Circulation* 2003; **107:** 690–695.
8. Cipollone F, Marini M, Fazia M, Pini B, Iezzi A, Reale M, et al. Elevated circulating levels of monocyte chemoattractant protein-1 in patients with restenosis after coronary angioplasty. *Arterioscler Thromb Vasc Biol* 2001; **21:** 327–334.
9. Oshima S, Ogawa H, Hokimoto S, Nakamura S, Noda K, Saito T, et al. Plasma monocyte chemoattractant protein-1 antigen levels and the risk of restenosis after coronary stent implantation. *Jpn.Circ J* 2001; **65:** 261–264.
10. Deo R, Khera A, McGuire DK, Murphy SA, de PMNJ, Morrow DA, et al. Association among plasma levels of monocyte chemoattractant protein-1, traditional cardiovascular risk factors, and subclinical atherosclerosis. *J Am Coll Cardiol* 2004; **44:** 1812–1818.
11. McDermott DH, Yang Q, Kathiresan S, Cupples LA, Massaro JM, Keaney JF Jr, et al. CCL2 polymorphisms are associated with serum monocyte chemoattractant protein-1 levels and myocardial infarction in the Framingham Heart Study. *Circulation* 2005; **112:** 1113–1120.
12. Kokubo Y, Iwai N, Tago N, Inamoto N, Okayama A, Yamawaki H, et al. Association analysis between hypertension and *CYBA, CLCNKB,* and *KCNMB1* functional polymorphisms in the Japanese population: The Suita study. *Circ J* 2005; **69:** 138–142.
13. Iwai N, Katsuya T, Mannami T, Higaki J, Ogihara T, Kokame K, et al. Association between SAH, an acyl-CoA synthetase gene, and hypertriglyceridemia, obesity, and hypertension. *Circulation* 2002; **105:** 41–47.
14. Mannami T, Konishi M, Baba S, Nishi N, Terao A. Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city: The Suita study. *Stroke* 1997; **28:** 518–525.
15. Kajimoto K, Shioji K, Ishida C, Iwanaga Y, Kokubo Y, Tomoike H, et al. Validation of the association between the gene encoding 5-lipoxygenase-activating protein and myocardial infarction in a Japanese population. *Circ J* 2005; **69:** 1029–1034.
16. Kajimoto K, Shioji K, Tago N, Tomoike H, Miyazaki S, Nonogi H, et al. Assessment of MEF2A mutations in myocardial infarction in Japanese patients. *Circ J* 2005; **69:** 1192–1195.

Enhanced cardiac production of matrix metalloproteinase-2 and -9 and its attenuation associated with pravastatin treatment in patients with acute myocardial infarction

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ABSTRACT

Previous experimental studies have demonstrated that MMPs (matrix metalloproteinases) contribute to LV (left ventricular) remodelling. We hypothesized that cardiac MMPs are activated in patients with AMI (acute myocardial infarction) and, if so, MMP production may be attenuated by statins (3-hydroxy-3-methylglutaryl-CoA reductase inhibitors) through their cardiovascular protective actions. We studied 30 patients, ten control patients with stable angina pectoris and 20 patients with AMI, in whom LV catheterization at the chronic stage was performed 22 ± 12 days (value is mean ± S.D.) after the onset of AMI. Blood samples were collected from the CS (coronary sinus) and a peripheral artery. In patients with AMI, the levels of MMP-2 and MMP-9 were significantly ($P < 0.05$) higher in the CS than the peripheral artery (MMP-2, 853 ± 199 compared with 716 ± 127 ng/ml; MMP-9, 165 ± 129 compared with 98 ± 82 ng/ml), whereas no significant differences were observed in the patients with angina pectoris. The CS–arterial concentration gradients of MMP-2 and MMP-9 correlated positively with BNP (brain natriuretic peptide) levels (MMP-2, $R = 0.68$, $P < 0.01$; MMP-9, $R = 0.59$, $P < 0.05$) and LV end-diastolic volume index (MMP-2, $R = 0.70$, $P < 0.01$; MMP-9, $R = 0.70$, $P < 0.01$). When patients with AMI treated with 10 mg of pravastatin or without ($n = 10$ in each group) were compared, this statin therapy significantly ($P < 0.05$) decreased the CS–arterial concentration gradients of MMP-2 (69 ± 43 compared with 213 ± 185 ng/ml) and MMP-9 (14 ± 27 compared with 119 ± 84 ng/ml). In conclusion, the enhanced production of cardiac MMP-2 and MMP-9 is associated with LV enlargement and elevated BNP levels in patients with AMI. A pleiotropic effect of statins appears to be associated with the modulation of cardiac MMP activation, which may be potentially beneficial in the attenuation of post-infarction LV remodelling.

Key words: acute myocardial infarction, angina pectoris, brain natriuretic peptide (BNP), metalloproteinase (MMP), remodelling, statin, tissue inhibitor of metalloproteinases (TIMP).

Abbreviations: ACE-I, angiotension-converting enzyme inhibitor; AMI, acute myocardial infarction; Ang II, angiotensin II; AP, angina pectoris; BNP, brain natriuretic peptide; CK, creatine kinase; CRP, C-reactive protein; CS, coronary sinus; LDL, low-density lipoprotein; LV, left ventricular; LVEDVI, LV end-diastolic volume index; LVEF, LV ejection fraction; MMP, matrix metalloproteinase; TGF-β, transforming growth factor-β; TIMP, tissue inhibitor of metalloproteinases; WBC, white blood cell.

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INTRODUCTION

The loss of myocytes as a consequence of AMI (acute myocardial infarction) results in progressive changes in ventricular architecture [1,2]. This process, defined as post-infarction ventricular remodelling, is associated with a higher mortality and a higher incidence of complications, such as the development of heart failure, aneurysm formation and ventricular rupture [3,4]. During the remodelling process, as well as intrinsic changes in cardiac myocytes, it has been recognized that important alterations also occur within the extracellular matrix of the myocardium [5,6].

