proinflammatory cytokines, stimulates endothelial cells to express adhesion molecules, increases the synthesis of metalloproteinases, and inhibits the synthesis of proteoglycans in cartilage (39). TNF levels are chronically elevated in the blood and, more specifically, in the joints, of patients with RA (40), and it has been proven that the blocking of TNF-related pathways is a strong therapeutic tool in RA (41). RANKL has emerged as one of the essential pathogenic factors in the destruction of cartilage and bone in RA (42–45). RANKL is part of the TNF ligand family and is an important regulator of both osteoclastogenesis and functions of the immune system, including lymph node organogenesis and lymphocyte development. Even though the roles of RANKL in the pathogenesis of RA during the chronic stage have not yet been elucidated, it is known that it is expressed both by synovial fibroblasts and by activated T lymphocytes derived from synovial tissue from patients with RA (43–45). Moreover, blocking of the RANKL pathway at the onset of adjuvant-induced arthritis prevents bone and cartilage destruction (42). Therefore, it is a noteworthy finding that OCH stimulation induces much lower levels of gene expression of TNFα and RANKL compared with stimulation by other glycolipid antigens such as α-GC.

We demonstrated in this study that OCH was effective in the treatment of established CIA in autoimmune-prone mice of the SJL strain, which have a quantitative and functional NKT cell deficiency; this suggests that OCH might be useful for the treatment of patients with various autoimmune diseases associated with reduced numbers of NKT cells. Furthermore, in contrast to classic MHC molecules, CD1d molecules are nonpolymorphic and are remarkably well conserved among the population and may become extremely valuable in the development of HLA-independent treatment approaches for autoimmune conditions. These findings highlight the potential use of OCH for therapeutic intervention in autoimmune diseases such as RA.

ACKNOWLEDGMENTS

We thank Masaru Taniguchi (Chiba University, Graduate School of Medicine, Chiba, Japan) for providing $J_{\alpha}281$-knockout mice, and Karen Mullane-Robinson (Boston, MA) for critical reading of the manuscript.

REFERENCES

1. Van Roon JAG, Lafeber FPJG, Bijlsma JWJ. Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. Arthritis Rheum 2001;44:3–12.
2. Walmsley M, Katsikis PD, Abney E, Parry S, Williams RO, Maini RN, et al. Interleukin-10 inhibition of the progression of established collagen-induced arthritis. Arthritis Rheum 1996;39: 495–503.
3. Joosten LAB, Lubberts E, Durez P, Helsen MMA, Jacobs MJM, Goldman M, et al. Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis: protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction. Arthritis Rheum 1997;40:249–60.
4. Horsfall AC, Butler DM, Marinova L, Warden PJ, Williams RO, Maini RN, et al. Suppression of collagen-induced arthritis by continuous administration of IL-4. J Immunol 1997;159:5687–96.
5. Apparailly F, Verwaerde C, Jacquet C, Auriault C, Sany J, Jorgensen C. Adenovirus-mediated transfer of viral IL-10 gene inhibits murine collagen-induced arthritis. J Immunol 1998;160: 5213–20.
6. Ma Y, Thornton S, Duwel LE, Boivin GP, Giannini EH, Leiden JM, et al. Inhibition of collagen-induced arthritis in mice by viral IL-10 gene transfer. J Immunol 1998;161:1516–24.
7. Lubberts E, Joosten LAB, van den Bersselaar L, Helsen MMA, Bakker AC, van Meurs JBJ, et al. Adenoviral vector-mediated overexpression of IL-4 in the knee joint of mice with collagen-induced arthritis prevents cartilage destruction. J Immunol 1999; 163:4546–56.
8. Setoguchi K, Misaki Y, Araki Y, Fujio K, Kawahata K, Kitamura T, et al. Antigen-specific T cells transduced with IL-10 ameliorate experimentally induced arthritis without impairing the systemic immune response to the antigen. J Immunol 2000;165:5980–6.
9. Lubberts E, Joosten LAB, Chabaud M, van den Bersselaar L, Oppers B, Coenen-de Roo CJJ, et al. IL-4 gene therapy for collagen arthritis suppresses synovial IL-17 and osteoprotegerin ligand and prevents bone erosion. J Clin Invest 2000;105:1697–710.
10. Kim SH, Kim S, Evans CH, Ghivizzani SC, Oligino T, Robbins PD. Effective treatment of established murine collagen-induced arthritis by systemic administration of dendritic cells genetically modified to express IL-4. J Immunol 2001;166:3499–505.
11. Morita Y, Yang J, Gupta R, Shimizu K, Shelden EA, Endres J, et al. Dendritic cells genetically engineered to express IL-4 inhibit murine collagen-induced arthritis. J Clin Invest 2001;107:1275–84.
12. Bendelac A, Rivera MN, Park SH, Roark JH. Mouse CD1-specific NK1 T cells: development, specificity, and function. Annu Rev Immunol 1997;15:535–62.
13. Hong S, Scherer DC, Singh N, Mendiratta SK, Serizawa I, Koezuka Y, et al. Lipid antigen presentation in the immune system: lessons learned from CD1d knockout mice. Immunol Rev 1999;169:31–44.
14. Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. NKT cells: facts, functions and fallacies. Immunol Today 2000;21: 573–83.
15. Sumida T, Sakamoto A, Murata H, Makino Y, Takahashi H, Yoshida S, et al. Selective reduction of T cells bearing invariant Vα24JαQ antigen receptor in patients with systemic sclerosis. J Exp Med 1995;182:1163–8.
16. Wilson SB, Kent SC, Patton KT, Orban T, Jackson RA, Exley M, et al. Extreme Th1 bias of invariant Vα24JαQ T cells in type 1 diabetes. Nature 1998;391:177–81.
17. Illes Z, Kondo T, Newcombe J, Oka N, Tabira T, Yamamura T. Differential expression of NK T cell Vα24JαQ invariant TCR chain in the lesions of multiple sclerosis and chronic inflammatory demyelinating polyneuropathy. J Immunol 2000;164:4375–81.
18. Kojo S, Adachi Y, Keino H, Taniguchi M, Sumida T. Dysfunction of T cell receptor AV24AJ18+,BV11+ double-negative regulatory natural killer T cells in autoimmune diseases. Arthritis Rheum 2001;44:1127–38.
19. Yoshimoto T, Bendelac A, Hu-Li J, Paul WE. Defective IgE

production by SJL mice is linked to the absence of CD4+, NK1.1+ T cells that promptly produce interleukin 4. Proc Natl Acad Sci U S A 1995;92:11931–4.
20. Mieza MA, Itoh T, Cui JQ, Makino Y, Kawano T, Tsuchida K, et al. Selective reduction of Vα14+ NK T cells associated with disease development in autoimmune-prone mice. J Immunol 1996;156:4035–40.
21. Gombert JM, Herbelin A, Tancrede-Bohin E, Dy M, Carnaud C, Bach J-F. Early quantitative and functional deficiency of NK1$^+$-like thymocytes in the NOD mouse. Eur J Immunol 1996;26: 2989–98.
22. Hong SH, Wilson MT, Serizawa I, Wu L, Singh N, Naidenko OV, et al. The natural killer T-cell ligand α-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. Nat Med 2001;7:1052–6.
23. Singh AK, Wilson MT, Hong S, Olivares-Villagomez D, Du C, Stanic AK, et al. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. J Exp Med 2001;194:1801–11.
24. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of Vα14. Nature 1998;391:177–81.
25. Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, Dellabona P, et al. CD1d-mediated recognition of an α-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. J Exp Med 1998;188:1521–8.
26. Spada FM, Koezuka Y Porcelli SA. CD1d-restricted recognition of syntheic glycolipid antigens by human natural killer T cells. J Exp Med 1998;188:1529–34.
27. Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing Th2 bias of natural killer T cells. Nature 2001;413:531–4.
28. Pal EA, Tabira T, Kawano T, Taniguchi M, Miyake S, Yamamura T. Costimulation-dependent modulation of experimental autoimmune encephalomyelitis by ligand stimulation of Vα14+ NK T cells. J Immunol 2001;166:662–8.
29. Courtenay JS, Dallman MJ, Dayan AD, Martin A, Mosedale B. Immunization against heterologous type II collagen induces arthritis in mice. Nature 1980;283:666–8.
30. Campbell IK, Rich MJ, Bischof RJ, Dunn AR, Grail D, Hamilton JA. Protection from collagen-induced arthritis in granulocyte-macrophage colony-stimulating factor-deficient mice. J Immunol 1998;161:3639–44.
31. Campbell IK, Hamilton JA, Wicks IP. Collagen-induced arthritis in C57BL/6 (H-2^b) mice: new insights into an important disease model of rheumatoid arthritis. Eur J Immunol 2000;30:1568–75.
32. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, et al. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. Science 1997;278:1623–6.
33. Sharif S, Arreaza GA, Zucker P, Mi Q-S, Sondhi J, Naidenko OV, et al. Activation of natural killer T cells by α-galactosylceramide treatment prevents the onset and recurrence of autoimmune type 1 diabetes. Nat Med 2001;7:1057–62.
34. Mempel M, Ronet C, Suarez F, Gilleron M, Puzo,G, van Kaer L, et al. Natural killer T cells restricted by the monomorphic MHC class 1b CD1d1 molecules behave like inflammatory cells. J Immunol 2002;168:365–71.
35. Hammond KJL, Poulton LD, Palmisano LJ, Silveria PA, Godfrey DJ, Bazter AG. α/β-T cell receptor (TCR)+CD4-CD8-(NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. J Exp Med 1998;187:1047–56.
36. Lehuen A, Lantz O, Beaudoin L, Laloux V, Carnaud C, Bendelac A, et al. Overexpression of natural killer T cells protects Vα14-Jα281 transgenic nonobese diabetic mice against diabetes. J Exp Med 1998;188:1831–9.
37. Wang B, Geng YB, Wang CR. CD1-restricted NK T cells protect nonobese diabetic mice from developing diabetes. J Exp Med 2001;194:313–9.
38. Keffer J, Probert L, Cazlaris H, Georgopoulos S, Kaslaris E, Kioussis D, et al. Transgenic mice expressing human tumor necrosis factor: a predictive genetic model of arthritis. EMBO J 1991;10:4025–31.
39. Choy EH, Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med 2001;344:907–16.
40. Brennan FM, Maini RN, Feldmann M. TNF alpha: a pivotal role in rheumatoid arthritis? Br J Rheumatol 1992;31:293–8.
41. Criscione LG, St Clair EW. Tumor necrosis factor-α antagonists for the treatment of rheumatic diseases. Curr Opin Rheumatol 2002;14:204–11.
42. Kong Y-Y, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 1999; 402:304–9.
43. Gravallese EM, Manning C, Tsay A, Naito A, Pan C, Amento E, et al. Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. Arthritis Rheum 2000;43:250–8.
44. Shigeyama Y, Pap T, Kunzler P, Simmen BR, Gay R, Gay S. Expression of osteoclast differentiation factor in rheumatoid arthritis. Arthritis Rheum 2000;43:2523–30.
45. Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, et al. Involvement of receptor activator of nuclear factor κB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. Arthritis Rheum 2000;43:259–69.

