Erratum

The authors apologize for an error in the paper by Li Chen, Jinsong Zhu, Guoqiang Sun and Alexander S Raikhel which appeared in the December 2004 issue of *Journal of Molecular Endocrinology* **33** 743–761, titled 'The early gene *Broad* is involved in the ecdysteroid hierarchy governing vitellogenesis of the mosquito *Aedes aegypti*'.

Page 757 read:
and JH blocks their appearance in larval *Manduca* (Zhou & Riddiford 2002).

When it should have read:
and JH blocks their appearance in larval *Manduca* (Zhou *et al.*, 1998).

Page 759 read:
misexpression of Z1 causes the reappearance of a pu
cuticle gene *Edg78E* and suppresses a larval cuticle ge
Lcp65A-b.

When it should have read:
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Lcp65A-b (Zhou & Riddiford, 2002).

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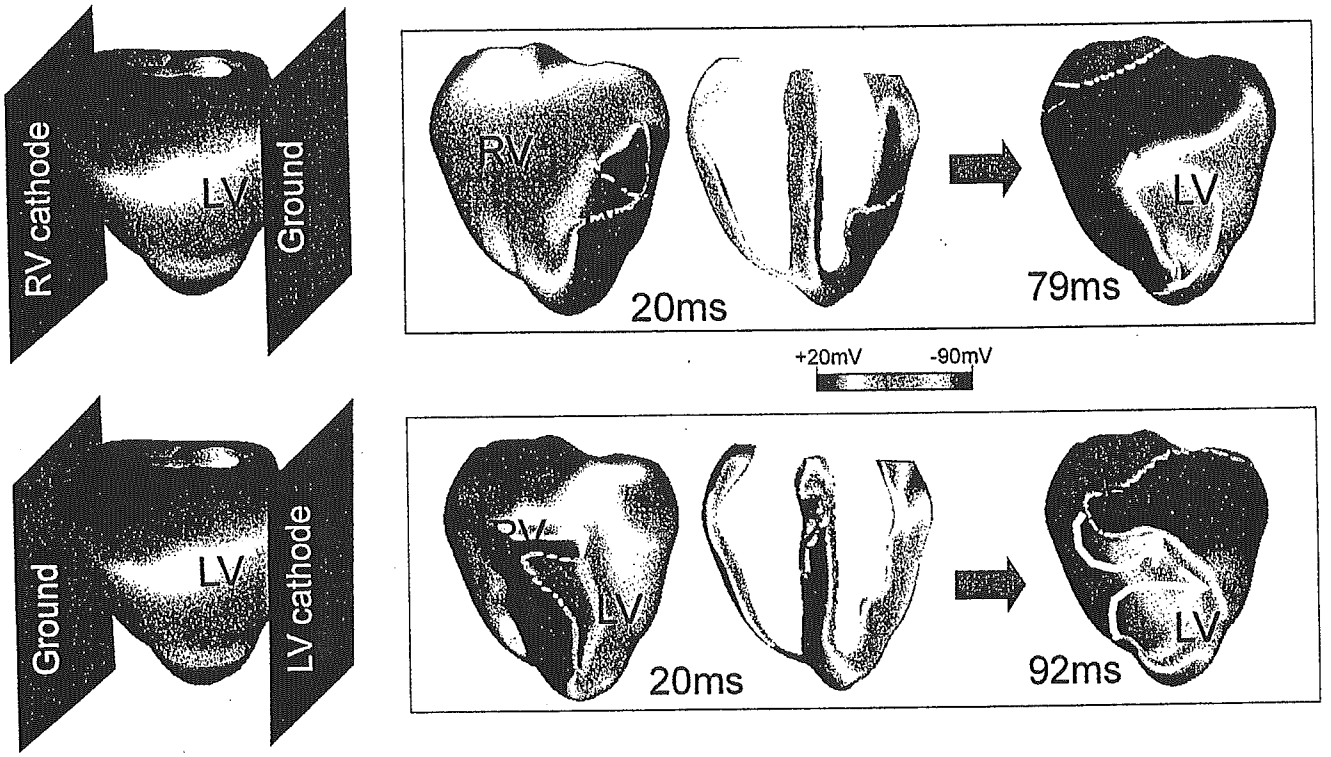

Abstracts

Second Annual Symposium of the American Heart Association Council on Basic Cardiovascular Sciences

Targeting Heart Failure—New Science, New Tools, New Strategies

July 24–27, 2005
Keystone Conference Center
Keystone, CO

Sponsored by the American Heart Association Council on Basic Cardiovascular Sciences
Co-sponsored by the American Heart Association Interdisciplinary Working Group on Functional Genomics & Translational Biology and the National Heart, Lung, and Blood Institute

Heart failure remains a leading cause of death in the adult population, affecting more than 5 million individuals in the United States alone. This second annual meeting of the American Heart Association Council on Basic Cardiovascular Sciences highlights new research that has aimed at expanding our knowledge of the underlying mechanisms, diagnostic approaches, and therapeutic strategies to treat this common disorder. The meeting features both invited presentations and poster abstract presentations, with participants from 21 different countries.

Abstracts for the poster presentations are provided in this special online supplement.

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following I/R in hearts with defective TLR4. To determine the role of ROS in TLR4-mediated inflammatory response, hearts were stimulated with hydrogen peroxide (0.25 mM/L). While hydrogen peroxide induced the production of MIP-2 and TNF-alpha in hearts isolated from control mice, its effects were greatly attenuated in hearts with defective TLR4. Further experiments using isolated macrophages confirmed that hydrogen peroxide induces NF-kappaB intranuclear translocation and inflammatory mediator production in a TLR4-dependent manner. We conclude that TLR4 signaling plays a novel role in the regulation of post-ischemic cardiac inflammatory response and that ROS may mediate this response through activation of TLR4.