MMPs (matrix metalloproteinases) belong to a family of zinc-containing endoproteinases responsible for extracellular protein degradation, and are inhibited by specific tissue inhibitors [TIMP (tissue inhibitor of metalloproteinases)] [5,6]. In experimental myocardial infarction, MMPs are up-regulated in myocardial tissues, and are the driving force in extracellular matrix remodelling and infarct expansion [7,8]. Among the MMPs, the importance of MMP-9 during the processes of infarct healing and LV (left ventricular) remodelling has been demonstrated in previous studies using genetically modified mice [9,10]. Infarcted mice with the targeted deletion of MMP-9 had a decreased incidence of early myocardial rupture [9] and progressive LV dilation [10]. However, in the clinical setting, there has been little evidence regarding the production of MMPs in the infarcted human heart.

Statins have various cardiovascular protective actions, including anti-inflammatory and anti-apoptotic actions, independent of their effects on cholesterol levels. A study using a mouse AMI model demonstrated that statin treatment attenuated LV remodelling [11], which was associated with decreased MMP activity [12].

In the present study, we hypothesized that cardiac MMP activation may be associated with the degree of LV enlargement and the level of BNP (brain natriuretic peptide), a biochemical marker of post-infarction remodelling [13,14]. If so, MMP production may be attenuated by statin treatment in patients with AMI.

MATERIALS AND METHODS

Patients

This study included 30 male patients. All of the patients gave their written informed consent prior to participation in the study. The Institutional Ethical Committee on Human Research approved the study protocol. Patients with the following disorders were excluded from the study: prior myocardial infarction, and liver (elevated activities of aminotransferases), kidney (elevated level of creatinine or urea) or lung dysfunction (restrictive or obstructive pattern in spirometry).

The control group consisted of ten patients with stable AP (angina pectoris), who complained of symptoms consistent with Canadian Cardiovascular Society Classification of angina level I, II or III, with evidence of myocardial ischaemia. All of the control patients had no evidence of a previous AMI, and had severe coronary artery stenosis and therefore underwent coronary angioplasty (with adjunctive stenting in five patients). The treated sites were the left anterior descending artery in four patients (40 %), the right or left circumflex artery in four patients (40 %), and both the left anterior descending and right coronary arteries in two patients (20 %).

We also studied 20 patients with AMI who fulfilled the following criteria: typical chest pain > 30 min of duration, ST segment elevation > 0.1 mV in two or more ECG leads with the subsequent evolution of a typical infarct pattern, and increased serum CK (creatine kinase) level. A total of 14 patients underwent PTCA (percutaneous transluminal coronary angioplasty) of the infarct-related artery (with adjunctive stenting in nine patients), and the remaining six patients received an intravenous administration of a tissue-type plasminogen activator and/or heparin in the acute phase. In all the patients, coronary angiography immediately after treatment showed a TIMI 3 grade flow in the infarct-related artery. The elapsed time to reperfusion was 4.6 h on average. The infarct sites were in the anterior wall in ten patients (50 %), the inferior wall in seven patients (35 %) and the postero-lateral wall in three patients (15 %). In this study, all of the patients with AMI were treated with the ACE-I (angiotension-converting enzyme inhibitor) enalapril (5 mg) after their hospital admission. Among them, ten patients with hyperlipidaemia (total cholesterol level > 220 mg/dl) were treated with 10 mg of pravastatin; the remaining ten patients did not have hyperlipidaemia and thus did not receive pravastatin. A recent Management of Elevated Cholesterol in the Primary Prevention Group of Adult Japanese (MEGA) trial [14a] has shown a similar decrease in coronary artery disease incidence following treatment with 10–20 mg of pravastatin used in Asia to that observed for 20–40 mg doses used in Europe and the United States.

Cardiac catheterization and analysis of LV function

In patients with AMI, chronic-stage cardiac catheterization was repeated approx. 3–4 weeks after the onset of AMI. A 5 French multipurpose catheter (Cathex) was introduced into the CS (coronary sinus) through the left subclavian vein under fluoroscopic guidance [14]. The position of the catheter tip was confirmed by the injection of contrast medium. Blood samples were collected from the CS before the intravenous administration of heparin. Following the collection of blood samples from the right brachial artery (as peripheral blood samples) through a 6 French sheath, heparin was administered and coronary

angiography and left ventriculography were performed, according to the conventional Judkins' technique. LV pressure was measured using a 2 French high-fidelity micromanometer catheter (Miller Instruments) advanced into the left ventricle via the lumen of a 6 French pig-tail catheter. The restenosis of a treated artery was defined as an arterial narrowing of $>75\%$, as determined by coronary angiography.

LV volume was evaluated angiographically by a cardiologist who was blinded to the results of the biochemical assays. Ventricular silhouettes in a 30° right anterior oblique projection were digitized using an ANCHOR ventriculography analysis system (Siemens-Elema). Using the area-length method, LV end-systolic volume index, LVEDVI (LV end-diastolic volume index) and LVEF (LV ejection fraction) were calculated.

Biochemical assessment

Blood samples were centrifuged and serum was stored at −80 °C until assay. A sandwich enzyme immunoassay was performed to determine MMP-2 level (Fuji Chemical Industries) [15]. In addition, the level of MMP-9, another gelatinase-like MMP-2, and that of MMP-13, an interstitial collagenase, were analysed using MMP Biotrak enzyme-linked immunoadsorbent assay kits (Amersham Biosciences). The levels were back-calculated from the standard curve determined with the enzyme-linked immunoadsorbent assay kits using a 96-well microplate reader (Emax; Molecular Devices). These kits detect the pro-enzyme and the pro-enzyme complexed with TIMP. The detection limits were 0.5 ng/ml for MMP-2, 0.6 ng/ml for MMP-9 and 0.03 ng/ml for MMP-13.

We also measured levels of TIMP-1 (Fuji Chemical Industries) and TIMP-2 (Amersham Biosciences) using sandwich enzyme immunoassays [15]. The detection limits for TIMP-1 and TIMP-2 were 1.2 and 8.0 ng/ml respectively.

BNP was measured using specific immunoradiometric assay kits (Shionogi). The sensitivity of these kits was 2 pg/ml. Ang II (angiotensin II) and TGF-β (transforming growth factor-β) levels were also measured, as reported previously [16].