Original Paper

Pharmacology

Pharmacology 2004;72:213–219
DOI: 10.1159/000080375

Received: January 5, 2004
Accepted: April 22, 2004

Hemodynamic Effects of Carvedilol Infusion and the Contribution of the Sympathetic Nervous System in Rats with Heart Failure

Yuichi Abe[a] Kenichi Watanabe[a] Shinji Sato[b] Yusuke Nagai[a]
Fadia A. Kamal[a] Mir I.I. Wahed[a] Juan Wen[a] Gurusamy Narasimman[a]
Meilei Ma[a] Palaniyandi Suresh[a] Toshihiro Takahashi[c] Hitoshi Tachikawa[d]
Takeshi Kashimura[d] Naohito Tanabe[d] Makoto Kodama[d]
Yoshifusa Aizawa[d] Kenichi Yamaguchi[e] Makoto Miyazaki[f] Masao Kakemi[f]

Departments of [a]Clinical Pharmacology and [b]Functional Food and Analytical Food Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata, [c]Radioisotope Center, [d]First Department of Medicine, and [e]Department of Homeostatic Regulation and Development, Niigata University School of Medical and Dental Sciences, Niigata, and [f]Department of Pharmaceutics, Osaka University of Pharmaceutical Sciences, Osaka, Japan

Key Words
Carvedilol · Sympathetic nervous system · Norepinephrine · Chronic heart failure

Abstract
We investigated the contribution of the sympathetic nervous system (SNS) in maintaining the blood pressure and in regulating the cardiac function during and after carvedilol administration in rats with heart failure (group F). Left ventricular end-diastolic pressure, percent functional shortening, and rates of intraventricular pressure rise were significantly changed by carvedilol infusion as compared with the basal values in group N (normal rats), but not in group F. The left ventricular end-diastolic pressure was elevated, corresponding to the enhancement of the plasma norepinephrine (NE) concentration caused by carvedilol infusion, in group N. The enhancement of the plasma NE concentration induced by carvedilol administration in group F was higher than that in group N. The value for the maximal hypertensive effect of NE intravenous infusion (E_{max}) was decreased, and the plasma NE concentration at half-maximal effect (EC_{50}) was increased in group F as compared with the values in group N. These results indicate that the SNS (presynaptic) activity is increased and that the SNS receptor sensitivity in the cardiovascular regulation system is decreased in heart failure.

Introduction

Carvedilol is a third-generation nonselective β- and α-adrenergic blocker and can potentially blunt the release of norepinephrine (NE) by blocking presynaptic β_2-adrenergic receptors [1–3]. β-Adrenergic antagonists have been used in clinical practice not only for the treatment of hypertension, but also for the prevention of heart failure [4–7]. Carvedilol has been shown to reduce morbidity and mortality in patients and experimental animals with heart failure [7–10]. Furthermore, it has recently been reported that carvedilol has been associated with a greater increase in survival rate and left ventricular ejec-

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0031–7012/04/0724–0213$21.00/0

Accessible online at:
www.karger.com/pha

Kenichi Watanabe
Department of Clinical Pharmacology
Niigata University of Pharmacy and Applied Life Sciences
Kamishinei-cho 5-13-2, Niigata 950-2081 (Japan)
Tel. +81 25 268 1362, Fax +81 25 268 1230, E-Mail watanabe@niigata-pharm.ac.jp

tion fraction than metoprolol which is a selective β_1-blocker [11, 12]. We previously reported that low-dose, but not high-dose, carvedilol exerted beneficial effects in rats with dilated cardiomyopathy after myocarditis [10].

Plasma catecholamines have been used as an appropriate means to evaluate the baroreflex-mediated sympathetic nervous system (SNS) activity, because circulating catecholamines have been thought to consist mainly of catecholamines liberated from the synapses of the SNS in proportion to their degree of activity [13–15]. Imai et al. [13] reported that the blood pressure reduction caused by the administration of calcium antagonists correlated significantly with the increase in the plasma NE concentration.

This study investigated the contribution of the SNS in maintaining the blood pressure and its influence on the cardiovascular system during and after carvedilol administration in rats suffering from cardiomyopathy after autoimmune giant cell myocarditis [13–16]. We also examined whether the SNS hyperactivity induced by high-dose carvedilol administration is closely related to the severity of heart failure.

Materials and Methods

Reagents

Carvedilol was kindly donated by Daiichi Pharmaceutical (Tokyo, Japan) and was used without further purification. It was dissolved in N,N-dimethylformamide, 10% acetic acid, and isotonic sodium chloride solution and administered by intravenous infusion. All other chemicals were of reagent grade and were obtained commercially.

Animals

Nine-week-old male Lewis rats were used (Charles River Japan, Kanagawa, Japan). Cardiac myosin, prepared from the ventricular muscle of pig hearts according to a procedure described previously [10, 16], was injected into the rats. The morbidity of experimental autoimmune myocarditis was 100% in the rats immunized using this protocol [10, 16]. The rats in the myosin-immunized group became ill and immobile after 14 days, then their activity gradually recovered at the beginning of the 4th week. Thirty-one percent of the rats in the myosin-immunized group died between day 19 and day 42. Six weeks after immunization, 23 rats were used for the study (group F; heart failure). Twenty normal Lewis rats (group N) were used as the age-matched control group. Indwelling cannulae (PE10 and PE50; Becton Dickinson, Franklin Lakes, N.J., USA) were implanted into right jugular vein and left femoral artery of 17 rats in group F and of 15 rats in group N under light ether anesthesia 1 day before the experiments. Throughout the studies, all animals were treated in accordance with our institute's guidelines on animal experimentation [10].

Experimental Protocol

To characterize the alterations in blood pressure and plasma NE concentrations, either carvedilol or NE was injected into the right jugular vein by five consecutive 20-min infusions at increasing rates (model KDS220P infusion pump; Muromachi Kikai, Tokyo): 0.13, 0.25, 0.64, 1.27, and 2.54 mg h^{-1} rat^{-1} (300 g body weight) for the carvedilol study and 1.5, 3.0, 7.5, 15, and 30 μg h^{-1} rat^{-1} (300 g body weight) for the NE study. To avoid the effects of anesthesia, conscious rats were fixed in Bollman cages (Natsume, Tokyo) and were allowed to rest for 1 h before starting the experiments. Arterial blood pressure and heart rate were measured through the left femoral artery using a transducer and an amplifier (Powerlab, Tokyo). Blood samples (0.4 ml) were intermittently collected from the left femoral artery cannulae until 460 min (carvedilol study) and 220 min (NE study) after the initiation of drug administration. Blood was then replaced by injection of an equal volume of citrated blood from a donor rat. All these procedures were performed in a quiet environment to avoid the influence of any disturbance to animals. The obtained plasma samples were stored at −80 °C until analysis of the plasma NE and epinephrine concentrations. The plasma NE and epinephrine concentrations were determined by a modified high-performance liquid chromatography electrochemical assay with 3,4-dihydroxybenzylamine as an internal standard [17, 18].

The rats that had not been cannulated were anesthetized with pentobarbital (40 mg kg^{-1} i.p.), and pentobarbital (5 mg kg^{-1} i.p.) was constantly added during the experiments to maintain anesthesia. The central venous pressure (CVP), the left ventricular end-diastolic pressure (LVEDP), and the rates of intraventricular pressure rise and decline ($\pm dP\ dt^{-1}$) were recorded as described previously [10]. Echocardiographic studies were carried out by means of an SSD-5500 echocardiograph (Aloka, Tokyo) using a 7.5-MHz transducer (Aloka). The left ventricular dimensions in diastole and in systole and the percent fractional shortening (%FS) were measured by M-mode echocardiogram. The heart weight was measured, and the ratio of heart weight (HW) to body weight (BW) [HW BW^{-1} (g kg^{-1})] was calculated.

β-Adrenergic Receptors in the Myocardium

The β-adrenergic receptor binding assay was carried out in duplicate using [^{125}I]iodocyanopindolol. The membrane-enriched fraction of the myocardium was incubated for 30 min at 23 °C in a total volume of 0.5 ml containing 60 mmol/l Tris-HCl and 20 mmol/l $MgCl_2$ (pH 7.4). Values of the myocardial β-adrenoceptor density (B_{max}) and the dissociation constant for β-adrenoceptor (K_d) were calculated using Scatchard analysis, and the radioligand concentrations ranged from 0.1 to 0.8 nmol/l, as previously reported [19].

Data Analysis of the Sigmoidal E_{max} Model

A sigmoidal E_{max} model was used to quantitatively show the relationship between the plasma NE concentration and the hypertensive effect of NE during the NE infusion [20, 21]. The equation for the sigmoidal E_{max} model is as follows:

$$E = E_{max} \cdot C^n / C^n + EC_{50}^n$$

where E is the predicted hypertensive effect of NE, E_{max} is the maximal hypertensive effect of NE, EC_{50} is the plasma concentration that produces 50% of the maximal effect, and n is a measure

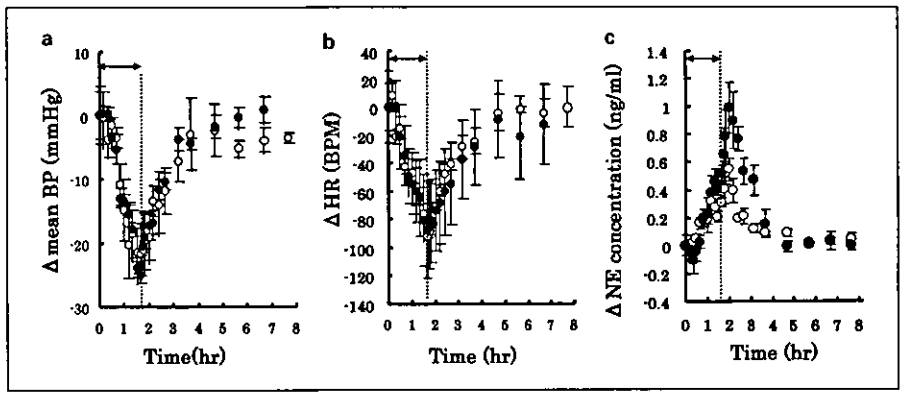

Fig. 1. Time courses of hypotensive effect (**a**), heart rate (**b**), and plasma NE concentration (**c**) during and after carvedilol infusion in group N (open circles) and group F (solid circles) using conscious rats. The arrows indicate the carvedilol infusion period. Each experimental point represents a change in value from that at baseline. Each value is expressed as mean ± SE. (This applies to the values of the other figures as well.) BP = Blood pressure; HR = heart rate; NE = norepinephrine.

of the curve gradient. The values of these parameters were estimated using the computer program WinNonlin [21].

Statistics

Data are presented as the mean ± SE. Statistical assessment of the groups was performed by Student's t test, followed by Tukey's method. Comparisons with the baseline values were performed by one-way analysis of variance followed by Dunnett's method. Differences were considered significant at $p < 0.05$. Constitutive plasma NE concentration and HW BW^{-1} were examined using linear regression analysis.

Results

Steady-State Physiological Levels

The constitutive levels of body weight, heart weight, HW BW^{-1}, hemodynamic parameters, echocardiographic parameters, plasma catecholamine concentrations, myocardial β-adrenoceptor parameters (B_{max} and K_d), and parameters of the hypertensive effect of NE infusion (E_{max} and EC_{50}) in group F and group N are shown in table 1. The values of all hemodynamic parameters, echocardiographic parameters, and plasma catecholamine concentrations were significantly changed in group F as compared with those in group N.

Changes in Hypotensive Effect and Plasma NE Concentration Induced by Intravenous Carvedilol Infusion

The changes in blood pressure, heart rate, and plasma NE concentration induced by intravenous carvedilol infusion were determined in group F and in group N. The changes in values for mean blood pressure, heart rate, and plasma NE concentration at steady state (Δ values) are shown in figure 1. The hypotensive effect of carvedilol in group F did not significantly differ from that in group N

Table 1. Values of steady-state physiological levels and parameters for the sigmoid E_{max} model in group N (normal) and group F (heart failure)

	Group N	Group F
BW, g	388 ± 6.1	300 ± 9**
HW, g	0.89 ± 0.05	1.37 ± 0.06**
HW/BW, g/kg	2.6 ± 0.04	5.0 ± 0.24**
BP, mm Hg	111 ± 4	91 ± 3.3**
HR, beats/min	369 ± 13	384 ± 1.2*
LVEDP, mm Hg	3.3 ± 2.3	17.4 ± 6.3*
CVP, mm Hg	1.5 ± 0.7	4.9 ± 1.1*
$_{max}$dP/dt, mm Hg/s	13,100 ± 1,400	5,100 ± 600**
$_{min}$dP/dt, mm Hg/s	−15,400 ± 1,400	−4,700 ± 600**
FS, %	43.8 ± 2.7	13.9 ± 5.1**
LVDd, mm	7.8 ± 0.3	9 ± 0.3*
LVDs, mm	4.4 ± 0.3	7.9 ± 0.8*
NE, pg/ml	203 ± 27.5	526 ± 65.3**
EPI, pg/ml	71 ± 3.5	209 ± 44.3**
B_{max}, fmol/mg protein	53 ± 2	32 ± 4**
K_d, nmol/l	0.45 ± 0.08	0.49 ± 0.09
E_{max}, mm Hg[a]	28.6 ± 1.1	20.8 ± 2.7
EC_{50}, ng/ml[a]	1.5 ± 0.1	4.5 ± 0.85
n[a]	2.7 ± 0.4	1.8 ± 0.31

BW = Body weight; HW = heart weight; BP = arterial blood pressure; HR = heart rate; LVEDP = left ventricular end-diastolic pressure; CVP = central venous pressure; $_{max/min}$dP/dt = rates of intraventricular pressure rise and decline, respectively; FS = fractional shortening; LVDd = left ventricular dimension in diastole; LVDs = left ventricular dimension in systole; NE = plasma norepinephrine concentration; EPI = plasma epinephrine concentration; B_{max} = cardiac β-adrenoceptor density; K_d = dissociation constant for β-adrenoceptor. All values are expressed as the mean ± SE.