X. Meng, None; **L. Ao**, None; **J. Cha**, None; **M. Wang**, None; **D.A. Fullerton**, None.

P 98

Role of Dual-Specificity Phosphatases in the Heart as Revealed by Gene Targeting

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The mitogen-activated protein kinases (MAPK) have been shown to participate in diverse biological processes in the heart including cardiac hypertrophy, myocyte survival, and apoptosis. MAPKs are tightly regulated through the addition or removal of phosphate groups in threonine and tyrosine residues located in the activation loop domain. Inactivation of MAPKs is controlled by removal of these phosphate groups and is mediated through a family of proteins known as dual-specificity phosphatases (DSPs), which have a high degree of specificity for individual MAPK substrates. DSPs directly bind to their MAPK substrate and remove phosphate groups from both threonine and tyrosine residues located in the activation loop domain, allowing MAPKs to be recycled. The potential role of the downstream counter-acting DSPs has not been explored in the heart. Therefore, in order to evaluate the function of DSPs in the heart as well as the consequences of increased MAPK signaling, we have systematically inactivated by gene-targeting several of the most critical and heart-expressed counter-acting DSPs including, MKP-1, MKP-2 and MKP-5. This approach will provide the first "physiologic" assessment of sustained and/or prolonged MAPK activation and its consequences on the heart. Initial observations show that DSP-targeted mice display an overall increased MAPK activation in the heart following agonist and pressure overload stimulation. Moreover, analysis of MKP-1-/- x MKP-2-/- double mutant mice showed a significant increase in cardiac hypertrophy following chronic isoproterenol infusion and myocardial infarction. However, these double mutant mice subjected to chronic angiotensin II / phenylephrine infusion or 14 days of pressure overload induced by TAC did not show any alteration in the hypertrophic response when compared with wild-type animals. These preliminary results suggest that in DSP-deficient mice, the nature of the hypertrophic stimulus will determine the levels of activity in each of the MAPK branches and eventually establish the resulting phenotype.

O.F. Bueno, None; **R.A. Kaiser**, None; **J. Xu**, None; **B.J. Wilkins**, None; **J.D. Molkentin**, None.

P 99

Genome-Wide Expression Profiling of NFAT Transcriptional Activity in a Ventricular Muscle Cell Line

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Cardiac hypertrophy is dependent upon activation of calcineurin-Nuclear Factor of Activated T cells (NFAT) signaling, but limited information is available on the nature and number of downstream NFAT target genes. Recently, cell lines were isolated from ventricular sarcomas from juvenile transgenic mice harboring a Nkx2.5-floxed SV40 T antigen (TAg) construct. NKL-TAg cells were isolated from sarcomas, which actively proliferate, and withdraw from the cell cycle and adopt a cardiac muscle cell fate upon Cre mediated loxP excision of the SV40 TAg (Rybkin et al. J Biol Chem 2003). We established a cellular system to allow inducible NFAT activation by stably transfecting the NKL-TAg cell line with the tetracyclin-sensitive transcriptional repressor (TetR) by hygromycin selection, allowing a doxycyclin (DOX)-on system in culture. Individual clones were tested by transient transfection with a luciferase reporter under control of two tetracycline operators (tetO). Stimulation with DOX for 24 hrs resulted in derepression of the reporter gene and over 100-fold activation of luciferase expression in four distinct TetR stable clones. Using two distinct TetR clones, a construct harboring an active form of NFAT under control of tetO operators was stably transfected in TetR cells using zeocin selection. Double stable clones were chosen using NFAT-reporter genes and western blots before and after DOX stimulation as selection criteria. Two double-stable TetR-NFAT clones were selected to control for potential cell based variations, AdCre infected to mortalize and adopt a cardiac muscle fate, and subjected to the Agilent microarray system and 44k mouse whole genome arrays. After DOX-stimulation for 24 hrs, 175 genes were differentially expressed in both clones with a 3 fold-change in expression. Gene ontology analysis revealed that NFAT target genes profoundly affect the cardiac transcriptome, signal transduction, translation efficiency and extracellular matrix. Conclusively, this novel cellular model allows for rapid genome wide screening of target genes of transcription factors in ventricular muscle, and will provide more entry points to our fundamental understanding of the calcineurin/NFAT transcriptional pathway in cardiac hypertrophy.

M. Bourajjaj, None; **A. Armand**, None; **R. Bassel-Duby**, None; **E.N. Olson**, None; **L.J. De Windt**, None.

P100

Differential Transcriptional Regulation of Cardiac Metabolic Genes by Myocyte Enhancer Factor-2 and Peroxisome Proliferator-Activated Receptors

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During the progression to heart failure, changes in energy utilization occur in the heart, with the failing heart being more reliant on glucose utilization to the expense of fatty acid oxidation. Peroxisome proliferators-activated receptor (PPAR) family members are key regulators of cardiac fatty acid oxidation. In the regulatory region of the the PPAR target gene carnithine palmitoyl transferase-I (CPT-I), conserved PPAR response elements (PPRE) and myocyte enhancer factor-2 (MEF2) binding sites are present. We analyzed the transcriptional relationship between different PPAR isoforms (alpha, beta and gamma) and MEF2A by combinatorial site directed mutagenesis of the PPRE and MEF2 binding sites, and demonstrate a preferential relationship between PPARbeta and gamma with MEF2A, and that disruption of the PPRE increases activation of the CPT-I gene dramatically after stimulation with beta and gamma specific agonists. Furthermore, disruption of the MEF2 binding site completely abolished the activation of the reporter gene, irrespective whether the PPRE site was intact or not, suggesting that PPAR activity strongly depends on MEF2 transcriptional activity. Finally, a micro array analysis on cardiac muscle cell lines, which inducibly express a constitutively active form of MEF2, revealed that 20% of the differentially expressed genes were involved in mitochondrial metabolism/fatty acid oxidation. Remarkably, only a minority of these genes had PPRE sites and were responsive to PPAR agonists. Using RNAi-mediated downregulation of each PPAR isoform and a dominant negative MEF2 adenovirus, we confirmed the dominant role of MEF2 in the transcriptional regulation of endogenous metabolic genes. The combined observations suggest that for a select number of genes PPAR and MEF2 differentially converge to regulate adaptive fatty acid oxidation gene expression, with a dominant role for the bonafide pro-hypertrophic transcription factor MEF2. Further, MEF2 is also capable of altering the metabolic gene profile in cardiac muscle independent of PPAR activity. These data also suggest that the early hypertrophic phase is associated with an increased rather than decreased fatty acid utilization.