The serum CRP (C-reactive protein) level was measured by N Latex CRP II monoassay using a nephelometric analyser (BN II; Dade Behring). The lower detection limit of this test was 0.06 mg/dl. Total cholesterol, triacylglycerol (triglyceride) and HDL (high-density lipoprotein) cholesterol concentrations were determined by enzymatic methods using a Toshiba TBA 80M analyser. LDL (low-density lipoprotein) was calculated using Fredewald's formula. We also measured WBC (white blood cell) number.

Statistical analysis

The two groups were compared by Student's t test. Measurements from the CS and the peripheral artery were compared within a group by ANOVA. When a significant difference among groups was indicated by the initial analysis, individual paired comparisons were determined using the Student–Newman–Keuls method. A linear regression line was calculated by the least-square method to assess the correlation between two parameters. To investigate independent predictors, we used multivariate logistic regression analysis. In all cases, differences were considered significant at $P < 0.05$. Results are presented as means ± S.D., or medians.

Table 1 Clinical characteristics

*$P = 0.05$ and **$P < 0.01$ compared with control (patients with stable AP).

Characteristic	Patients with AMI ($n = 20$)	Patients with stable AP ($n = 10$)
Age (years)	66 ± 9	67 ± 6
Peak CK (units/l)	1986 (801–8574)	–
Cardiac function		
LVEF (%)	48 ± 7**	58 ± 7
LVEDVI (ml/m^2)	95 ± 18**	55 ± 21
Vessels > 75 % stenosed (n)	1.5 ± 0.7	1.6 ± 0.7
Risk factors (n)		
Hypertension	11 (55 %)	7 (70 %)
Diabetes mellitus	15 (75 %)	6 (60 %)
Hyperlipidaemia	10 (50 %)	6 (60 %)
Smoking	12 (60 %)	6 (60 %)
Biochemical parameters†		
Total cholesterol (mg/dl)	193 ± 27	198 ± 20
LDL (mg/dl)	120 ± 30	122 ± 31
WBC count (cells/μl)	6615 ± 1571	5600 ± 1063
CRP (mg/dl)	0.34 ± 0.33*	0.13 ± 0.06
Medication used (n)		
ACE-I	20 (100 %)	4 (40 %)
β-Blockers	11 (55 %)	6 (60 %)
Statins	10 (50 %)	6 (60 %)
Calcium antagonists	7 (35 %)	5 (50 %)
Nitrates	4 (20 %)	2 (20 %)
Aspirin	20 (100 %)	10 (100 %)

† Data obtained on the day when cardiac catheterization was performed.

RESULTS

The baseline clinical characteristics of the patients with AMI and the control patients with AP (without evidence of AMI) are summarized in Table 1. In the patients with AMI, cardiac function data were obtained at chronic-stage cardiac catheterization performed 22 ± 12 days after the onset of AMI. Coronary angiography revealed 90 % stenosis of the infarct-related artery in two patients and 100 % stenosis in three patients. These five patients with restenosis had received intravenous thrombolysis alone in the acute stage. In the remaining 15 patients, the treated

Table 2 Comparisons of BNP, MMP and TIMP levels in the CS and peripheral artery

*$P < 0.05$ compared with levels in artery; †$P < 0.05$ compared with control (patients with stable AP).

	Patients with AMI ($n = 20$)		Patients with stable AP ($n = 10$)	
Peptide	CS	Artery	CS	Artery
BNP (pg/ml)	400 ± 376*†	126 ± 176	54 ± 25	52 ± 25
MMP-2 (ng/ml)	853 ± 199*†	716 ± 127	631 ± 44	630 ± 46
MMP-9 (ng/ml)	165 ± 129*†	98 ± 82	68 ± 25	71 ± 24
MMP-13 (ng/ml)	0.05 ± 0.04	0.05 ± 0.02	0.04 ± 0.02	0.04 ± 0.02
TIMP-1 (ng/ml)	155 ± 59	150 ± 53	130 ± 33	134 ± 32
TIMP-2 (ng/ml)	112 ± 18	108 ± 14	94 ± 11	97 ± 16

sites remained patent. With the exception of cardiac function (LVEF and LVEDVI) and the prevalence of ACE-I use, clinical characteristics were similar between patients with AMI and AP.

Enhancement of cardiac MMP production in patients with AMI

Table 2 shows the comparison of BNP, MMP and TIMP levels between blood samples from the CS and peripheral artery. In patients with AMI, levels of BNP, MMP-2 and MMP-9 were significantly ($P < 0.05$) higher in the CS than in the peripheral artery, whereas the levels of MMP-13, TIMP-1 and TIMP-2 were similar. In control patients with AP, no significant differences in the levels of BNP, MMPs and TIMPs were observed between the CS and peripheral artery. These findings indicate that the production of MMP-2 and MMP-9, as well as that of BNP, is enhanced in an infarcted heart.

Correlation of cardiac MMP production with post-infarction LV remodelling

In patients with AMI, the CS–arterial concentration gradients of MMP-2 and MMP-9 correlated positively with those of BNP and LVEDVI respectively (Figure 1), but not with LVEF, peak CK level and circulating WBC counts. These myocardial gradients were not different between patients with and without progression to restenosis (MMP-2, 87 ± 32 compared with 152 ± 173 ng/ml; MMP-9, 83 ± 86 compared with 61 ± 82 ng/ml).

Comparisons between pravastatin-treated patients with AMI and non-pravastatin-treated patients with AMI

We then compared levels of MMPs between ten patients treated with 10 mg of pravastatin and ten patients not treated with pravastatin (Table 3). Although the total cholesterol level before treatment was higher ($P < 0.05$) in the pravastatin-treated patients with AMI (223 ± 7 mg/dl in treated patients compared with 195 ± 17 mg/dl in