[a] The values of these parameters were estimated by fitting the hypertensive effect of NE data using the computer program WinNonlin (see text).

* $p < 0.05$ and ** $p < 0.01$ vs. group N.

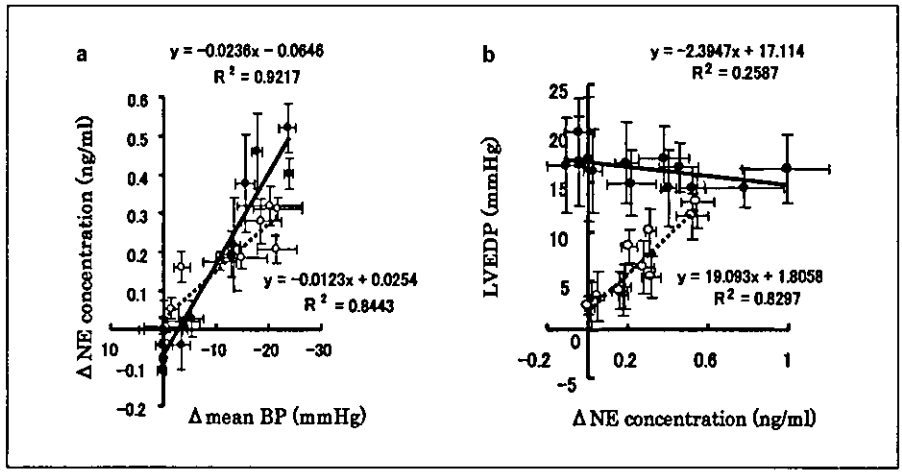

Fig. 2. Relationships between enhancement of plasma NE concentration and decrease in blood pressure (BP) (**a**) and change of LVEDP and enhancement of plasma NE concentration (**b**) with carvedilol infusion in group N (open circles and dotted line) and group F (solid circles and solid line).

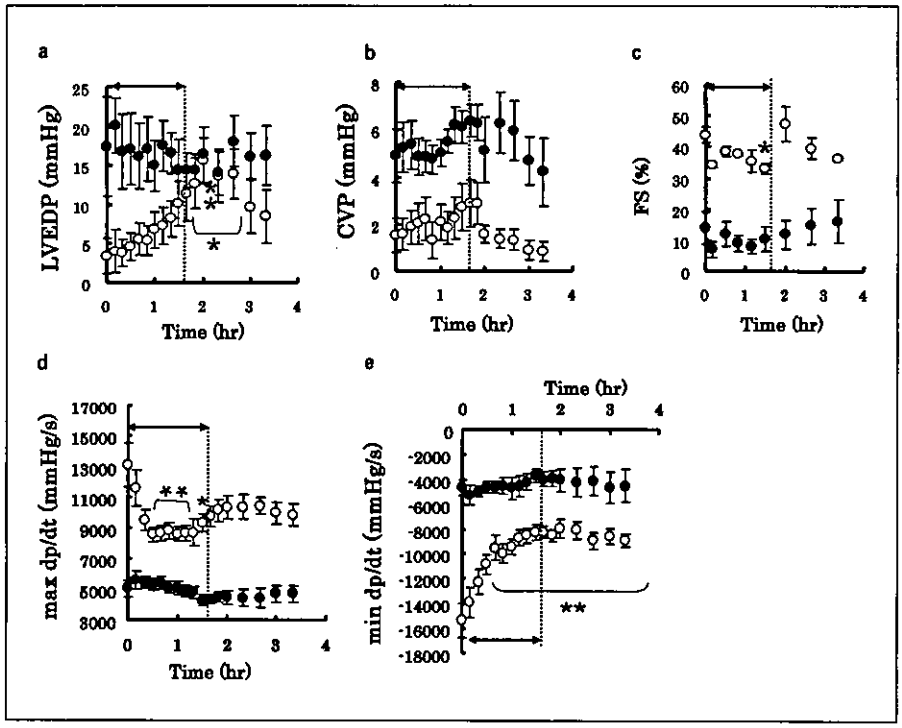

Fig. 3. Time courses of hemodynamics during and after carvedilol infusion in group N (open circles) and group F (solid circles) using unconscious rats. The arrows indicate the carvedilol infusion period. * $p < 0.05$ and ** $p < 0.01$ vs. baseline. LVEDP (**a**) = Left ventricular end-diastolic pressure; CVP (**b**) = central venous pressure; FS (**c**) = fractional shortening; max/min dP/dt (**d**, **e**) = rates of intraventricular pressure rise and decline, respectively.

(fig. 1a). However, the enhancement of the plasma NE concentration induced by carvedilol administration in group F was higher than that in group N ($p = 0.055$; fig. 1c). The slope of the enhanced plasma NE concentration in group F was twice as high as that in group N (fig. 2a).

Effects of Carvedilol Infusion on Echocardiographic and Hemodynamic Parameters

The changes in echocardiographic and hemodynamic parameters induced by intravenous carvedilol infusion were determined (fig. 3). LVEDP and CVP were significantly increased and %FS and $\pm dP\ dt^{-1}$ decreased at baseline in group F as compared with the values in group N ($p < 0.01$ for all parameters). Relative to baseline values, the LVEDP was significantly increased, while $\pm dP\ dt^{-1}$ and %FS were significantly reduced by carvedilol infusion in group N but not in group F; however, there was no significant change in CVP. A rebound phenomenon, an increase in %FS, was observed after carvedilol infusion in group N; these responses corresponded to NE enhancement following carvedilol infusion. Besides, the LVEDP was increased, corresponding to the plasma NE

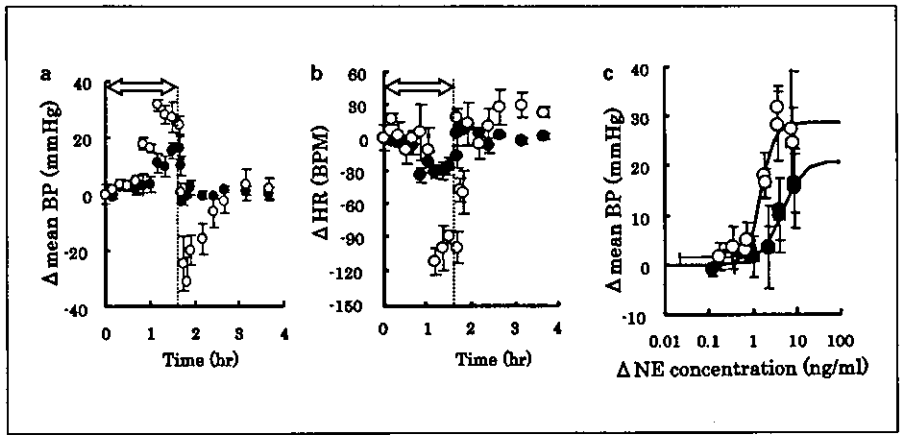

Fig. 4. Time courses of hypertensive effect (**a**) and heart rate (**b**) during and after NE infusion in group N (open circles) and group F (solid circles). **c** Relationship between hypertensive effect and plasma concentration of NE during NE infusion in both groups. The arrows in **a** and **b** indicate the NE infusion period. Each experimental point represents a change in value from that at baseline. BP = Blood pressure; HR = heart rate. The solid lines in **c** represent values calculated using the sigmoid E_{max} model.

concentration enhancement induced by carvedilol infusion (fig. 2b).

Effects of NE Infusion on Blood Pressure and Heart Rate

The plasma NE concentration and its hypertensive effects during intravenous infusion were measured to clarify the changes in the sensitivity to NE in both groups (fig. 4). A sigmoidal E_{max} model was used to show quantitatively the relationship between the plasma NE concentration and its hypertensive effect (fig. 4c). The values for the maximal NE hypertensive effect (E_{max}) were lower and the plasma NE concentrations at the half-maximal NE hypertensive effect (EC_{50}) higher in group F as compared with those in group N, as shown in table 1.

Discussion

Carvedilol, a nonselective β- and α-adrenergic receptor blocker, can potentially blunt the release of NE by blocking presynaptic β_2-adrenergic receptors [1–3]. In fact, the enhancement of the plasma NE concentration in response to the reduced blood pressure was greater with β_1-selective blockers than that with carvedilol, as previously reported [1, 2]. However, there are no previous data comparing carvedilol effects in normal and failing hearts. Although the clinical effects of carvedilol therapy on ejection fraction and mortality have been reported [6, 9], β-blockers have potential negative inotropic effects. Thus, clinical therapy with β-blockers should be initiated using very small doses (3.125 mg of carvedilol twice a day), followed by a doubling of the dose not more frequently than every 1–2 weeks (to a target dose of 2 × 25 mg carvedilol). We have previously reported [10] that carvedilol improved the cardiac function in the group receiving a low dose (2 mg kg^{-1} day^{-1}), but not in the group receiving a high dose (20 mg kg^{-1} day^{-1}) in rats with heart failure. A more cautious dosing schedule may be required in patients with severe heart failure [22].

In the present study, we examined the effects of carvedilol on the SNS in rats with heart failure (group F). The maximal infusion rate of carvedilol was 2.54 mg h^{-1} rat^{-1} (300 g body weight), such that the maximal plasma carvedilol concentration attained at such an infusion rate is the same as that obtained during oral carvedilol treatment at 20 mg kg^{-1} day^{-1} (high dose) in rats with heart failure. The changes in the mean blood pressure and in the plasma NE concentrations in group F and group N, induced by intravenous carvedilol infusion, were determined in conscious rats to avoid the influence of anesthesia. In normal rats, %FS, blood pressure, and $\pm dP\ dt^{-1}$ were significantly and dose dependently reduced by carvedilol infusion. At the same time, the plasma NE concentration was enhanced in response to the blood pressure reduction. Zhang et al. [23] have shown that plasma NE enhancement leads to a rise in LVEDP which might be a possible explanation of the dose-dependent increase of the LVEDP. However, this rise of LVEDP may not only be the result of plasma NE enhancement, but may also be due to the impairment of the ventricular compliance, as previously explained by Zhang et al. [23]. This impairment was clearly reflected by the dose-dependent reduction of %FS and $\pm dP\ dt^{-1}$.

On the other hand, in group F rats, there were no significant changes in %FS and $\pm dP\ dt^{-1}$ by carvedilol infusion. Only the blood pressure was significantly reduced, in a manner similar to that in normal rats. The plasma

NE concentration was also enhanced in response to the blood pressure reduction; however, this enhancement was greater than that in normal rats which indicates that the SNS activity in response to a blood pressure reduction is enhanced in heart failure. Unlike group N rats, in group F rats, there was no significant change in LVEDP, in spite of the greater enhancement of plasma NE concentrations. This might have been induced by one or both of the following: decreased SNS receptor sensitivity (as it will be explained below) and the fact that severe heart failure has raised the LVEDP to maximal values that were difficult to be exceeded by carvedilol infusion.