H. el Azzouzi, None; **R.J. van Oort**, None; **M. Bourajjaj**, None; **M. van Bilsen**, None; **L.J. De Windt**, None.

P101

Reduced-Energy Diet Improves the Survival of Viral Myocarditis in Obese Mice: Relation to Cardiac Adiponectin Expression

Tsugiyasu Kanda, Seiichiro Saegusa, Takashi Takahashi, Yu Fei, Shigeto Morimoto, Takeshi Nakahashi, Kunimitsu Iwai, Masayuki Matsumoto, Kanazawa Med Univ, Kahoku Ishikawa, Japan

Obesity is an important risk factor for heart diseases. Whether weight loss affects the extent of viral myocarditis is a matter of debate. We hypothesized that weight loss could improve cardiac function induced by cardiac expression of cardioprotective cytokine, adiponectin and reduction of cardiac TNF-alpha and also altered

immune reaction. We examined the relationship between weight loss and heart failure due to viral myocarditis in obese KKAy mice, comparing with wild type C57BL mice (WT). We intraperitoneally injected encephalomyocarditis virus (500 plaque-forming units/mouse) for KKAy feed ad libitum (100F) or 60% of the food intake (60F) and WT (n = 30 for each). Ten-day survival rate was 0% in 100F, whereas it was 17% in 60F and 40% in WT. Body weight in 60F was lower than in 100F (21.1 + 0.3 vs. 29.1 + 0.24 g, $P<0.05$, n = 4 for each) on Day 0 and continuously lower on Day 5. Heart weight/body weight ratio in 60F was lower than in 100F on Day 5 after viral inoculation (4.73 + 0.18 vs. 4.96 + 0.88 mg/g, $P<0.05$, n = 4 for each). Histological scores (0 to 5) for myocardial necrosis and inflammation on Day 5 were significantly lower in 60F than in 100F (necrosis; 1.8 + 0.3 vs. 3.2 + 0.7, $P<0.05$, inflammation; 1.4 + 0.5 vs. 2.2 + 0.4, $P<0.05$, n = 4 for each). Circulating adiponectin levels on Day 0 were significantly elevated in 60F compared with 100F (32 + 9 vs. 22 + 2 microg/mL, $P<0.05$) and those in 60F on Day 5 were also higher than in 100F. Comparative expression of cardiac adiponectin mRNA in 60F was significantly higher than in 100F (5.1 +0.3 vs. 1 + 0.2, $P<0.05$, n = 4 for each).and cardiac TNF-alpha mRNA in 60F were significantly decreased compared with in 100F on Day 5 (0.23 +0.03 vs. 1 + 0.3, $P<0.05$, n = 4 for each). Cardiac adiponectin mRNA was negatively correlated with cardiac TNF-alpha mRNA ($r=-0.53$, $P<0.01$, n=12). On Day 0, thymus weight/ body weight and spleen weight/body weight in 60F were significantly ($P<0.05$) lower than in 100F and WT. Weight loss improved the survival and myocardial damages in obese mice with viral myocarditis and also induced the cardiac expression of adiponectin. Therapeutic modulation of cardiac adiponectin might provide benefit to the cardioprotective effect against acute heart failure due to viral myocarditis in obese subjects.

T. Kanda, None; **S. Saegusa**, None; **T. Takahashi**, None; **Y. Fei**, None; **S. Morimoto**, None; **T. Nakahashi**, None; **K. Iwai**, None; **M. Matsumoto**, None.

P102

PPARα Activation Upregulates the Fatty Acid Oxidation Pathway and Reduces Cardiac Ceramide Content in Heart Failure but Does Not Affect LV Dysfunction

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Peroxisome proliferator activated receptor α (PPARα) overexpression in transgenic mice increases the lipotoxic intermediate ceramide and causes LV remodeling and dysfunction. Furthermore, in advanced heart failure (HF), the expression of genes regulated by PPARα is down-regulated causing decreased fatty acid oxidation (FAO), which may function as a positive compensatory response. We tested the hypothesis that up-regulation of the FAO pathway with a direct PPARα agonist would exacerbate left ventricular (LV) dysfunction and dilation in HF. Rats underwent left coronary artery ligation or sham surgery (n=10), and 8 weeks later were assigned treatment with the PPARα agonist fenofibrate (Inf+Feno, 150 $mg \cdot kg^{-1} \cdot day^{-1}$; n =13) or left untreated (Inf, n=10). Twenty weeks post ligation, LV function was assessed by echocardiography and catherization. LV systolic function was decreased and end diastolic area increased to a similar extent in both infarcted groups. Treatment with the PPARα-agonist up-regulated the mRNA expression of the PPARα regulated genes, and medium chain acyl-CoA dehydrogenase (MCAD) protein expression and activity were increased in the Inf+Feno group compared to sham and Inf groups (see Table). However, the mRNA and protein expression of PPARα and retinoid X receptor α were unchanged. Treatment with fenofibrate significantly increased LV mass/body mass ratio compared to sham and Inf groups. Although cardiac ceramide content was increased following infarction, PPARα activation reduced ceramide content. In conclusion, PPARα activation increased mRNA expression of FAO enzymes, increased MCAD protein expression and activity and reduced myocardial ceramide content. These results suggest that PPARα activation of the FAO pathway and ceramide content do not contribute to LV dysfunction and remodeling in a rat model of coronary artery ligation-induced HF.

LV Function, MCAD Activity & Tissue Ceramide in Sham, Inf and Inf+Feno Rats

	SHAM	INF	INF+FENO
LV End Diastolic Area (cm 2)	0.83 ± 0.08	1.21 ± 0.17	1.23 ± 0.09
Fractional Area of Shortening (%)	59 ± 2	28 ± 3	28 ± 4
MCAD Activity (umol/mg/min)	8.2 ± 0.5	7.8 ± 0.7	15.4 ± 1.0
Tissue Ceramide (nmol/g wet wt)	1.37 ± 0.07	1.99 ± 0.29	1.45 ± 0.08

M.P. Chandler, None; **E.E. Morgan**, None; **J.H. Rennison**, None; **T.A. McElfresh**, None; **T.A. Kung**, None; **M.E. Young**, None; **B.D. Hoit**, None; **W.C. Stanley**, None.

P103

Structural and Functional Changes in Heart Mitochondria from Sucrose-Fed Hypertriglyceridemic Rats

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In the heart of sugar-induced hypertriglyceridemic (HTG) rats, cardiac performance is impaired with glucose as fuel, but not with fatty acids. Accordingly, the glycolytic flux and the transfer of energy diminish in the HTG heart, in comparison to control heart. To further explore the biochemical nature of such alteration in the HTG heart, the components of the systems involved were evaluated. Total creatine kinase (CK) activity in the myocardial tissue was depressed by 30% in the HTG heart whereas the activity of mitochondrial CK (mitCK) isoenzyme decreased in isolated HTG heart mitochondria by 45%. Adenylate kinase (AK) was 20% lower in the HTG heart. In contrast, respiratory rates with 2-oxoglutarate (2-OG) and pyruvate/malate (pyr) were significantly higher in HTG heart mitochondria than in control mitochondria. 2-OG dehydrogenase activity was also higher in HTG mitochondria. Respiration with succinate was similar in both groups. Content of cytochromes b, c $+c_1$ and $a+a_3$, and cytochrome c oxidase activity, were also similar in the two kinds of mitochondria. A larger content of saturated and monounsaturated fatty acids was found in the HTG mitochondrial membranes with no changes in phospholipids composition or cholesterol content. Mitochondrial membranes from HTG hearts were more rigid, which correlated with the generation of higher electrochemical H^+ gradient across the inner mitochondrial membrane. As the mitochondrial function was preserved or even enhanced in the HTG heart, these results indicated that deficiency in energy transfer was mainly due to dysfunction in the mitCK and AK. This situation brought about uncoupling between the site of ATP production and the site of ATP consumption (contractile machinery), in spite of compensatory increase in mitochondrial oxidative capacity and H^+ gradient generation.