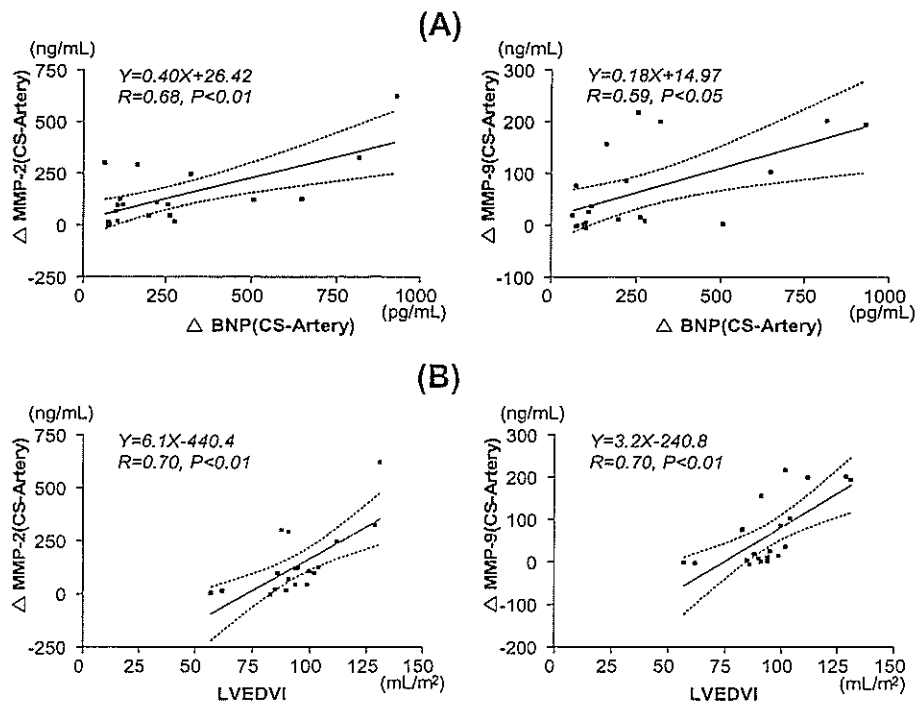

Figure 1 Correlations between CS–arterial concentration gradients of MMP-2 and -9 and BNP (A) and LVEDVI (B) in 20 patients with AMI

Table 3 Comparisons of MMPs between pravastatin-treated and non-pravastatin-treated patients

*$P < 0.05$ compared with levels in artery; †$P < 0.05$ compared with levels in non-pravastatin-treated patients. CS–artery, CS–arterial concentration gradient.

	Patients with AMI						Patients with stable AP					
	Pravastatin-treated ($n = 10$)			Non-pravastatin-treated ($n = 10$)			Pravastatin-treated ($n = 6$)			Non-pravastatin-treated ($n = 4$)		
MMP (ng/ml)	CS	Artery	CS–artery	CS	Artery	CS–artery	CS	Artery	CS–artery	CS	Artery	CS–artery
MMP-2	808 ± 182	739 ± 158	69 ± 43†	897 ± 216*	684 ± 84	213 ± 185	631 ± 53	624 ± 51	7 ± 23	629 ± 32	639 ± 43	−9 ± 53
MMP-9	94 ± 61†	80 ± 59	14 ± 27†	236 ± 142*	117 ± 100	119 ± 84	68 ± 20	72 ± 16	−4 ± 4	68 ± 20	69 ± 29	0 ± 5
MMP-13	0.06 ± 0.06	0.03 ± 0.03	0.03 ± 0.06	0.03 ± 0.02	0.05 ± 0.03	−0.01 ± 0.03	0.03 ± 0.04	0.04 ± 0.02	−0.01 ± 0.01	0.04 ± 0.02	0.03 ± 0.02	0.01 ± 0.03

non-treated patients), no significant differences were observed after treatment between the two groups (183 ± 31 mg/dl in treated patients compared with 201 ± 20 mg/dl in non-treated patients). Levels of CRP (0.18 ± 0.13 mg/dl in treated patients compared with 0.50 ± 0.40 mg/dl in non-treated patients; $P = 0.03$) and the CS–arterial concentration gradients of MMP-2 and MMP-9 (Table 3) were significantly different between the two groups. However, the concentration gradients of TGF-β and Ang-II were similar between patients treated with pravastatin and those not treated (Ang-II, 19.5 ± 20.2 compared with 36.9 ± 32.4 pg/ml respectively; TGF-β, 1.2 ± 3.3 compared with 2.1 ± 4.7 pg/ml respectively).

We then performed multivariate analysis for the predictors of CS–arterial concentration gradients of MMP levels, including age, sex, coronary risk factors, peak CK, infarct site (anterior wall), CRP, TIMP, pravastatin treatment, LVEF and LVEDVI. The association between pravastatin treatment and cardiac MMP-2 production was modest, with an odds ratio of 0.074 (95 % confidence interval, 0.005–1.109; $P = 0.06$), and did not reach statistical significance.

DISCUSSION

The major findings of the present clinical study are that after AMI, the cardiac production of MMP-2 and MMP-9 is enhanced and associated with LV enlargement and BNP secretion, and that the pleiotropic effect of statins appears to be associated with the modulation of cardiac MMP activation.

Among the MMP species, MMP-2 and MMP-9 play an important role in LV remodelling, as these MMPs are activated in the myocardium and it has been reported that the targeted deletion of these MMPs prevents post-infarction cardiac dysfunction and rupture [9,10]. In the clinical setting, circulating MMP-2 and MMP-9 levels have been measured in previous studies of patients with AMI [17–19]; however, these results were conflicting. Squire et al. [17] reported that circulating MMP levels were inversely correlated with LV dilatation, whereas Matsunage et al. [18] and Nakaya et al. [19] found that serum MMP levels and activity were positively correlated with LV dilatation. In addition, circulating MMP levels could be affected at the acute stage following reperfusion therapy and by the clinically vulnerable state [20–23]. In the present study, we focused on cardiac production of MMP [14], and the measurement was performed at the clinically stable stage following AMI. As shown in Table 2, despite similar levels of TIMPs, significant differences in levels of BNP, MMP-2 and MMP-9 were observed between the CS and the peripheral artery in patients with AMI. To our knowledge, this is the first study demonstrating the enhanced production of MMP-2 and MMP-9 in a human infarcted heart. Moreover, as shown

in Figure 1, the CS–arterial concentration gradients of MMP-2 and MMP-9 correlated positively with those of BNP and LVEDVI. Taking into account the delicate balance between MMPs and TIMPs in tissue remodelling, the present findings indicate that excessive cardiac production of MMPs may play an important pathological role in the progression of post-infarction LV dysfunction.