In order to clarify the change in the sensitivity to NE in the blood pressure regulation system, the relationship between the plasma NE concentration and its hypertensive effect during its intravenous infusion was investigated in both groups, and the sigmoidal E_{max} model was applied to demonstrate this relationship quantitatively (fig. 4c). The values of the maximal NE hypertensive effect (E_{max}) were lower and those of the plasma NE concentration at half-maximal hypertensive effect (EC_{50}) higher in group F as compared with those in group N (table 1). These results indicate that the SNS receptor sensitivity to the hypertensive effect of NE was decreased in group F. Besides, a reduction in the cardiac β-adrenocepter density (B_{max}) in group F rats was demonstrated by the β-adrenergic receptor-binding assay using [^{125}I]iodocyanopindolol (table 1). The results of the in vitro receptor-binding studies are supported by the in vivo experiment, where a rebound phenomenon, an increase in %FS, was induced after carvedilol infusion only in group N and not in group F, probably owing to reduced SNS receptor sensitivity and β-receptor density.

Others have reported that carvedilol causes less enhancement of the plasma NE concentration than β_1-selective blockers. However, no study has been done to compare the effects of carvedilol in normal and failing hearts. In our study, we have demonstrated in a rat model of heart failure that the SNS (presynaptic) activity is enhanced along with a reduction in the sensitivity of the SNS receptors in the cardiovascular regulatory system. We have also demonstrated that high-dose carvedilol causes much greater enhancement of the plasma NE levels in heart failure than in normal rats. This implies that, although the plasma NE enhancement in carvedilol treatment is less than that in β_1-selective blocker treatment, proper care and more consideration should be executed while using high doses of carvedilol in the treatment of heart failure, as it may worsen the condition of the failing heart.

Acknowledgments

This work was supported by a Yujin Memorial grant and grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Promotion and Mutual Aid Corporation for Private Schools of Japan.

References

1 Herman RB, Jesudason PJ, Mustafa AM, Husain R, Choy AM, Lang CC: Differential effects of carvedilol and atenolol on plasma noradrenaline during exercise in humans. Br J Clin Pharmacol 2003;55:134–138.
2 Newton GE, Parker JD: Acute effects of β_1-selective and nonselective β-adrenergic receptor blockade on cardiac sympathetic activity in congestive heart failure. Circulation 1996;94:353–358.
3 Gilbert EM, Abraham WT, Olsen S, Hattler B, White M, Meary P, Larrabee P, Bristow MR: Comparative hemodynamic, left ventricular functional, and antiadrenergic effects of chronic treatment with metoprolol versus carvedilol in the failing heart. Circulation 1996;94:2817–2825.
4 Waagstein F, Hjalmarson A, Varnauskas E, Wallentin I: Effect of chronic beta-adrenergic receptor blockade in congestive cardiomyopathy. Br Heart J 1975;37:1022–1036.
5 The Metoprolol in Dilated Cardiomyopathy (MDC) Trial Study Group: 3-year follow-up of patients randomized in the metoprolol in dilated cardiomyophathy trial. Lancet 1998;351:1180–1181.
6 Australia/New Zealand Heart Failure Research Collaborative Group: Randomized, placebo-controlled trial of carvedilol in patients with congestive heart failure due to ischaemic heart disease. Lancet 1997;349:375–380.
7 Doughty RN, Rodgers A, Sharpe N, MacMahon S: Effects of beta-blocker therapy on mortality in patients with heart failure: A systematic overview of randomized controlled trials. Eur Heart J 1997;18:560–565.
8 Feuerstein GZ, Ruffolo RR: Carvedilol, a novel multiple action antihypertensive agent with antioxidant activity and the potential for myocardial and vascular protection. Eur Heart J 1995;16(suppl F):38–42.
9 Packer M, Bristow MR, Cohn JN, Colucci WS, Fowler MB, Gilbert EM, Shusterman NH: The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. N Engl J Med 1996;334:1349–1357.
10 Watanabe K, Ohta Y, Nakazawa M, Higuchi H, Hasegawa G, Naito M, Fuse K, Ito M, Hirono S, Tanabe N, Hanawa H, Kato K, Kodama M, Aizawa Y: Low dose carvedilol inhibits progression of heart failure in rats with dilated cardiomyopathy. Br J Pharmacol 2000;130:1489–1495.
11 Carvedilol Or Metoprolol European Trial Investigators: Comparison of carvedilol and metoprolol on clinical outcomes in patients with chronic heart failure in the Carvedilol or Metoprolol European Trial (COMET): Randomized controlled trial. Lancet 2003;362:7–13.

12 Packer M, Antonopoulos GV, Berlin JA, Chittams J, Konstam MA, Udelson JE: Comparative effects of carvedilol and metoprolol on left ventricular ejection fraction in heart failure: Results of a meta-analysis. Am Heart J 2001; 141:899–907.
13 Imai K, Higashidate S, Prados PR, Santa T, Adachi-Akahane S, Nagao T: Relation between blood pressure and plasma catecholamine concentration after administration of calcium antagonists to rats. Biol Pharm Bull 1994;17:907–910.
14 Prados P, Santa T, Homma H, Doi H, Narita H, Del Castillo B, Martin MA, Imai K: Comparison of the sympathetic nervous system activity between spontaneously hypertensive and Wistar-Kyoto rats to respond to blood pressure reduction. Biol Pharm Bull 1997;20:341–344.
15 Bol CJJG, Danhof M, Stanski DR, Mandema JW: Pharmacokinetic-pharmacodynamic characterization of the cardiovascular, hypnotic, EEG and ventilatory responses to dexmedetomidine in the rat. J Pharmacol Exp Ther 1997; 283:1051–1058.
16 Kodama M, Hanawa H, Saeki M, Hosono H, Inomata T, Suzuki K, Shibata A: Rat dilated cardiomyopathy after autoimmune giant cell myocarditis. Circ Res 1994;75:278–284.
17 Watanabe K, Hirokawa Y, Shibata A: Determination of catecholamines by high-performance liquid chromatography with electrochemical detection. Bull Coll Biomed Technol Niigata Univ 1987;3:30–35.
18 Holmes C, Eisenhofer G, Goldstein DS: Improved assay for plasma dihydroxyphenylacetic acid and other catechols using high-performance liquid chromatography with electrochemical detection. J Chromatogr B Biomed Appl 1994;653:131–138.
19 Watanabe K, Shibata A, Wakabayashi H, Shimada K, Tsuchihashi H, Nagatomo T: Alterations of binding characteristics of α_1-, β_1-adrenoceptors and Ca^{2+} binding sites in the myocardium of spontaneously hypertensive rats (SHR) by chronically administered bunazosin, atenolol, ketanserin and verapamil. Biol Pharm Bull 1993;16:480–482.
20 Sato S, Koitabashi T, Koshiro A: Pharmacokinetic and pharmacodynamic studies of *L*-dopa in rats. I. Pharmacokinetic analysis of *L*-dopa in rat plasma and striatum. Biol Pharm Bull 1994;17:1616–1621.
21 Gabrielsson J, Weiner D (eds): Pharmacokinetic and Pharmacodynamic Data Analysis: Concepts and Applications, ed 2. Stockholm, Swedish Pharmaceutical Press, 1997.
22 Vanderhoff BT, Ruppel HM, Amsterdam PB: Carvedilol: The new role of beta blockers in congestive heart failure. Am Fam Physician 1998;58:1627–1634, 1641–1642.
23 Zhang J, Pfaffendorf M, van Zwieten PA: Hemodynamic effects of angiotensin II and the influence of angiotensin receptor antagonists in pithed rabbits. J Cardiovasc Pharmacol 1995;25:724–731.

Circ J 2004; **68:** 1223 – 1226

Plasma Concentrations of Cytokines and Neurohumoral Factors in a Case of Fulminant Myocarditis Successfully Treated With Intravenous Immunoglobulin and Percutaneous Cardiopulmonary Support

Satoru Abe, MD; Yuji Okura, MD; Makoto Hoyano, MD; Ryu Kazama, MD; Satoru Watanabe, MD†; Takuya Ozawa, MD; Takashi Saigawa, MD; Manabu Hayashi, MD; Tsuyoshi Yoshida, MD; Hitoshi Tachikawa, MD; Takeshi Kashimura, MD; Keisuke Suzuki, MD††; Masayuki Nagahashi, MD**; Junzo Watanabe, MD**; Kouji Shimada, MD**; Go Hasegawa, MD*; Kiminori Kato, MD; Haruo Hanawa, MD; Makoto Kodama, MD; Yoshifusa Aizawa, MD

A 53-year-old Japanese man with fulminant myocarditis was referred. Percutaneous cardiopulmonary support (PCPS) was introduced immediately and intravenous immunoglobulin (IVIG) therapy followed for 2 days. Cardiac function showed signs of recovery on the 4th hospital day and the patient was weaned from PCPS on the 7th hospital day. Creatine kinase-MB peaked at 12 h after admission and was 176 ng/ml. Endomyocardial biopsy showed active myocarditis. A marked increase of the neutralizing antibody titer suggested coxsackievirus B3 infection. Plasma concentrations of cytokines and neurohumoral factors were analyzed. Proinflammtory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF-α), and anti-inflammatory cytokines, such as IL-1 receptor antagonist, soluble TNF receptor-1 and IL-10, were elevated on admission and all had decreased on the 7th hospital day. Brain natriuretic peptide and noradrenaline were already elevated upon admission (1,940 pg/ml and 4.6 ng/ml, respectively) and decreased thereafter. Although IVIG therapy under PCPS is a common treatment for fulminant myocarditis, the immunological response in vivo remains unclear. This case demonstrated suppression of serum cytokines after IVIG and PCPS treatment. Immunological parameters in those who have been treated with IVIG and PCPS and survived without complications are of great value for evaluation of the therapy. Further analysis with more cases in a multicenter study is necessary. (*Circ J* 2004; **68:** 1223 – 1226)

Key Words: Cytokines; Fulminant myocarditis; Intravenous immunoglobulin; Percutaneous cardiopulmonary support

Cardiopulmonary support is regarded as most important for rescue in cases of fulminant myocarditis with circulatory crisis[1] Despite some case reports suggesting the potential therapeutic efficacy of intravenous immunoglobulin (IVIG) therapy in fulminant myocarditis[2,3] this indication remains controversial and because of the huge cost of that therapy, its biological validity should be examined in humans from an immunological viewpoint[4] However, the scarcity of fulminant myocarditis itself, vascular trouble associated with percutaneous cardiopulmonary support (PCPS), or multi-organ failure (MOF) that complicates the general condition lessen the opportunity to examine the pure immunological response to fulminant myocarditis[1] Therefore, investigating the immunological parameters in those who had been treated with IVIG and survived the cardiopulmonary crisis without complication is of great value for evaluation of IVIG therapy. Herein, we report a rather typical case of fulminant myocarditis in a patient who survived the cardiopulmonary crisis with PCPS and IVIG therapy.

(Received July 18, 2003; revised manuscript received October 24, 2003; accepted November 18, 2003)
*Division of Cardiology, Hematology and Endocrinology/Metabolism, *Department of Cellular Function Division of Cellular and Molecular Pathology, **The Division of Thoracic and Cardiovascular Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata, †Division of Cardiology, National Defense Medical College, Tokorozawa, ††The Division of Cardiology and Internal Medicine, Sado General Hospital, Kanaimachi, Japan*
Mailing address: Yuji Okura, MD, The Division of Cardiology, Niigata University Graduate School of Medical and Dental Sciences, 1-754 Asahimachi, Niigata 951-8510, Japan

Case Report

A 53-year-old Japanese man presented with sore throat, epigastric pain and a fever of 40.0°C at Sado General Hospital in August 2002. Ibuprofen, cefixime, and cimetidine were prescribed with a diagnosis of viral infection. One week later, the patient developed nausea, vomiting and general fatigue and visited the hospital again.