K. Carvajal, None; **M. El Hafidi**, None; **A. Marín-Hernández**, None; **R. Moreno-Sánchez**, None.

P104

Glucosamine-Induced Cardioprotection Mediated by the Hexosamine Biosynthesis Pathway and Increased Levels of O-Linked N-Acetylglucosamine on Nucleocytoplasmic Proteins

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Increased levels of O-linked N-acetylglucosamine (O-GlcNAc) on nucleocytoplasmic proteins are associated with decreased calcium entry into cardiomyocytes and improved tolerance of mammalian cells to stress. Therefore we tested the hypothesis that in the heart glucosamine (GlcN) treatment would increase hexosamine biosynthesis pathway (HBP) flux and protein O-GlcNAc levels, resulting in improved recovery following ischemia/reperfusion (I/R). In the isolated perfused rat heart the level of UDP-GlcNAc, a precursor for synthesis of O-GlcNAc was assessed by HPLC and protein O-GlcNAc levels were assessed by immunoblot analysis using CTD110, an anti O-GlcNAc antibody. Under normoxic conditions the addition of 5mM GlcN significantly increased UDP-GlcNAc levels from 72±7 to 126±7 nmols/g ($p<0.05$) and increased protein O-GlcNAc levels relative to untreated hearts (236±33% Vs 100±20%, $p<0.05$). Low flow ischemia (LFI, 0.3mls/min for 30min)

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Inhibition of cyclooxygenase-2 enhances myocardial damage in a mouse model of viral myocarditis

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Abstract

To determine critical role of cyclooxygenase-2 (COX-2) for development of viral myocarditis, a mouse model of encephalomyocarditis virus-induced myocarditis was used. The virus was intraperitoneally given to COX-2 gene-deficient heterozygote mice (*COX-2*$^{+/-}$) and wild-type mice (WT). We examined differences in heart weights, cardiac histological scores, numbers of infiltrating or apoptotic cells in myocardium, cardiac expression levels of COX-2, tumor necrosis factor-α (TNF-α), and adiponectin mRNA, immunoreactivity of COX-2, TNF-α, and adiponectin in myocytes, cardiac concentrations of TNF-α and adiponectin, prostaglandin E_2 (PGE_2) levels in hearts, and viral titers in tissues between *COX-2*$^{+/-}$ and WT. We observed significantly decreased expression of COX-2 mRNA and reactivity in hearts from *COX-2*$^{+/-}$ on day 8 after viral inoculation as compared with that from WT, together with elevated cardiac weights and severe inflammatory myocardial damage in *COX-2*$^{+/-}$. Cardiac expression of TNF-α mRNA, reactivity, and protein on day 8 was significantly higher in *COX-2*$^{+/-}$ than in WT, together with reciprocal expression of adiponectin mRNA, reactivity, and protein in hearts. Significantly reduced cardiac PGE_2 levels on day 8 were found in *COX-2*$^{+/-}$ compared with those in WT. There was no difference in local viral titers between both groups on day 4. Infected WT treated with a selective COX-2 inhibitor, NS-398, also showed the augmented myocardial damage on day 8. These results suggest that inhibition of COX-2 may enhance myocardial damage through reciprocal cardiac expression of TNF-α and adiponectin in a mouse model of viral myocarditis.

Keywords: Cyclooxygenase-2; Myocardial damage; Viral myocarditis; Prostaglandin E_2; Tumor necrosis factor-α; Adiponectin

Introduction

Cyclooxygenase (COX) catalyzes oxidation of arachidonic acid, producing prostaglandin (PG) H_2, which is then isomerized to biologically active eicosanoids and thromboxanes. Two distinct COX isoforms are identified: COX-1 is present in most cells and is constitutively expressed, whereas COX-2 is both inducible and the major isoform of inflammatory cells (DeWitt et al., 1993). COX-1 gene is more than 22 kb, and is located on chromosome arm 9q32–q33.3 in humans (Kosaka et al., 1994). The COX-2 gene is encoded by a gene more than 8 kb in size located on the long arm of chromosome 1q25.2–q25.3 (Kosaka et al., 1994). This gene is very similar to the mouse and chicken COX-2 genes (Kosaka et al., 1994). Mice with disrupted COX-1 or COX-2 genes have been generated using gene-targeting strategies (Langenbach et al., 1995; Morham et al., 1995), and the characteristics of these mice have been reviewed (Langenbach et al., 1999).

While a role of COX-1 in inflammation is largely unknown, COX-2 is likely to play a role in the inflammatory process and has been implicated as a mediator of various inflammatory diseases. Reduction of PG production by inhibition of COX is thought to be main mechanism of action of most non-steroidal anti-inflammatory drugs. It has also been described that COX-2-deficient mice are resistant to endotoxin-induced inflammation and death (Ejima et al., 2003). On the other hand, COX-2 deficiency has been reported to fail in altering inflammatory responses in several standard models (Dinchuk et al., 1995).

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Viral infection, i.e. infection of encephalomyocarditis (EMC) virus is shown to stimulate COX-2 expression and PGE_2 accumulation through activation of nuclear factor-kappa B (NF-κB) in macrophages (Steer et al., 2003), and inhibitors of COX-2 enzymatic activity attenuate viral replication (Chen et al., 2000; Zhu et al., 2002), suggesting that the expression of COX-2 and the increased production of PG may possess an important role in viral replication. COX-2 enzyme is also described to be protective against pulmonary fibrogenesis, indicating that COX-2 generation of PGE_2 is a critical factor in resolving inflammation (Bonner et al., 2002).