A previous experimental study of an AMI model using BNP-transgenic mice demonstrated a potential interaction of BNP with inflammation [24]. The overexpression of BNP leads to neutrophil infiltration and MMP-9 expression in the infarct region and increases the incidence of cardiac rupture. These findings suggest the significance of inflammatory reaction in the heart accompanied by changes in LV function. 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors, such as statins, exert various cardiovascular protective effects beyond their lipid-level lowering actions [12,25]. These pleiotropic effects include the inhibition of inflammatory responses. In the present study, we have shown that the CS–arterial concentration gradients of MMP-2 and MMP-9 were smaller in the pravastatin-treated group than in the non-pravastatin-treated group, which was accompanied by a decrease in CRP level. These findings indicate that pravastatin may modulate cardiac MMP production in patients with AMI, probably via its anti-inflammatory effects. Similar observations of decreased circulating MMP-2 levels in patients with AMI treated with 10 mg of paravastain have been reported previously [19].

There are several potential limitations of the present study. First, this study was not randomized. Pravastatin was administered to a small number of patients with AMI with hyperlipidaemia. In such a pro-inflammatory state, tissue MMPs might have been activated before treatment [26], which could affect the results. Therefore prospective studies will be required to determine if pravastatin has a causal role in reducing cardiac MMP production in patients with AMI. Secondly, the present study was carried out over the short term, whereas ventricular remodelling is known to progress over months or years. Thirdly, previous studies have shown that the renin–angiotensin system is also involved in the induction of post-infarction ventricular remodelling [27] and can be inhibited by statins [28,29]. However, we have shown that the CS–arterial concentration gradients of Ang II were similar between pravastatin-treated patients and non-pravastatin-treated patients. This may be related, in part, to the fact that all our patients with AMI had been treated with 5 mg of enalapril.

In conclusion, the present study demonstrates the enhancement of MMP production in an infarcted heart. Pleiotropic effects of statins may be associated with the modulation of cardiac MMP activation, which is potentially beneficial in the attenuation of post-infarction LV remodelling.

REFERENCES

1 Sutton, M. G. and Sharpe, N. (2000) Left ventricular remodeling after myocardial infarction: pathophysiology and therapy. Circulation 101, 2981–2988
2 Yousef, Z. R., Redwood, S. R. and Marber, M. S. (2000) Postinfarction left ventricular remodelling: where are the theories and trials leading us? Heart 83, 76–80
3 White, H. D., Norris, R. M., Brown, M. A., Brandt, P. W., Whitlock, R. M. and Wild, C. J. (1987) Left ventricular end-systolic volume as the major determinant of survival after recovery from myocardial infarction. Circulation 76, 44–51
4 Weisman, H. F. and Healy, B. (1987) Myocardial infarct expansion, infarct extension, and reinfarction: pathophysiologic concepts. Prog. Cardiovasc. Dis. 30, 73–110
5 Creemers, E. E., Cleutjens, J. P., Smits, J. F. and Daemen, M. J. (2001) Matrix metalloproteinase inhibition after myocardial infarction: a new approach to prevent heart failure? Circ. Res. 89, 201–210
6 Spinale, F. G. (2002) Matrix metalloproteinases: regulation and dysregulation in the failing heart. Circ. Res. 90, 520–530
7 Cleutjens, J. P., Kandala, J. C., Guarda, E., Guntaka, R. V. and Weber, K. T. (1995) Regulation of collagen degradation in the rat myocardium after infarction. J. Mol. Cell. Cardiol. 27, 1281–1292
8 Rohde, L. E., Ducharme, A., Arroyo, L. H. et al. (1999) Matrix metalloproteinase inhibition attenuates early left ventricular enlargement after experimental myocardial infarction in mice. Circulation 99, 3063–3070
9 Heymans, S., Luttun, A., Nuyens, D. et al. (1999) Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. Nat. Med. 5, 1135–1142
10 Ducharme, A., Frantz, S., Aikawa, M. et al. (2000) Targeted deletion of matrix metalloproteinase-9 attenuates left ventricular enlargement and collagen accumulation after experimental myocardial infarction. J. Clin. Invest. 106, 55–62
11 Nahrendorf, M., Hu, K., Hiller, K. H. et al. (2002) Impact of hydroxymethylglutaryl coenzyme a reductase inhibition on left ventricular remodeling after myocardial infarction: an experimental serial cardiac magnetic resonance imaging study. J. Am. Coll. Cardiol. 40, 1695–1700
12 Hayashidani, S., Tsutsui, H., Shiomi, T. et al. (2002) Fluvastatin, a 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitor, attenuates left ventricular remodeling and failure after experimental myocardial infarction. Circulation 105, 868–873
13 Nagaya, N., Nishikimi, T., Goto, Y. et al. (1998) Plasma brain natriuretic peptide is a biochemical marker for the prediction of progressive ventricular remodeling after acute myocardial infarction. Am. Heart J. 135, 21–28
14 Yasuda, S., Goto, Y., Baba, T. et al. (2000) Enhanced secretion of cardiac hepatocyte growth factor from an infarct region is associated with less severe ventricular enlargement and improved cardiac function. J. Am. Coll. Cardiol. 36, 115–121
14a Nakamura, H., Arakawa, K., Itakura, H. et al. (2006) Primary prevention of cardiovascular disease with pravastatin in Japan (MEGA study): a prospective randomised control trial. Lancet 368, 1155–1163
15 Fujimoto, N. and Iwata, K. (2001) Use of EIA to measure MMPs and TIMPs. Methods Mol. Biol. 151, 347–358
16 Yasuda, S., Goto, Y., Sumida, H. et al. (1999) Angiotensin-converting enzyme inhibition restores hepatocyte growth factor production in patients with congestive heart failure. Hypertension 33, 1374–1378
17 Squire, I. B., Evans, J., Ng, L. L., Loftus, I. M. and Thompson, M. M. (2004) Plasma MMP-9 and MMP-2 following acute myocardial infarction in man: correlation with echocardiographic and neurohumoral parameters of left ventricular dysfunction. J. Card. Failure 10, 328–333