The patient was alert, but his pulse was weak and the rate was 95 beats/min. Blood pressure was 80 mmHg by palpation and a diastolic gallop was noted at the apex of the heart. There was a moist rale in the bilateral lower lung field. The extremities were very cold, but there was no pretibial edema. An electrocardiogram (ECG) showed accelerated idioventricular rhythm at the rate of 98 beats/min. The chest

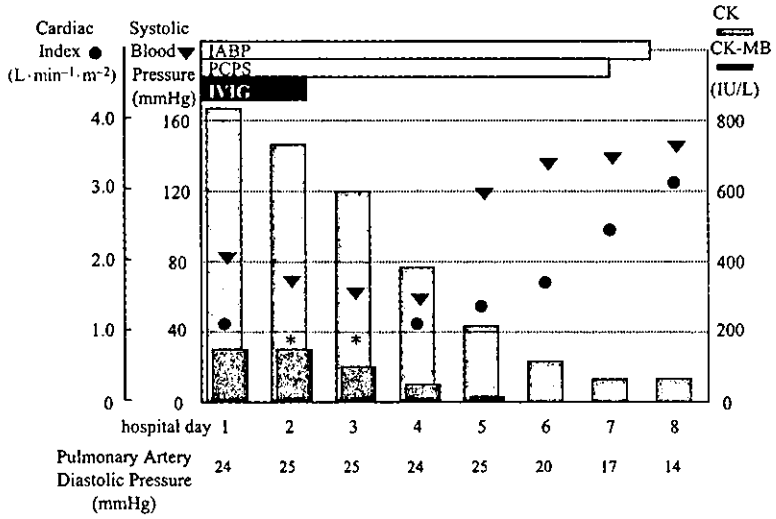

Fig 1. Clinical course. Elevated creatine kinase normalized and the reduced cardiac function improved after intravenous immunoglobulin treatment. *The cardiac index could not be measured with a Swan-Ganz catheter because of extremely low cardiac output during the second and third hospital days. CK, creatine kinase; IVIG, intravenous immunoglobulin; PCPS, percutaneous cardiopulmonary support; IABP, intra-aortic balloon pump.

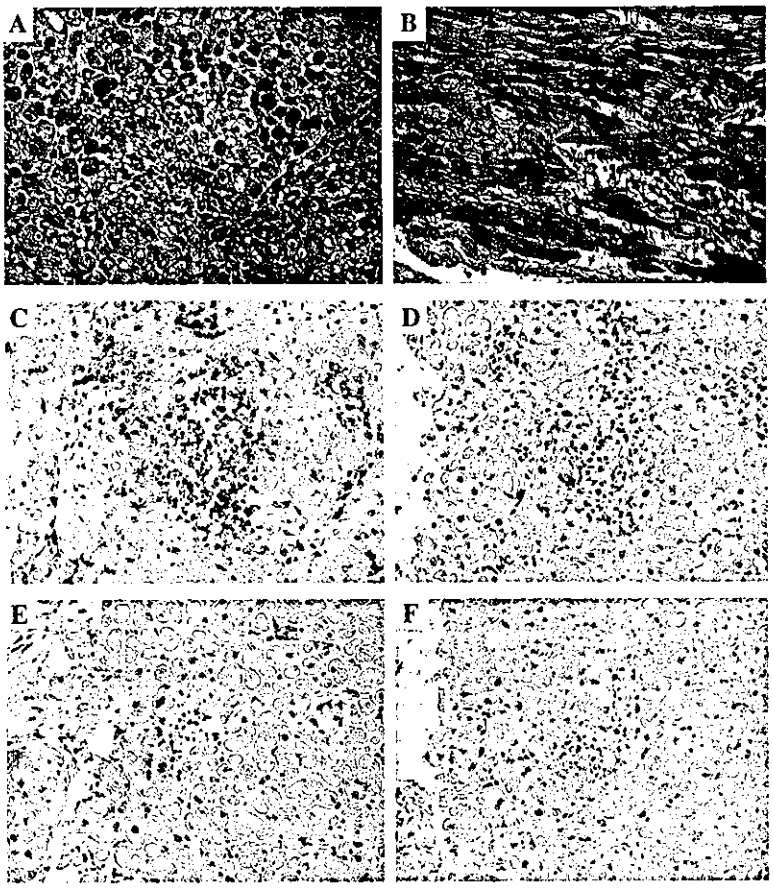

Fig 2. Histopathology and immunohistochemistry of the endomyocardial biopsy on the 10th hospital day. Histopathology (A, B). Myocyte necrosis and degeneration associated with infiltration of the mononuclear cells (H&E; original magnification, ×40). Immunohistochemistry (C, D). Most of the infiltrating cells are positive for CD45RO (T cells) or CD68 (macrophages). (E, F) Only a few cells are positive for CD20 (B cells) or CD1a (dendritic cells) (Original magnification, ×40).

roentgenogram showed cardiomegaly (cardiothoracic ratio 63%) and pulmonary congestion. The leukocyte count was 9,400/μl. Serum concentrations of aspartate aminotransferase (619 U/L), lactic dehydrogenase (1,639 U/L), creatine kinase (825 IU/L), and creatine kinase-MB (149 ng/ml) were also elevated. The echocardiogram showed severe diffuse hypokinesis and wall thickening of the left ventricle with slight pericardial effusion. The thickness of the interventricular septum and posterior wall was 13 mm and 12 mm, respectively. The left ventricular end-diastolic dimension, end-systolic dimension and ejection fraction (EF) calculated by the Teichholz method were 48 mm, 46 mm, and 10%, respectively. Cardiac valves were normal. In the afternoon of the day of admission. he developed cardiac arrest and intravenous administration of inotropic agents, temporary pacing and intra-aortic balloon pump (IABP) failed to produce hemodynamic improvement. He was transferred to the university hospital in the evening by helicopter with a clinical diagnosis of fulminant myocarditis.

On his arrival, the cardiac index was 1.2 L·min^{-1}·m^{-2}. We immediately started PCPS and IVIG treatment after ob-

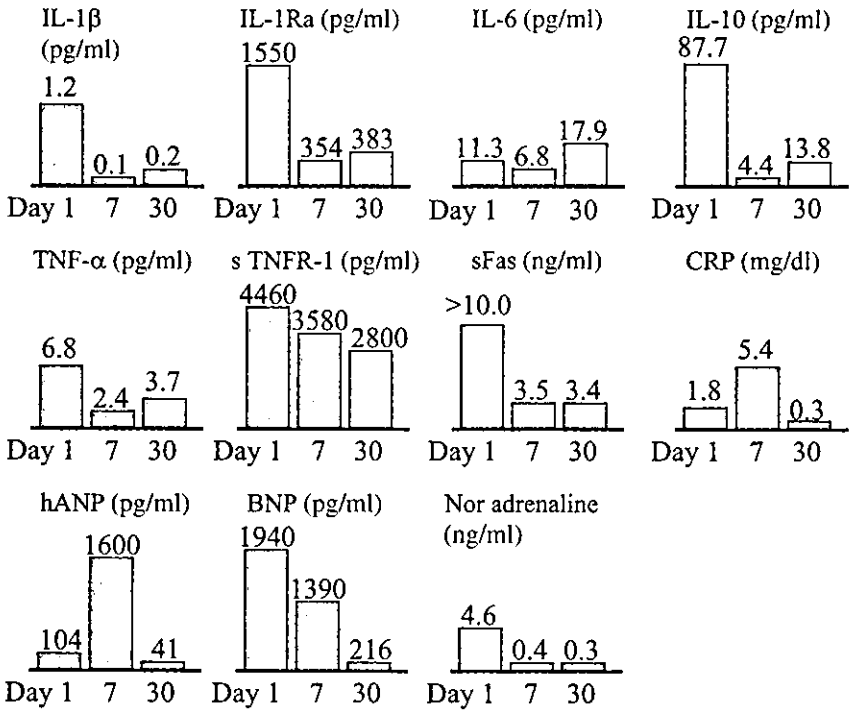

Fig 3. Serum concentrations of cytokines and neurohumoral markers during the clinical course. Day 1, first hospital day. Immunoglobulin was administered on the first and second hospital day. On day 7, the patient was weaned off percutaneous cardiopulmonary support (PCPS) without complications.

taining written informed consent. Gamma-Venin (Aventis Corporation, Japan) of $0.5 g \cdot kg^{-1} \cdot day^{-1}$ was administered for the first 2 days after admission (Fig 1). However, the cardiac index decreased and could not be measured with a Swan-Ganz catheter during the second and third hospital days because of extremely low cardiac output. His ECG showed no spontaneous excitation for the first 3 days. Cardiac arrest continued until the 4th hospital day, when the echocardiogram showed recovery of left ventricular wall motion. On the 7th hospital day, the patient was weaned off the system without complications of PCPS, MOF or serious infection. Endomyocardial biopsy on the 10th hospital day showed infiltrates of mononuclear cells associated with myocyte necrosis (Fig 2). Neither polymorphonuclear cells nor giant cells were observed. Immunohistochemistry using antibodies to CD45RO and CD68 (DAKO Cytomation, Corp) showed that the mononuclear cells were mostly T lymphocytes or macrophages. There were very few CD20 and CD1a positive cells (DAKO Cytomation, Corp), which represent B lymphocytes and dendritic cells, respectively.

One month later, coronary angiography and left ventriculography (LVG) were performed and no significant stenosis in the coronary arteries was observed. The left ventricular ejection fraction (LVEF) improved to 49%. Complete atrioventricular block remained, but the patient was discharged with a permanent pacemaker. Neutralizing antibody titers for influenza virus, echovirus, adenovirus and parainfluenza virus did not rise significantly between admission and discharge. However, the neutralizing antibody titer for coxsackievirus B3 showed 256-fold increase on admission and reached a plateau of 512-fold on discharge.

Plasma concentrations of neurohumoral factors and cytokines were examined at 3 time points: on admission (day 1), after weaning from the PCPS (day 7) and on the day of discharge (day 30) (Fig 3). Samples were analyzed at Mitsubishi Kagaku Bio-Clinical Laboratories, Inc in Tokyo, Japan. Samples were collected and stored in accordance with the direction of the laboratories. Proinflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF-α), were elevated on day 1 and decreased on day 7. Anti-inflammatory cytokines, such as IL-1 receptor antagonist (IL-1Ra), soluble TNF receptor-1 (sTNFR1) and IL-10, demonstrated similar changes to the proinflammatory cytokines. All cytokine concentrations except for sTNFR1 and soluble Fas (sFas) demonstrated a transient fall on day 7, followed by a slight increase that was below that of day 1. C-reactive protein (CRP) also increased in the peripheral blood and peaked not on day 1, but on day 7. Brain natriuretic peptide and noradrenaline were already elevated on admission (1,940 pg/ml and 4.6 ng/ml, respectively) and decreased thereafter. In contrast, human atrial natriuretic peptide (hANP) peaked on day 7. Most data had almost normalized by day 30.

Discussion

The effect of IVIG has been examined in chronic heart failure[5-7] but a clinical trial of IVIG therapy in fulminant myocarditis does not exist. Intervention in a Myocarditis and Acute Cardiomyopathy (IMAC) trial failed to demonstrate evidence of the therapeutic efficacy of IVIG on recent onset dilated cardiomyopathy (DCM). Forty-five percent of the IMAC population was given β-blockers and improvement in the LVEF was greater than those in a case series by Dec et al[8] and also one by Steimle et al[9] both of which were conducted before β-blockers were widely used in the management of patients with systolic dysfunction. The effect of IVIG in the IMAC trial proved much slighter than that of β-blockers, angiotensin converting enzyme inhibitors, or angiotensin II receptor antagonists in the management of DCM[5] However, intervention of the immune system may be more important than that of the neurohumoral system in acute heart failure caused by viral infection, such as fulminant myocarditis.

Lieberman et al first classified myocarditis as either fulminant or acute on the basis of the clinicopathological

criteria, including the severity of illness at presentation.[10] McCarthy et al reported that fulminant myocarditis as an entity has a good long-term outcome and they emphasized the importance of intensive cardiopulmonary support in the event of circulatory crisis.[11] However, the Japanese National Survey demonstrated that MOF, life-threatening arrhythmia and complication of PCPS, such as leg ischemia, often occurred during a circulatory crisis and reduced the survival rate to 58%! A combination of PCPS and IVIG is a promising therapy, but immunological markers that reflect heart inflammation are currently not detectable in the peripheral blood. In addition to the small number of cases of fulminant myocarditis, the low survival rate lessens the opportunity to examine the mechanism of the disease. Therefore, to examine the immunological parameters in those who were treated with IVIG and survived the cardiopulmonary crisis without complication is of great value for verifying IVIG therapy.