COX-2 is reported to be up-regulated in an animal model of cardiac failure (Adderley and Fitzgerald, 1999), and its induction and activation of NF-κB has been detected in cardiomyocytes of patients with congestive heart failure (Wong et al., 1998). Deletion of the COX-2 gene may result in myocardial fibrosis (Dinchuk et al., 1995). Several manuscripts suggest that COX-2 plays a crucial role in mediating cardioprotective effects of ischemic preconditioning, which is the most potent anti-ischemic intervention known to date in terms of endogenous protection of ischemic myocardium (Shinmura et al., 2000; Bolli et al., 2002), since PGE_1 of COX-2 metabolites exerts cardioprotective action such as reduction in infarct size (Simpson et al., 1988).

We hypothesized that endogenous cardiac expression of COX-2 could play a cardioprotective role in establishment of severe inflammatory conditions induced by EMC virus. Therefore, we determined degrees of myocardial damage in the COX-2-deficient mice with acute viral myocarditis based on the histopathological findings and cardiac expression of tumor necrosis factor-alpha (TNF-α) mRNA, immunoreactivity, and protein in comparison with those in the wild-type mice with myocarditis.

Materials and methods

Animals

B6;129S7-*Ptgs2*^{tm1Jed} (*COX-2*$^{+/-}$) mice and C57BL/6 wild-type (WT) mice (3–5 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) as described previously (Yokota et al., 2002). High mortality and unavailability precluded use of homozygous *COX-2*$^{-/-}$ animals in this experiment.

Virus

A myocarditic variant of EMC virus was obtained from Y. Seto, Ph.D. (Keio University, Tokyo, Japan). Virus preparations were stored at −80 °C in Eagle's minimum essential medium (MEM) supplemented with 0.1% fetal bovine serum until the time of use.

Infection protocol

Animals were intraperitoneally inoculated with 500 plaque-forming units of EMC virus suspended in 0.1 ml of saline.

Treatment protocol

We administered EMC virus to WT ($n=12$) and *COX-2*$^{+/-}$ ($n=12$) mice. A normal WT mouse without viral inoculation was also included. Cardiac tissues were immediately extracted after sacrifice by cervical dislocation in both groups on days 4 and 8 after the EMC virus inoculation.

Histological examinations of hearts

Hearts were immediately weighed after sacrifice. Body weights were also recorded before sacrifice. Ratios of heart weight to body weight were calculated in both groups. Halves of cardiac tissues were fixed in 10% buffered formalin and stained with hematoxylin–eosin (H&E), while the other halves were immediately frozen in liquid nitrogen and stored at −80 °C for the studies of COX-2, PGE_2, and cytokine. Two transverse sections of the ventricular myocardium were graded for the severity of necrosis and mononuclear cell infiltration by an experienced pathologist, who had no knowledge of our study design, according to the following scale: grade 1, lesions involving <25% of the ventricular myocardium; grade 2, lesions involving 25% to 50% of the myocardium; grade 3, lesions involving 50% to 75% of the myocardium; and grade 4, lesions involving >75% of the myocardium. We also performed the staining on myosin as well as the H&E staining to identify the myocyte necrosis accurately. In addition, the pathologist randomly selected five high power fields (HPF) (×400 magnification) from each transverse section of the myocardium, and counted the infiltrating cells. The number of apoptotic cells in the randomly selected 5 HPF (×400 magnification) per section in the transverse sections of the myocardium was determined with in situ TUNEL as previously described (Kanda et al., 1999).

Comparative expression levels of COX-2, TNF-α, and adiponectin mRNA in hearts

RNA extraction for each half of frozen cardiac tissues was performed as described by the manufacturer (RNeasy Mini Kit, QIAGEN Inc., Tokyo, Japan). Procedure of DNAase was performed during the RNA extraction to avoid DNA contaminations. The total RNA concentrations were determined by measuring the optical density at 260 and 280 nm. Aliquots of 20 μl RNA from the each tissue were applied for production of cDNA. Comparative expression levels of COX-2, TNF-α, and adiponectin mRNA in cardiac tissues from both groups were determined using a quantitative real-time reverse transcriptase–polymerase chain reaction (RT–PCR) as described previously (Takeda et al., 2004). We applied TaqMan MGB Probe (Applied Biosystems Inc., CA, USA) for the real-time PCR. Primers and probe for quantification of COX-2 transcript (synthesized by Hokkaido System Science Co. Ltd., Hokkaido, Japan) were designed using a primer design software Primer Express (Applied Biosystems Inc.). We used a commercially available kit for TNF-α and

adiponectin RT-PCR (Mm00443258 m1 and Mm00456425 m1, Applied Biosystems Inc.). Each threshold cycle number up to 50 cycles (C_t value) within the RT–PCR was examined for the COX-2, TNF-α, and adiponectin mRNA levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous internal standard, and was amplified with specific primers for the number of cycles. A negative control without template cDNA was always included. ΔC_t values referred to differences between the C_t values for the each target gene and the GAPDH gene. After confirming that efficiencies of amplification of the each molecule and GAPDH transcripts were approximately equal, amount of the COX-2, TNF-α, or adiponectin transcript relative to the GAPDH transcript was determined using the comparative C_t method described in Perkin Elmer Applied Biosystems User Bulletin #2 (1997). Data are expressed as fold-increases relative to the baseline values in heart from a normal WT mouse without viral inoculation.

Immunoreactivity of COX-2, TNF-α, and adiponectin in cardiomyocytes

Immunohistochemical staining by streptavidin biotin complex method (#K0675 and #E0466 or #E0353, DAKO Cytomation Co. Ltd., Kyoto, Japan) was performed for the sections of transverse ventricular myocardium obtained from both groups on days 4 and 8 after viral infection. As a normal control, the immunoreactivity of COX-2, TNF-α, and adiponectin was determined with heart from a normal WT mouse without viral inoculation. We used the following commercially available primary antibodies; goat polyclonal anti-mouse COX-2 antibody at dilution of 1:100 (#sc-1747, Santa Cruz Biotechnology Inc., CA, USA), goat polyclonal anti-mouse TNF-α antibody at dilution of 1:50 (#RC410, DAKO Cytomation Co. Ltd.), and rabbit polyclonal anti-mouse adiponectin antibody at dilution of 1:50 (#ACRP303-A, Alpha Diagnostic International Inc., TX, USA). Control slides were treated with normal diluted goat or rabbit serum. The slides were blindly reviewed by the same pathologist, and were semiquantitatively graded according to the positive degrees of immunoreactivity as 0 for absence of staining and 1+ for weak, 2+ for moderate, and 3+ for strong staining (Kanazawa et al., 1996). They were compared with the respective control slides to exclude nonspecific staining. The positive degrees of COX-2, TNF-α, and adiponectin reactivity were assessed for randomly selected 30 myocytes corresponding to the surviving cells found on the respective H&E and myosin-stained slides.