18 Matsunaga, T., Abe, N., Kameda, K. et al. (2005) Circulating level of gelatinase activity predicts ventricular remodeling in patients with acute myocardial infarction. Int. J. Cardiol. 105, 203–208
19 Nakaya, R., Uzui, H., Shimizu, H. et al. (2005) Pravastatin suppresses the increase in matrix metalloproteinase-2 levels after acute myocardial infarction. Int. J. Cardiol. 105, 67–73
20 Wagner, D. R., Delagardelle, C., Ernens, I., Rouy, D., Vaillant, M. and Beissel, J. (2006) Matrix metalloproteinase-9 is a marker of heart failure after acute myocardial infarction. J. Card. Failure 12, 66–72
21 Higo, S., Uematsu, M., Yamagishi, M. et al. (2005) Elevation of plasma matrix metalloproteinase-9 in the culprit coronary artery in patients with acute myocardial infarction: clinical evidence from distal protection. Circ. J. 69, 1180–1185
22 Kai, H., Ikeda, H., Yasukawa, H. et al. (1998) Peripheral blood levels of matrix metalloproteases-2 and -9 are elevated in patients with acute coronary syndromes. J. Am. Coll. Cardiol. 32, 368–372
23 Fukuda, D., Shimada, K., Tanaka, A. et al. (2006) Comparison of levels of serum matrix metalloproteinase-9 in patients with acute myocardial infarction versus unstable angina pectoris versus stable angina pectoris. Am. J. Cardiol. 97, 175–180
24 Kawakami, R., Saito, Y., Kishimoto, I. et al. (2004) Overexpression of brain natriuretic peptide facilitates neutrophil infiltration and cardiac matrix metalloproteinase-9 expression after acute myocardial infarction. Circulation 110, 3306–3312
25 Davignon, J. (2004) Beneficial cardiovascular pleiotropic effects of statins. Circulation 109, III39–III43
26 El Messal, M., Beaudeux, J. L., Drissi, A. et al. (2005) Elevated serum levels of proinflammatory cytokines and biomarkers of matrix remodeling in never-treated patients with familial hypercholesterolemia. Clin. Chim. Acta 366, 185–189
27 Bader, M. (2002) Role of the local renin-angiotensin system in cardiac damage: a minireview focusing on transgenic animal models. J. Mol. Cell. Cardiol. 34, 1455–1462
28 Dechend, R., Fiebler, A., Lindschau, C. et al. (2001) Modulating angiotensin II-induced inflammation by HMG Co-A reductase inhibition. Am. J. Hypertens. 14, 55S–61S
29 Luo, J. D., Zhang, W. W., Zhang, G. P., Guan, J. X. and Chen, X. (1999) Simvastatin inhibits cardiac hypertrophy and angiotensin-converting enzyme activity in rats with aortic stenosis. Clin. Exp. Pharmacol. Physiol. 26, 903–908

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Prevention of Life-Threatening Ventricular Tachyarrhythmia by a Novel and Pure Class-III Agent, Nifekalant Hydrochloride

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Abstract: Nifekalant hydrochloride (NIF) is a novel intravenous class-III antiarrhythmic agent with a pirimidinedione structure that purely blocks the K^+channel without inhibiting β-adrenergic receptors. The authors investigated the efficacy of NIF for refractory ventricular tachycardia/fibrillation (VT/VF). They studied 30 patients treated with an intravenous infusion of NIF [26 men, 4 women; age: 63 ± 17 (mean ± SD) years] at a dose of 0.19 ± 0.14 mg/kg body weight per hour. Sixteen were patients with acute coronary syndrome (ACS), and 14 were patients with chronic structural heart disease (Chr-HD). Amiodarone and sotalol had already been administered to 9 patients with Chr-HD before the administration of NIF. The QT and T peak-end (Tp-e) intervals were measured and corrected by Bazett's method (QTc, cTp-e). The left ventricular ejection fraction was depressed (28 ± 9%). NIF was effective for preventing VT/VF without proarrhythmia and hemodynamic deterioration in 21 patients (70%; 12 with ACS; 9 with Chr-HD), but ineffective in 4 patients (all with Chr-HD). The QTc prolongation in the responders was more pronounced than in the nonresponders (25% ± 15% versus 5% ± 7% increase; $P < 0.05$). Proarrhythmic torsade de pointes (TdP) developed transiently in the remaining 5 patients in whom the cTp-e was markedly increased compared with that in the responders (93% ± 49% versus 37% ± 41% increase; $P < 0.05$). In conclusion, these findings indicate that the intravenous administration of NIF is useful in the emergent treatment of inhibiting drug-refractory VT/VF, although proarrhythmic TdP owing to an enhancement of transmural dispersion of repolarization needs to be taken into account.

Key Words: antiarrhythmia agents, electrocardiography, potassium, tachyarrhythmia, torsade de pointes

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INTRODUCTION

Nifekalant hydrochloride (NIF) is a class-III antiarrhythmic agent having a pirimidinedione structure.[1] NIF inhibits HERG channels, suggesting the selective inhibition of the rapid component of the delayed rectifier K^+current (IKr).[2] The major adverse effect of NIF is proarrhythmic torsade de pointes (TdP).[3,4] As class-III antiarrhythmic agents, NIF and amiodarone are similar, but they do have some differences. NIF is characterized as a pure K^+ channel blocker with a minimal negative inotropic effect,[5,6] which amiodarone has via a β-blocking action.[7] Negative inotropic effect of amiodarone is disadvantageous, particularly when amiodarone is administered rapidly to a failing heart. Moreover, NIF decreases the defibrillation threshold;[8] whereas, amiodarone does not.[9] On the basis of these unique features of NIF, we hypothesized that NIF may effectively inhibit refractory ventricular tachycardia/fibrillation (VT/VF) in patients with severe left ventricular dysfunction. We also investigated the electrocardiographic characteristics related to antiarrhythmic and proarrhythmic actions of NIF in electrocardiography. Therapy using NIF did not include the general advice of the Cardiopulmonary Resuscitation and Emergency Cardiovascular Care to administer amiodarone,[10] but instead was used as a replacement for this guideline.