Gullestad et al demonstrated an IVIG-induced change in the balance between pro- and anti-inflammatory cytokines in congestive heart failure.[6] Contrary to their observation that improvement of LVEF was associated with an enhanced anti-inflammatory net effect, in the present case both types of cytokines increased at admission and showed a transient fall after IVIG therapy. Because both IL-1 and TNF-α can impair myocardial performance, it is conceivable that inhibition of the effects of these cytokines may be beneficial. Interestingly, both pro- and anti-inflammatory cytokines showed a significant decrease when the patient was successfully weaned of PCPS, despite hemodynamic markers remaining at a high level. That may reflect immunological recovery in vivo proceeding hemodynamic recovery. However, we can not conclude that the immunological effect of IVIG was the main cause of the change in the circulating cytokines concentrations. Recovery of heart failure, dependent or independent of the IVIG effect, might contribute to those alternations. Further observations under various conditions are needed.

Kishimoto et al examined the effects of immunoglobulin on murine myocarditis induced by coxsackievirus B3, encephalomyocarditis virus, and in rat autoimmune myocarditis.[12–14] They reported that immunoglobulin therapy suppressed acute viral myocarditis by an anti-viral effect, an anti-inflammatory effect and improvement in extracellular matrix changes.[12,13] Moreover, immunoglobulin therapy suppressed experimental giant cell myocarditis in rats, associated with the suppression of the expression of dendritic cell via inhibitory Fc receptor.[14] Some immunomodulatory effect of IVIG might have worked favorably in the present case.

We have reported the immunological parameters in those who were treated with IVIG and PCPS and survived without complications. According to our retrospective study conducted before IVIG were routinely used in the management of patients with fulminant myocarditis,[15] the prognosis of the present case was classified as poor because of the high sFas concentrations. Therefore, the addition of IVIG therapy may be one of the recent improvements in the treatment for fulminant myocarditis. Our observations may show that there is an immunological effect of IVIG; however, it does not assure the clinical efficacy of IVIG therapy in fulminant myocarditis. Accordingly, a large number of patients should be studied and more attention should be paid when interpreting the effect of IVIG on the hemodynamic and immunological variables.

References

1. Aoyama N, Izumi T, Hiramori K, Isobe M, Kawana M, Hiroe M, et al. National survey of fulminant myocarditis in Japan: Therapeutic guidelines and long-term prognosis of using percutaneous cardiopulmonary support for fulminant myocarditis. *Circ J* 2002; **66:** 133–144.
2. Takeda Y, Yasuda S, Miyazaki S, Daikoku S, Nakatani S, Nonogi H. High-dose immunoglobulin G therapy for fulminant myocarditis. *Jpn Circ J* 1998; **62:** 871–872.
3. Shioji K, Matsuura Y, Iwase T, Kitaguchi S, Nakamura H, Yodoi J, et al. Successful immunoglobulin treatment for fulminant myocarditis and serial analysis of serum thioredoxin: A case report. *Circ J* 2002; **66:** 977–980.
4. Izumi T. Clinical presentation of fulminant myocarditis. *Nippon Naika Gakkai Zasshi* 2003; **92:** 463–470.
5. McNamara DM, Holubkov R, Starling RC, Dec GW, Loh E, Torre-Amione G, et al. Controlled trial of intravenous immune globulin in recent-onset dilated cardiomyopathy. *Circulation* 2001; **103:** 2254–2259.
6. Gullestad L, Aass H, Fjeld JG, Wikeby L, Andreassen AK, Ihlen H, et al. Immunomodulating therapy with intravenous immunoglobulin in patients with chronic heart failure. *Circulation* 2001; **103:** 220–225.
7. McNamara DM, Rosenblum WD, Janosko KM, Trost MK, Villaneuva FS, Demetris AJ, et al. Intravenous immune globulin in the therapy of myocarditis and acute cardiomyopathy. *Circulation* 1997; **95:** 2476–2478.
8. Dec GW Jr, Palacios IF, Fallon JT, Aretz HT, Mills J, Lee DC, et al. Active myocarditis in the spectrum of acute dilated cardiomyopathies: Clinical features, histologic correlates, and clinical outcome. *N Engl J Med* 1985; **312:** 885–890.
9. Steimle AE, Stevenson LW, Fonarow GC, Hamilton MA, Moriguchi JD. Prediction of improvement in recent onset cardiomyopathy after referral for heart transplantation. *J Am Coll Cardiol* 1994; **23:** 553–559.
10. Lieberman EB, Hutchins GM, Herskowitz A, Rose NR, Baughman KL. Clinicopathologic description of myocarditis. *J Am Coll Cardiol* 1991; **18:** 1617–1626.
11. McCarthy RE 3rd, Boehmer JP, Hruban RH, Hutchins GM, Kasper EK, Hare JM, et al. Long-term outcome of fulminant myocarditis as compared with acute (nonfulminant) myocarditis. *N Engl J Med.* 2000; **342:** 690–695.
12. Takada H, Kishimoto C, Hiraoka Y. Therapy with immunoglobulin suppresses myocarditis in a murine coxsackievirus B3 model: Antiviral and anti-inflammatory effects. *Circulation* 1995; **92:** 1604–1611.
13. Kishimoto C, Takamatsu N, Kawamata H, Shinohara H, Ochiai H. Immunoglobulin treatment ameliorates murine myocarditis associated with reduction of neurohumoral activity and improvement of extracellular matrix change. *J Am Coll Cardiol* 2000; **36:** 1979–1984.
14. Shioji K, Kishimoto C, Sasayama S. Fc receptor-mediated inhibitory effect of immunoglobulin therapy on autoimmune giant cell myocarditis: Concomitant suppression of the expression of dendritic cells. *Circ Res* 2001; **89:** 540–546.
15. Fuse K, Kodama M, Okura Y, Ito M, Hirono S, Kato K, et al. Predictors of disease course in patients with acute myocarditis. *Circulation* 2000; **102:** 2829–2835.

Linkage Between Mechanical and Electrical Alternans in Patients with Chronic Heart Failure

MAKOTO KODAMA, M.D., KIMINORI KATO, M.D., SATORU HIRONO, M.D., YUJI OKURA, M.D., HARUO HANAWA, M.D., TSUYOSHI YOSHIDA, M.D., MANABU HAYASHI, M.D., HITOSHI TACHIKAWA, M.D., TAKESHI KASHIMURA, M.D., KENICHI WATANABE, M.D.,* and YOSHIFUSA AIZAWA, M.D.

From the Division of Cardiology, Department of Cardiovascular and Vital Control, Niigata Graduate School of Medical & Dental Sciences, and *Department of Clinical Pharmacology, Niigata College of Pharmacy, Niigata, Japan

Mechanoelectrical Alternans in Chronic Heart Failure. ***Introduction:*** **Progressive heart failure and ventricular fibrillation are major causes of death in patients with chronic heart failure. Mechanical alternans (pulsus alternans) has been observed in patients with severe congestive heart failure. Visible T wave alternans occasionally is a precursor of ventricular fibrillation. We investigated the occurrence of both cardiac alternans in 94 patients with chronic heart failure.**

Methods and Results: **Mean left ventricular ejection fraction (LVEF) of the study population was 35 ± 10%. Mechanical alternans was detected in left ventricular pressure during diagnostic cardiac catheterization. Only sustained mechanical alternans was included in the study. Visible T wave alternans, not microvolt alternans, was noted on standard surface ECG. Cardiac alternans was examined at rest, during physiologic tachycardia, and during stepwise dobutamine loading (2–4–8 μg/kg/min). Prevalences of mechanical and electrical alternans were 19.1% and 4.4% at rest, 45.5% and 8.0% during physiologic tachycardia, and 62.1% and 9.5% under dobutamine loading. Overall, 70 patients (74.5%) showed mechanical alternans and 10 patients (10.6%) showed T wave alternans. T wave alternans always appeared with large mechanical alternans. Among patients with mechanical alternans, cases with T wave alternans showed lower LVEF than those without (27.5 ± 4.4 and 35.1 ± 10.2, $P < 0.002$).**

Conclusion: **Visible T wave alternans was detectable in patients with chronic heart failure, especially under tachycardia or catecholamine exposure. Investigating mechanical and mechanoelectrical alternans may bring new insights into the management of patients with chronic heart failure.** *(J Cardiovasc Electrophysiol, Vol. 15, pp. 295-299, March 2004)*

chronic heart failure, dilated cardiomyopathy, tachycardia, mechanical alternans, T wave alternans

Introduction

Patients with chronic heart failure have a poor prognosis. The two major causes of death in this patient population are progressive heart failure and unexpected sudden death due to ventricular fibrillation. The mortality of patients with chronic heart failure increases in accordance with the deterioration of cardiac functional class. However, almost half of the total deaths results from progression of heart failure; the remainder result from sudden death in any functional class.[1-3]

Mechanical alternans is a poorly understood phenomenon of alternating strong and weak beats with a constant beat-to-beat interval. The phenomenon has been observed in patients with severe congestive heart failure caused by global left ventricular dysfunction. It is considered a terminal sign in patients with chronic heart failure.[4-7] Although the precise origin of mechanical alternans remains uncertain, several experimental studies have suggested that mechanical alternans is derived from abnormal intracellular Ca^{2+} cycling in failing cardiomyocytes.[8-10]

Address for correspondence: Makoto Kodama, M.D., Division of Cardiology, Department of Cardiovascular and Vital Control, Niigata Graduate School of Medical & Dental Sciences, Asahimachi 1-754, Niigata 951-8510, Japan. Fax: 81-25-227-0774; E-mail: kodamama@med.niigata-u.ac.jp

Manuscript received 16 January 2003; Accepted for publication 16 October 2003.

doi: 10.1046/j.1540-8167.2004.03016.x

Electrical alternans is a phenomenon showing alternating beat-by-beat changes in ECG contour under a constant R-R interval. Electrical alternans is composed of QRS alternans and ST-T wave alternans. QRS alternans has been considered to be related to changes of the ventricular activation process or positional oscillation of the heart. On the other hand, ST-T wave alternans depends on oscillation of the action potential duration.[6] This form of electrical alternans leads to an increase in the dispersion of refractory periods. Indeed, ST-T wave alternans has been shown to be a precursor of ventricular fibrillation in patients with acute coronary syndrome and long QT syndrome.[11-14]

Mechanical alternans seems to be related to the risk for progressive heart failure, and T wave alternans may be related to the risk for ventricular fibrillation. However, correlation between both forms of cardiac alternans has not been fully examined in clinical situations. In this study, we examined the linkage between mechanical alternans and T wave alternans in patients with chronic heart failure

Materials and Methods

Patients

The study population consisted of 94 consecutive patients (71 men and 23 women; mean age 50.8 ± 13.8 years, range 18–71) with chronic heart failure due to mild-to-severe left ventricular dysfunction (Table 1). Underlying

TABLE 1
Characteristics of the Study Patients

Gender	
Male	71
Female	23
Age (years)	
18–39	19
40–49	24
50–59	24
60–71	27
Underlying heart disease	
Idiopathic dilated cardiomyopathy	78
Postmyocarditic dilated cardiomyopathy	8
Uremic cardiomyopathy	6
Previous mitral valve replacement	2
NYHA functional class	
I	5
II	55
III	34
Rhythm	
Sinus	68
Atrial fibrillation	26
Beta-blocker	
No	91
On titration*	3
Left ventricular end-systolic volume indices	
≤59	26
60–99	38
100–149	19
≥150	11
Ejection fraction	
50–59	5
40–49	27
30–39	27
20–29	23
≤19	12

*Low dose of beta-blocker was started but did not reach the maintenance dose.
NYHA = New York Heart Association.

heart diseases were idiopathic dilated cardiomyopathy, postmyocarditic cardiomyopathy, and other secondary dilated cardiomyopathies. Patients with active myocarditis, significant valvular diseases, hypertensive heart diseases, hypertrophic cardiomyopathy, or ischemic heart diseases were excluded from the study. Sixty-eight patients with sinus rhythm; the remainder had atrial fibrillation. Mean left ventricular end-diastolic volume index of the study population was 127 ± 42 mL/m^2, and mean left ventricular ejection fraction was 35 ± 10%. After admission to the hospital, congestive heart failure was controlled. After written informed consent was obtained, cardiac catheterization was performed in each patient. All study protocols were approved by the ethics committee of Niigata University.