Cardiac levels of TNF-α and adiponectin

Partial remnants of frozen cardiac tissues were applied to measurement of TNF-α and adiponectin tissue levels with the homogenate of each tissue. Enzyme-linked immunosorbent assay (ELISA) method, which used a polyclonal antibody specific for mouse TNF-α or adiponectin pre-coated onto a microtiter plate (ELISA kit for TNF-α; BioSource International Inc., CA, USA) (ELISA kit for adiponectin; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), was performed with cardiac samples according to the instructions of manufactures. As a normal control, cardiac levels of TNF-α and adiponectin were determined with heart from a normal WT mouse without viral infection. The ELISA kit applied for TNF-α concentrations showed that the limit of sensitivity and the within- and between-assay variations were 3.0 pg/ml, 6.5%, and 8.7%, respectively. The kit used for adiponectin levels demonstrated that the limit of sensitivity, the intra-assay variation, and the cross-reactivity were 0.25 ng/ml, less than 10%, and no responses for specimens from other animals including sheep, respectively.

Concentrations of PGE2 in hearts

Partial remnants of frozen cardiac samples from both groups on day 8 after viral infection were applied to measurement of PGE_2 tissue levels with the homogenate of each tissue as described previously (LaPointe et al., 2004). We also examined PGE_2 concentration with heart from a normal WT mouse without viral inoculation. Radioimmunoassay (RIA) method was performed using a commercially available kit with the tissue samples according to the instructions of manufactures (Prostaglandin E_2 [^{125}I] RIA Kit, Perkin Elmer Life Sciences Inc., MA, USA). This RIA kit used for plasma PGE_2 levels demonstrated that the sensitivity limit and the intra- and inter-assay variations were 0.5 pg/ml, 9.0–9.4%, and 6.7–11.6%, respectively.

Viral titers in hearts

EMC viral titers in hearts were determined in terms of the viral cytopathic effects, and were expressed as the tissue culture mean infectious dose ($TCID_{50}$). On day 4 after the inoculation ($n=6$ for each group), hearts were partially

Table 1
Body weight, cardiac weight, and ratio of cardiac weight to body weight in different mice groups after viral inoculation

	Body weight (g)			Cardiac weight (mg)		Ratio of cardiac weight to body weight	
	Day 0	Day 4	Day 8	Day 4	Day 8	Day 4	Day 8
WT	19.3±1.1	18.9±1.9	17.9±2.3	98±8	102±11	5.3±0.4	5.8±0.7
COX-2$^{+/-}$	19.5±1.6	19.0±2.2	16.2±2.5	102±10	116±13*	5.5±0.5	7.2±0.9*
NS-398	19.9±1.3	19.2±1.8	16.8±2.8	104±13	127±19*	5.6±0.7	7.5±1.1*

WT, wild-type mice; *COX-2*$^{+/-}$, mice with heterozygous deficiency of cyclooxygenase-2 gene; NS-398, wild-type mice treated with a selective inhibitor of cyclooxygenase-2, NS-398, at a dose of 3 mg/kg/day starting simultaneously with viral inoculation. Data are expressed as means±S.D.
* $P<0.05$ compared with WT mice group.

homogenized in 2 ml of MEM. After centrifugation, the supernatants were added into 96-well microtiter plates containing human amnion cells in MEM supplemented with 10% fetal calf serum as described previously (Kanda et al., 2000). The microtiter plates were observed daily for 5 days for the appearance of any cytopathic effects.

Reassessment study by a selective COX-2 inhibitor

In order to confirm the augmented myocardial damage, we orally administered WT mice ($n=12$) a selective COX-2 inhibitor, NS-398 (EMD Biosciences Inc., Darmstadt, Germany), at dosing of 3 mg/kg/day starting simultaneously with