MATERIALS AND METHODS

Study Patients

We studied 30 patients who were hospitalized between May 1999 and May 2004 in the National Cardiovascular Center (Suita, Japan) and were treated with intravenous administrations of NIF for refractory VT/VF [26 men, 4 women; age, 63 ± 17 (mean ± SD) years]. When VT/VF appeared in patients pretreated with oral amioradone or sotalol or when VT/VF did not disappear after intravenous administration of class Ia and Ib drugs, we considered it refractory to conventional treatments. Patients in whom VT/VF was

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refractory to direct counter shock were also included. All the patients gave their written informed consent, and the institutional ethical committee on human research approved the study protocol.

The left ventricular ejection fraction was depressed [28% ± 9% (mean ± SD)]. Sixteen patients had acute coronary syndrome (ACS). Ten had anterior acute myocardial infarction, and 6 had unstable angina pectoris. The remaining 14 had chronic structural heart diseases (chr-HD). Eight had dilated cardiomyopathy, 4 had old myocardial infarction (OMI), and 2 had valvular heart disease. All the patients with ACS underwent revascularization by percutaneous coronary intervention or coronary bypass surgery, and 7 underwent hemodialysis because of renal failure. Among the patients with chronic heart disease, 9 had been administered oral amiodarone or sotalol, and implantable cardioverter defibrillators (ICDs) had been implanted in 7 patients.

Study Design

Before and during the administration of NIF, we measured QT and the interval between the peak and the end of the T wave (Tp-e) in lead V4 or V5 reflecting the transmural dispersion of repolarization in the free wall of the left ventricle.[11] The QT and Tp-e intervals were corrected by Bazett's method to obtain corrected QT (QTc) and corrected Tp-e (cTp-e).

When original VT/VF remained even though the dose of NIF increased to at least 0.2 mg/kg/hr and were refractory to direct counter shock, we considered that the response to NIF was ineffective. When TdP developed even if original VT/VF disappeared, we considered that it was proarrhythmic effect of NIF.

Comparisons between 2 groups were made using 2-tailed Student's *t*-test. Differences were considered significant at $P < 0.05$. Data are presented as mean ± SD.

RESULTS

Efficacy of NIF Inhibiting VT/VF

When NIF was administered intravenously at a maintenance dose of 0.05 to 0.6 (0.19 ± 0.14) mg/kg/ hr, none of the patients had hemodynamic deterioration during administration. NIF was effective in preventing VT/VF without proarrhythmia and hemodynamic deterioration in 21 of the 30 patients (Figure 1). Thus, the overall efficacy rate was 70% in this study group. NIF was ineffective in 4 patients. In 5 patients, proarrhythmic TdP developed transiently, but it disappeared soon after NIF administration was discontinued without additional treatment. The group was limited to the 9 patients pretreated with oral amiodarone or sotalol, NIF was effective in 6 patients (67% of efficacy) (Figure 1).

Comparisons of Usage Dose and ECG Parameters Between Responders and Nonresponders to NIF

There was no significant difference in the dose of NIF used between responders and nonresponders to NIF (0.19 ± 0.15 vs. 0.22 ± 0.05 mg/kg/hr). In 4 nonresponders, 2 patients had been treated with oral amiodarone.

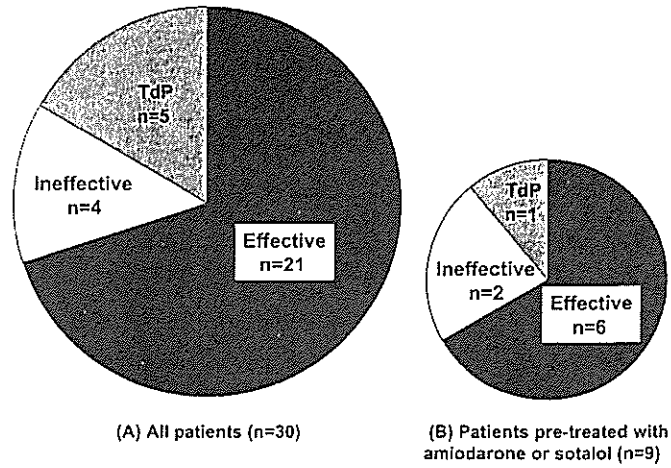

FIGURE 1. Efficacy of nifekalant hydrochloride (NIF) for preventing refractory ventricular tachycardia/fibrillation (VT/VF) in all patients studied (n = 30) and in patients pretreated with oral amiodarone or sotalol (n = 9).

Despite similar baseline QTc values (responders, 451 ± 64 msec; nonresponders, 478 ± 37 msec), the QTc after the administration of NIF differed between the 2 groups (responders, 559 ± 60 msec; nonresponders, 503 ± 68 msec; $P = 0.06$). The percent increase in QTc was 25% ± 15% in responders, whereas it was 5% ± 7% in nonresponders; these values were significantly different ($P = 0.018$), as shown in Figure 2. However, the cTp-e and the percent changes were similar between responders and nonresponders to NIF (Figure 3). Figure 4 shows representative cases.

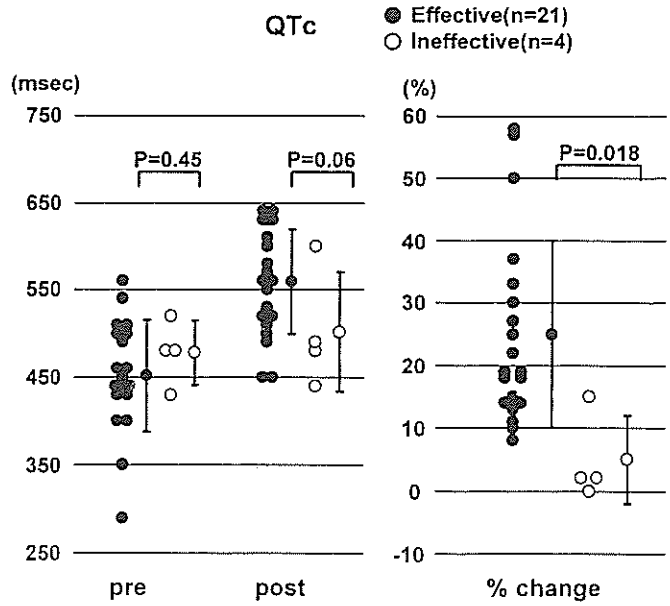

FIGURE 2. Comparisons of corrected QT between effective group (closed circles) and ineffective group (open circles). The QT interval was corrected by Bazett's method (QTc). % change = (post QTc − pre QTc) × 100/pre QTc.