Definitions and Assessment of Mechanical and Electrical Alternans

Mechanical alternans was detected by direct measurement of left ventricular pressure at diagnostic cardiac catheterization. The magnitude of the pressure difference between the strong and weak beats was defined as the alternating pressure. Patients with sustained mechanical alternans, which was defined as constant alternating pressure exceeding 4 mmHg that continued for more than 20 beats, were judged as positive for mechanical alternans. Patients with decay mechanical alternans were not included.[7] T wave electrical alternans was assessed by surface 3-lead ECG monitoring or standard 12-lead ECG, which was recorded simultaneously with left ventricular pressure. Visually apparent alternating beat-by-beat changes of ECG contour over 20 beats, not microvolt alternans, was included in T wave alternans. When two blinded cardiologists were in agreement, T wave alternans was judged as positive. Decay alternans of both forms were judged as negative in this study.

Pacing and Dobutamine Loading

In order to assess left ventricular functional reserve, our cardiac catheterization protocol was supplemented with physiologic tachycardia pacing and dobutamine infusion. The occurrence of cardiac alternans was determined under this protocol. Right atrial pacing at 110 beats/min was carried out in the patients with sinus rhythm. We assessed cardiac alternans at 100 beats/min in some patients when Wenckebach AV block appeared at 110 beats/min. For the patients with atrial fibrillation, right ventricular pacing at 110 beats/min was performed. Patients with tachycardia > 110 beats/min under base conditions or patients whose heart rate was not captured by right ventricular pacing were excluded prior to entry into the study. After the pacing study, stepwise dobutamine loading was performed. Dobutamine was intravenously infused, with upward titration of the infusion rate at 5-minute intervals, beginning with a dose of 2 μg/kg/min and increasing to 4 μg/kg/min and then 8 μg/kg/min. Three minutes after the start of dobutamine infusion, constant pacing at 110 beats/min was started, then left ventricular pressure and surface ECG were recorded at 5-minute interval.

Statistical Analysis

The clinical and hemodynamic parameters between the two groups were compared using the unpaired Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Prevalence of Mechanical and Electrical Alternans

The prevalence of mechanical and T wave alternans was 19.1% and 4.4% in 68 patients with sinus rhythm at rest. The frequencies were increased by physiologic tachycardia and by low doses of dobutamine infusion (Table 2). During pacing tachycardia, mechanical alternans was observed in 45.5% patients and T wave alternans in 8.0%. The frequencies of mechanical and T wave alternans increased in 62.1% and 9.5% under dobutamine loading. Overall, prevalence of mechanical alternans was 78% in patients with sinus rhythm (53/68 patients) and 65% in patients with atrial fibrillation (17/26 patients) under any condition. Consequently, 70 patients (74.5%) showed mechanical alternans, and 10 patients (10.6%) revealed T wave alternans. T wave alternans always appeared with large mechanical alternans (Fig. 1), and no case revealed T wave alternans without mechanical alternans. Alternating pressure of mechanical alternans accompanied by T wave alternans was larger than that not accompanied by T wave alternans (18.4 ± 9.6 mmHg vs 10.4 ± 5.5 mmHg, $P < 0.05$).

TABLE 2

Prevalence of Mechanical and T Wave Alternans in Patients with Chronic Heart Failure

Condition	No. of Patients	Mechanical Alternans	T Wave Alternans
At rest	68	13 (19.1%)	3 (4.4%)
Pacing	88	40 (45.5%)	7 (8.0%)
Dobutamine	74	46 (62.1%)	7 (9.5%)
Any condition	94	70 (74.5%)	10 (10.6%)

T wave alternans always appeared with mechanical alternans.

Left Ventricular Function of Patients With and Without Cardiac Alternans

Patients with mechanical alternans revealed larger left ventricular end-diastolic and end-systolic volumes than those without. Left ventricular ejection fraction of patients with mechanical alternans was lower than that of patients without mechanical alternans. Among patients with mechanical alternans, those with T wave alternans had lower left ventricular ejection fraction than those without T wave alternans (27.5 ± 4.4% vs 35.1 ± 10.2%, $P < 0.002$) (Table 3).

Follow-Up of the Study Population

The prognosis of the study patients was investigated. Twenty-three patients died during follow-up: 13 congestive heart failure, 8 sudden death, and 2 noncardiac causes. Mechanical alternans was observed in 9 of 13 patients who died of heart failure, 6 of 8 who died of sudden death, and 1 of 2 who died of noncardiac causes. Of the 23 patients who died, electrical alternans was observed in only 2 (1 heart failure and 1 sudden death). We could not draw any conclusion regarding the importance of mechanoelectrical alternans in predicting the prognosis of patients with chronic heart failure in this population.

Discussion

Mechanical alternans is highly prevalent in patients with chronic heart failure. Furthermore, visible T wave alternans, not microvolt alternans, also is detectable in this population, especially during tachycardia or catecholamine exposure. T wave alternans always appeared with large mechanical alternans. Patients who showed mechanoelectrical alternans had severely disturbed left ventricular function. The finding that T wave alternans was closely linked to large mechanical alternans may explain the linkage of ventricular fibrillation and progressive heart failure in patients with chronic heart failure.

The precise origin of mechanical alternans still has not been determined. Several theories have been proposed as the primary cause of mechanical alternans, such as a mechanism based on the Frank-Starling principle, an incomplete relaxation theory, a partial asystole theory, oscillation of the action potential duration, and oscillation of Ca^{2+} release from the sarcoplasmic reticulum (SR).[15-18] Recent experimental studies have suggested that oscillation of Ca^{2+} release from SR due to abnormal intracellular Ca^{2+} cycling in failing myocardium is the most reliable explanation for mechanical alternans.[8-10] We previously reported on clinical studies in which primary oscillation was associated with mechanical alternans during the isovolumic contraction period, so the Ca^{2+} oscillation theory also is likely in patients with chronic heart failure.[7] Abnormal Ca^{2+} cycling is one of the important intracellular events contributing to the pathogenesis of failing myocardium. Accordingly, the occurrence of mechanical alternans may be used as a clinical marker of the failing myocardium in patients with chronic heart failure.

The primary origin of T wave alternans also is uncertain. Spatial distribution of heterogeneous myocardium having long or short refractory periods leads to partial asystole

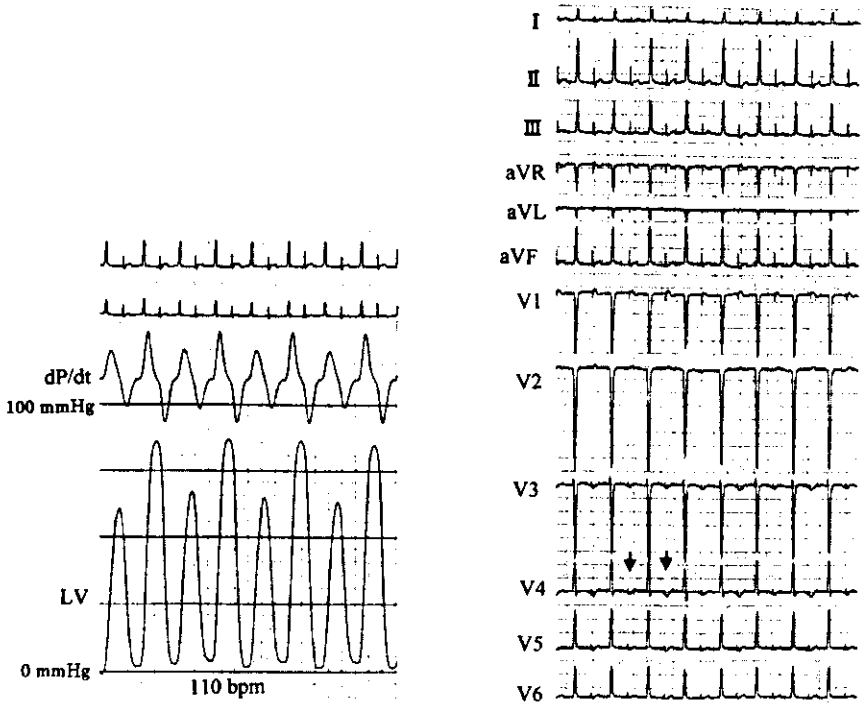

Figure 1. *Simultaneous recordings of left ventricular pressure and the surface ECG in a patient during right atrial pacing at 110 beats/min (bpm). The left ventricular pressure and first pressure derivatives (dP/dt) shows mechanical alternans. The alternating pressure is about 20 mmHg. Concurrently, T wave alternans is apparent in the lead V_4.*

TABLE 3
Left Ventricular Function in Patients With and Without Cardiac Alternans

	M Alternans (–) T Alternans (–)	M Alternans (–) T Alternans (+)	M Alternans (+) T Alternans (–)		M Alternans (+) T Alternans (+)
No. of patients	24	0	60		10
LVEDVI	116.8 ± 28.3	—	129.7 ± 46.6	NS	136.1 ± 38.6
LVESVI	74.5 ± 24.2	—	86.5 ± 41.3	NS	98.5 ± 34.0
LVEF	36.3 ± 11.1	—	35.1 ± 10.2	P < 0.002	27.5 ± 4.4
Alternating pressure	—	—	10.4 ± 5.5	P < 0.05	18.4 ± 9.6

LVEDVI = left ventricular end-diastolic volume index; LVEF = left ventricular ejection fraction; LVESVI = left ventricular end-systolic volume index.

under tachycardia and reveals electrical alternans that has features of both activation and repolarization alternans.[6,14] Another explanation of T wave alternans is beat-to-beat alternans of intracellular Ca^{2+} concentration, which affects the action potential duration.[13] Both theories agree that T wave alternans represents an abnormal and heterogeneous repolarization process in the myocardium, and that it is a predictor of polymorphic ventricular tachycardia or ventricular fibrillation. Recently, microvolt T wave alternans has been investigated extensively as a way to stratify the risk of ventricular fibrillation in various heart diseases.[19,20] We used visible T wave alternans in this study because a visually apparent T wave alternans, not a microvolt alternans, usually is a precursor to ventricular fibrillation.

Mechanical alternans can be provoked in isolated cardiomyocytes.[8] Several experimental studies using cardiomyocytes have revealed that mechanical alternans always is accompanied by alternating changes of the action potential duration through Ca^{2+}-dependent ion currents.[21,22] Alternating changes of the action potential duration leads to ST-T electrical alternans. Therefore, if the magnitude of mechanical alternans, namely, intracellular Ca^{2+} oscillation, is sufficiently enough, T wave alternans will be manifested on the surface ECG. In this study, T wave alternans appeared only with large mechanical alternans. Another explanation is that large mechanical alternans leads to movement alternans of the heart in the chest, which would lead to activation and repolarization alternans. Most of the small mechanical alternans was not accompanied by T wave alternans. If more sensitive methods (e.g., microvolt T wave alternans) were used, every mechanical alternans might be found to be accompanied by electrical alternans. Mechanical alternans, especially the large ones accompanied by T wave alternans, possibly can be used to predict the risk for sudden death in patients with chronic heart failure.

We previously reported that long-term beta-blocker therapy could improve left ventricular function and suppress the occurrence of mechanical alternans in patients with chronic heart failure.[23] Several multicenter clinical trials have revealed that beta-blocker therapy could improve the prognosis of patients with chronic heart failure.[24–26] Beta-blockers can improve left ventricular function with long-term use. Beta-blockers also can reduce the incidence of sudden death. It is unclear whether the two major beneficial effects of beta-blockers, namely, improvement of left ventricular function and reduction of the risk for ventricular fibrillation, are derived from an independent or a common mechanism of beta-blockers. Our study elicits a unique explanation that reduction of the risk for ventricular fibrillation may depend on suppression of mechanoelectrical alternans by beta-blockers.