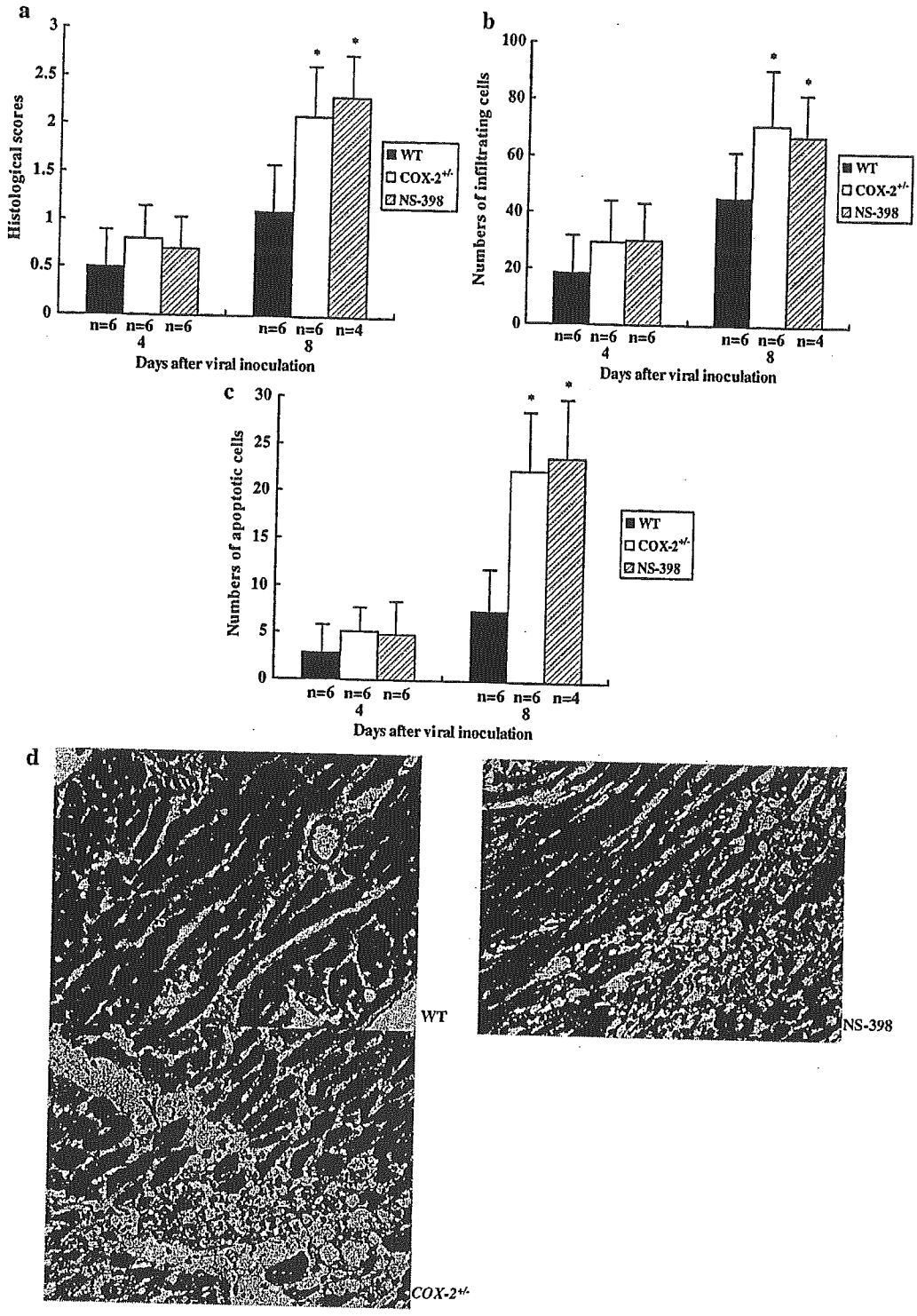

Fig. 1. Results of histological examination including histological scores in heart (a), numbers of infiltrating cells (b) or apoptotic cells (c) in myocardium from different mice on days 4 and 8 after viral inoculation, and photographic demonstration of myocardial damage in three groups on day 8 (d, original magnification). Data are expressed as mean±S.D. WT, wild-type mice; *COX-2*$^{+/-}$, mice with heterozygous deficiency of cyclooxygenase-2 gene; NS-398, wild-type mice treated with a selective inhibitor of cyclooxygenase-2, NS-398, at a dose of 3 mg/kg/day starting simultaneously with viral inoculation. *$P<0.05$ compared with the WT group.

viral inoculation as described previously (LaPointe et al., 2004). Histological examinations (cardiac histological scores and numbers of infiltrating or apoptotic cells in myocardium), as well as body weights, heart weights, and ratios of heart weight to body weight, were assessed with hearts obtained on days 4 and 8 after viral infection. Data were compared with those in infected WT mice without intervention of the COX-2 inhibitor.

Statistical analysis

Data are expressed as mean±standard deviations. A two-tailed analysis of variance was applied to evaluate differences in body and cardiac weights, ratios of heart weight to body weight, cardiac histological scores, numbers of infiltrating or apoptotic cells in myocardium among three groups. An unpaired two-tailed Student's *t*-test was used to assess differences in comparative expression levels of COX-2, TNF-α, and adiponectin mRNA in hearts, immunoreactivity of COX-2, TNF-α, and adiponectin in myocytes, cardiac levels of TNF-α and adiponectin, PGE_2 concentrations in hearts, and local EMC viral titers between the *COX-2*$^{+/-}$ mice and the WT mice. A value of $P<0.05$ was considered to be statistically significant.

Results

Mortality in different mice with viral myocarditis

There were no dead mice with viral myocarditis in the WT group and the *COX-2*$^{+/-}$ group. Numbers of mice in each group from which specimens of hearts were obtained on days 4 and 8 after EMC viral inoculation, were 6 on each day in the WT mice and 6 on each day in the *COX-2*$^{+/-}$ mice, respectively.

Body weights, cardiac weights, and ratios of cardiac weight to body weight

Body weights, heart weights, and ratios of heart weight to body weight in different groups are shown in Table 1. Cardiac weights in the *COX-2*$^{+/-}$ group on day 8 after viral infection were significantly increased as compared with those in the WT group ($P<0.05$, Table 1). There was also significant difference in ratios of cardiac weight to body weight between the *COX-2*$^{+/-}$ mice and the WT mice on day 8 ($P<0.05$, Table 1).

Histological findings in hearts

Histological scores and numbers of infiltrating or apoptotic cells per field in the hearts obtained from different mice on days 4 and 8 after viral inoculation are shown in Fig. 1a, b, and c, respectively. The hearts from the *COX-2*$^{+/-}$ group showed severe myocardial necrosis and mononuclear cell infiltration (Fig. 1d). The histological scores based on myocardial necrosis and cell infiltration on day 8 were significantly higher in the *COX-2*$^{+/-}$ mice than in the WT mice ($P<0.05$, Fig. 1a). The number of infiltrating cells per field in the myocardium from the *COX-2*$^{+/-}$ group on day 8