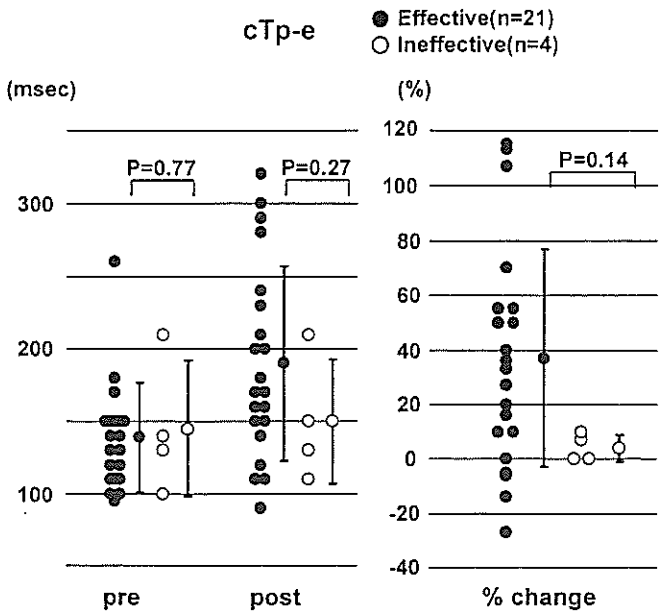

FIGURE 3. Comparisons of corrected T peak-end interval (cTp-e) between effective group (closed circles) and ineffective group (open circles). The Tp-e is the interval between the peak and the end of the T wave.

Comparisons of the Usage Dose and ECG Parameters Between Patients Effective to NIF and Those Developing Proarrhythmic TdP

The dose used in the TdP group was significantly lower than that used in the effective group (TdP, 0.09 ± 0.04 mg/kg/hr; effective, 0.19 ± 0.15 mg/kg/hr; $P = 0.01$). One of five patients in the TdP group was pretreated with oral amiodarone. ECG measurement before NIF administration was not performed in 1 patient with dilated cardiomyopathy who progressively failed in decompensate state due to incessant form of VT and VF.

Before NIF administration, the baseline QTc values of the 2 groups were similar (TdP, 485 ± 39 msec; effective, 452 ± 64 msec) (Figure 5). However, NIF increased QTc to be significantly longer in the TdP group than in the effective group (TdP, 636 ± 70 msec; effective, 559 ± 60 msec; $P = 0.02$). Also, percent increase in the QTc following NIF administration differed between the 2 groups (TdP, 33% ± 6%, effective, 25% ± 15%; $P = 0.09$).

Despite similar baseline cTp-e values (TdP, 133 ± 49 msec; effective, 139 ± 38 msec), the cTp-e after the administration of NIF differed between the 2 groups (TdP, 246 ± 50 msec; effective, 190 ± 67 msec; $P = 0.09$). The percent increase was 93% ± 49% in the TdP group, whereas it was 37% ± 41% in the effective group; these values were significantly different ($P = 0.02$) (Figure 6). Figure 7 shows representative cases.

Figure 8 shows serial changes in QTc and cTp-e in individual patient of TdP group. QTc prolongation was seen immediately after NIF administration. When TdP was induced, QTc interval increased more, accompanied by cTp-e prolongation. However, both QTc and cTp-e returned to the control (pre-) level after discontinuing NIF (-off).

Subclassification by Underlying Heart Diseases

Twenty-one responders consisted of 12 patients in the ACS group and 9 patients in the Chr-HD group. Four nonresponders belonged to the Chr-HD group; 2 patients had

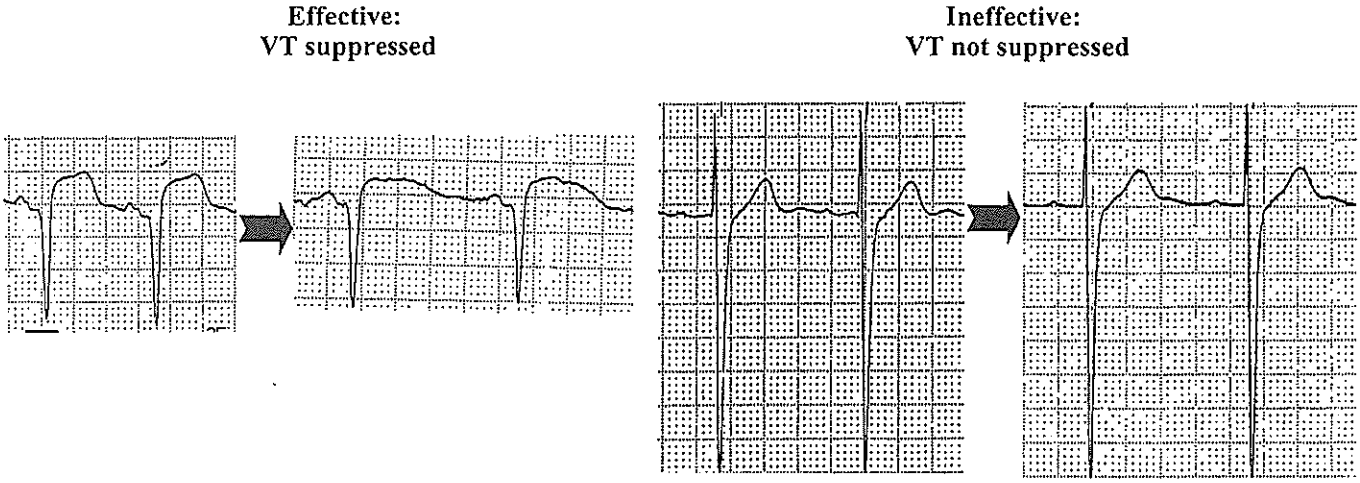

FIGURE 4. Representative patient in whom NIF was effective (left panel) and that in whom NIF was not effective (right panel). AMI indicates acute myocardial infarction; LVEF, left ventricular ejection fraction; MR, mitral regurgitation.