Mechanical alternans was highly prevalent in patients with chronic heart failure, especially during tachycardia and catecholamine exposure. Visible T wave alternans also was detectable in patients with chronic heart failure, and T wave alternans always appeared with large mechanical alternans. Among patients with mechanical alternans, cases with T wave alternans showed severely disturbed left ventricular function. This study demonstrated a linkage between mechanical alternans and electrical alternans in patients with nonischemic chronic heart failure.

References

1. The CONSENSUS Trial Study Group: Effects of enalapril on mortality in severe congestive heart failure. Results of the cooperative north Scandinavian enalapril survival study (CONSENSUS). N Engl J Med 1987;316:1429-1435.
2. The SOLVD Investigators: Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. N Engl J Med 1991;325:293-302.
3. The SOLVD Investigators: Effect of enalapril on mortality and the development of heart failure in asymptomatic patients with reduced left ventricular ejection fractions. N Engl J Med 1992;327:685-691.
4. White PD: Alternation of the pulse: a common clinical condition. Am J Med Sci 1915;150:82-97.
5. Lab MJ, Seed WA: Pulsus alternans. Cardiovasc Res 1993;27:1407-1412.
6. Surawicz B, Fisch C: Cardiac alternans: diverse mechanisms and clinical manifestations. J Am Coll Cardiol 1992;20:483-499.
7. Kodama M, Kato K, Hirono S, Okura Y, Hanawa H, Ito M, Fuse K, Shiono T, Watanabe K, Aizawa Y: Mechanical alternans in patients with chronic heart failure. J Card Failure 2001;7:138-145.
8. Lab MJ, Lee JA: Changes in intracellular calcium during mechanical alternans in isolated ferret ventricular muscle. Circ Res 1990;66:585-595.
9. Kihara Y, Morgan JP: Abnormal Ca^{++} handling is the primary cause of mechanical alternans: Study in ferret ventricular muscles. Am J Physiol 1991;261(Heart Circ Physiol 30):H1746-H1755.
10. Narayan P, McCune SA, Robitaille PML, Hohl CM, Altschuld RA: Mechanical alternans and the force-frequency relationship in failing rat hearts. J Mol Cell Cardiol 1995;27:523-530.
11. Rosenbaum DS, Jackson LE, Smith JM, Garan H, Ruskin JN, Cohen RJ: Electrical alternans and vulnerability to ventricular arrhythmias. N Engl J Med 1994;330:235-241.
12. Pastore JM, Girouard SD, Laurita KR, Akar FG, Rosenbaum DS: Mechanism linking T-wave alternans to the genesis of cardiac fibrillation. Circulation 1999;99:1385-1394.
13. Shimizu W, Antzelevitch C: Cellular and ionic basis for T-wave alternans under long-QT conditions. Circulation 1999;99:1499-1507.
14. Armoundas AA, Tomaselli GF, Esperer HD: Pathophysiological basis and clinical application of T-wave alternans. J Am Coll Cardiol 2002;40:207-217.
15. Mitchell JH, Sarnoff SJ, Sonnenblick EH: The dynamics of pulsus alternans: alternating end-diastolic fiber length as a causative factor. J Clin Invest 1963;42:55-63.

16. Freeman GL, Widman LE, Campbell JM, Colston JT: An evaluation of pulsus alternans in closed-chest dogs. Am J Physiol 1992;262(Heart Circ Physiol 31):H278-H284.
17. Nwasokwa ON: Mechanism of mechanical alternans in ischemia-reperfusion: role of deficient relaxation of the strong twitch. Am J Physiol 1995;269(Heart Circ Physiol 38):H169-H175.
18. Spear JF, Moore EN: A comparison of alternation in myocardial action potentials and contractility. Am J Physiol 1971;220:1708-1716.
19. Nearing BD, Huang AH, Verrier RL: Dynamic tracking of cardiac vulnerability by complex demodulation of the T wave. Science 1991;252:437-440.
20. Kitamura H, Ohnishi Y, Okajima K, Ishida A, Galeano EJ, Adachi K, Yokoyama M: Onset heart rate of microvolt-level-T-wave alternans provides clinical and prognostic value in nonischemic dilated cardiomyopathy. J Am Coll Cardiol 2002;39:295-300.
21. Hirayama Y, Saitoh H, Atarashi H, Hayakawa H: Electrical and mechanical alternans in canine myocardium in vivo. Dependence on intracellular calcium cycling. Circulation 1993;88:2894-2902.
22. Rubenstein DS, Lipsius SL: Premature beats elicit a phase reversal of mechanoelectrical alternans in cat ventricular myocytes. A possible mechanism for reentrant arrhythmias. Circulation 1995;91:201-214.
23. Kodama M, Kato K, Hirono S, Hanawa H, Okura Y, Ito M, Fuse K, Shiono T, Tachikawa H, Hayashi M, Abe S, Yoshida T, Aizawa Y: Changes in the occurrence of mechanical alternans after long-term β-blocker therapy in patients with chronic heart failure. Jpn Circ J 2001; 65:711-716.
24. CIBIS-II Investigators and Committees: The cardiac insufficiency bisoprolol study II (CIBIS-II): A randomised trial. Lancet 1999;353:9-13.
25. MERIT-HF Study Group: Effect of metoprolol CR/XL in chronic heart failure: Metoprolol CR/XL randomised intervention trial in congestive heart failure (MERIT-HF). Lancet 1999;353:2001-2007.
26. Packer M, Coats AJS, Fowler MB, Katus HA, Krum H, Mohacsi P, Rouleau JL, Tendera M, Castaigne A, Roecher EB, Schultz MK, DeMets DL, for the Carvedilol Prospective Randomized Cumulative Survival Study Group: Effect of carvedilol on survival in severe chronic heart failure. N Engl J Med 2001;344:1651-1658.

Tohoku J. Exp. Med., 2004, **202**, 155-161

A Novel Method to Assay Proteins in Blood Plasma after Intravenous Injection of Plasmid DNA

HARUO HANAWA, RITSUO WATANABE, MANABU HAYASHI, TSUYOSHI YOSHIDA, SATORU ABE, SATORU KOMURA, HUI LIU, RAAFAT ELNAGGAR, HE CHANG, YUJI OKURA, KIMINORI KATO, MAKOTO KODAMA, HIROKI MARUYAMA,[1] JUNICHI MIYAZAKI[2] and YOSHIFUSA AIZAWA

Division of Cardiology, [1]Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Science, Niigata 951-8510, and
[2]Divison of Stem Cell Regulation, Osaka University Graduate School of Medical Science, Osaka 565-0872

HANAWA, H., WATANABE, R., HAYASHI, M., YOSHIDA, T., ABE, S., KOMURA, S., LIU, H., ELNAGGAR, R., CHANG, H., OKURA, Y., KATO, K., KODAMA, M., MARUYAMA, H., MIYAZAKI, J. and AIZAWA, Y. *A Novel Method to Assay Proteins in Blood Plasma after Intravenous Injection of Plasmid DNA.* Tohoku J. Exp. Med., 2004, **202** (3), 155-161 —— Gene therapy is expected to lead to new and useful methods to treat diseases. The development of assays to quantitate gene-therapy-derived proteins circulating in blood will be essential to investigate the effects and side effects of the introduced proteins. The purpose of this study is to evaluate whether a protein circulating at trace concentrations in blood can be measured by tagging a peptide corresponding to glucagon residues 19-29 onto its C-terminal end. We constructed plasmids encoding chimeric proteins and transferred them into rats by hydrodynamics-based delivery. When plasmids encoding human IL8-glucagon 19-29 chimeric protein were injected into rats to evaluate the accuracy of this method, there was a high correlation between chimeric proteins measured by an enzyme-linked immunosorbent assay for human IL8 and one by a radioimmunoassay for glucagon. Furthermore, when plasmids coding rat IFN gamma receptor IgG-Fc glucagon 19-29 chimeric protein were injected to evaluate the time course of chimeric proteins in blood plasma, we could calculate the concentrations in blood from 10 μl plasma samples using glucagon 19-29 tag as follows: 2815±2318 ng/ml after 4 hours (mean±S.D.), 6061±2789 ng/ml after 8 hours, 5752±2270 ng/ml after 12 hours, 2870±1062 ng/ml after one day, 1440±334 ng/ml after three days, 1120±433 ng/ml after seven days, and 281±162 ng/ml after 16 days. Blood sugar levels which might have been increased by glucagon did not increase even at peak chime-

Received July 15, 2003; revision accepted for publication January 9, 2004.

Address for reprints: Haruo Hanawa, Division of Cardiology, Niigata University Graduate School of Medical and Dental Science, 1-754 Asahimachi, Niigata 951-8510, Japan.
e-mail: hanawa@med.niigata-u.ac.jp

ric protein concentrations. These results demonstrate a useful and convenient method to assay gene therapy products circulating in blood using a glucagon 19-29 tagging vector. ——— gene therapy; glucagon; pCAGGS; radioimmunoassay

Recently, numerous attempts to accomplish gene therapy by using viral or plasmid vector-based methods have been published (Liu et al. 1999; Lawson et al. 2000; Quattrocchi et al. 2000; Maruyama et al. 2002; Matsui et al. 2002; Watanabe et al. 2001). It is expected that these new methods will be developed further and will prove useful in the treatment of various diseases. In many of these studies, it is essential to measure the concentration of the synthesized protein in blood. In general, proteins in blood are measured by an enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA). However, if antibodies for these assays are not available, the protein levels can be difficult to measure (Lawson et al. 2000). The proteins synthesized by these types of vectors are often tagged with peptides containing 6-20 amino acids to aid in their purification, for example poly-His-tags, the c-myc-tag, the glutathione *S*-transferase tag, or the FLAG-tag (Denbow et al. 1996; Lu et al. 1997; Bouchard et al. 1999; Hefti et al. 2003). We have previously attempted to measure recombinant proteins using poly-His-tags and Tag-100 and they were able to be detected in transfected Cos-7 cell culture medium, not in the blood of rat after intravenous injection of plasmid DNA. The levels of tagged, recombinant proteins derived from these plasmids cannot yet be assayed with sufficient accuracy. We decided to develop super-sensitive assays by exploiting the tags on the proteins themselves, as they are often unique markers for the recombinant products.

In this study, we investigated whether a plasmid vector that adds a peptide tag consisting of glucagon residues 19-29 to the C-terminal end of proteins could be exploited to measure the concentration of the tagged protein in blood following gene therapy. This type of plasmid was injected rapidly into rats via the tail vein using published protocol (Liu et al. 1999; Maruyama et al. 2002) and the synthesized protein in blood was measured by a commonly used glucagon assay kit (Imagawa et al. 1979; Nishino et al. 1981) over a 16-day period following the injection. Glucagon consists of 29 amino acids, and this peptide hormone plays a physiological role by increasing blood sugar and its amino acid sequence is the same in mouse, rat and human (Lefebvre 1995; Irwin 2001). Therefore, the expressed glucagon 19-29 peptides already exist in living bodies and are thought to have low antigenicity in all three species. The recombinant chimeric protein contained a glucagon-derived peptide tag (residues 19-29) corresponding to 38% of the entire peptide hormone. Assays of blood sugar in rats treated by gene therapy using this protein indicated that possible glucagon-like side effects in fact did not occur. The synthesized protein would be expected to have no glucagon-like side effects in the other species either, due to the sequence identity of the glucagon peptide in all three species.

Materials and Methods

Plasmid DNA

To construct pCAGGS-IL8 glucagon 19-29, the first PCR products of glucagon 19-29 DNA were amplified using KOD Plus DNA polymerase (TOYOBO, Osaka) and the following primers: (5′-gaGAATTCATTTAAATgagaGCGGCCGCccc aggtaaagcccaagattttgtgcagtggttg-3′ with SwaI and NotI restriction sites and 5′-gagagagaGAATTCtcaggtattcatcaaccactgcacaaaatcttgggc-3′) (Heinrich et al. 1984). The amplified glucagon 19-29 DNA was inserted into the pCAGGS vector using the EcoRI sites. Escherichia coli JM109 competent cells were then transformed and recombinant plasmids, i.e., pCAGGS-glucagon 19-29, were