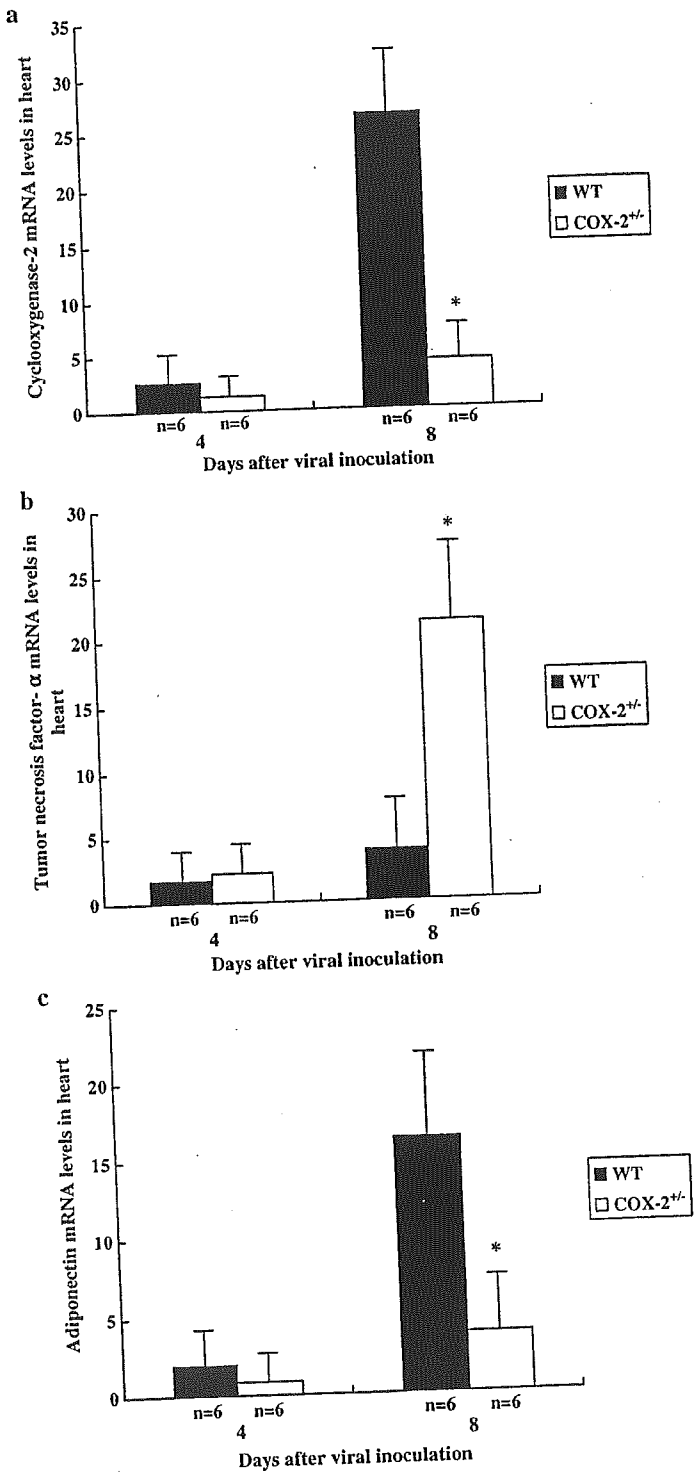

Fig. 2. Results of comparative expression levels of cyclooxygenase-2 (a), tumor necrosis factor-α (b), and adiponectin (c) mRNA using a quantitative real-time reverse transcriptase–polymerase chain reaction with hearts from different mice on days 4 and 8 after viral infection. Data are expressed as mean±S.D. WT, wild-type mice; *COX-2*$^{+/-}$, mice with heterozygous deficiency of cyclooxygenase-2 gene; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-α. *$P<0.05$ compared with the WT group.

was significantly elevated as compared with that in the WT group ($P<0.05$, Fig. 1b). The number of apoptotic cells per field in the hearts on day 8 was significantly higher in the *COX-2*$^{+/-}$ mice than in the WT mice ($P<0.05$, Fig. 1c). There were no significant differences in histological scores and numbers of infiltrating or apoptotic cells between the *COX-2*$^{+/-}$ group and the WT group on day 4.

Comparative expression levels of COX-2, TNF-α, and adiponectin mRNA in hearts

Tissue sample of heart from a normal WT mouse was assigned to each value of 1 for tissue expression levels of COX-2, TNF-α, and adiponectin mRNA, respectively. Cardiac levels of COX-2, TNF-α, and adiponectin mRNA from

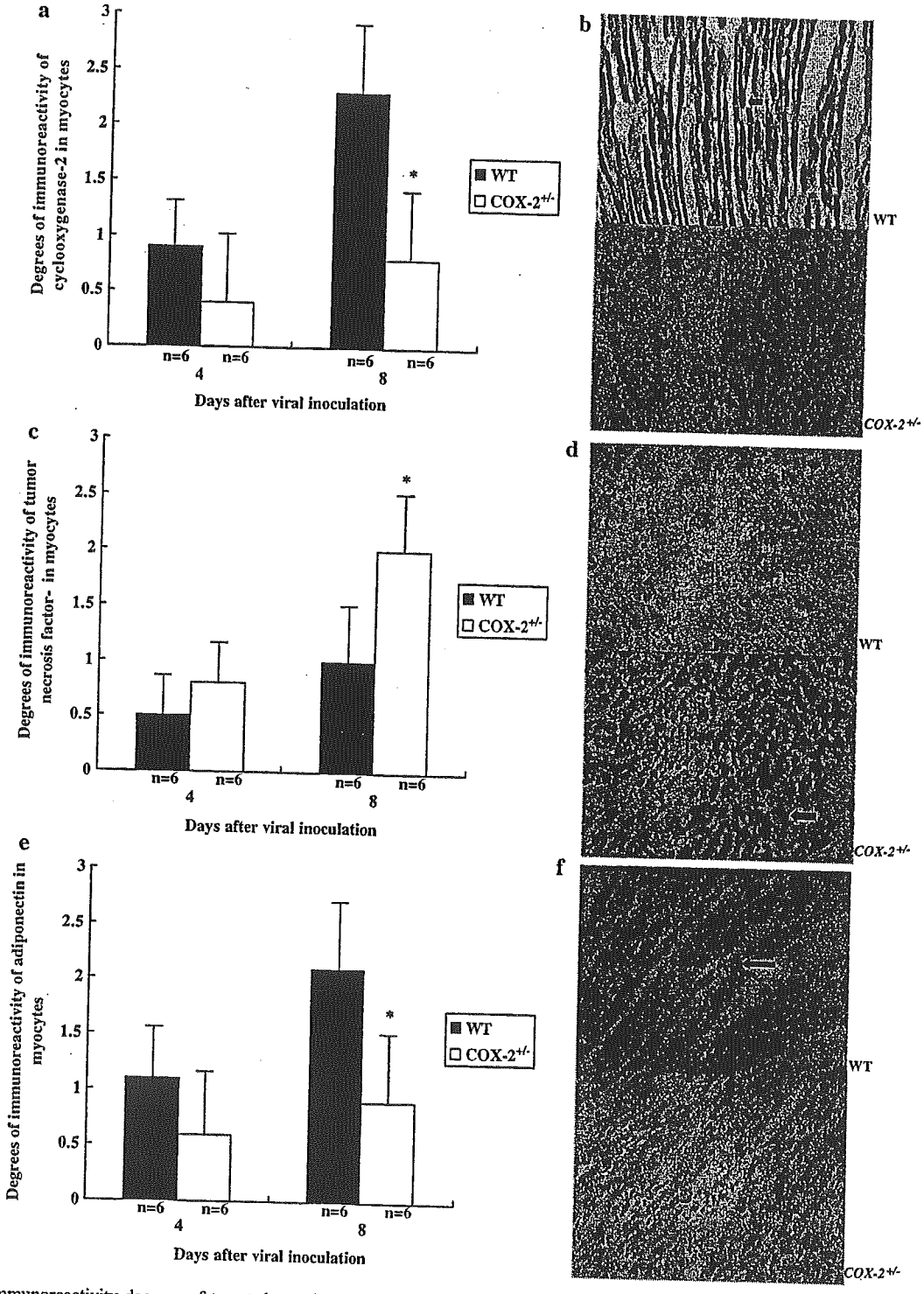

Fig. 3. Results of immunoreactivity degrees of targeted proteins including cyclooxygenase-2 (a), tumor necrosis factor-α (c), adiponectin (e) in myocytes from different mice on days 4 and 8 after viral inoculation, and photographic demonstration (arrows) of cyclooxygenase-2 (b, original magnification), tumor necrosis factor-α (d, original magnification), and adiponectin (f, original magnification) reactivity in myocardium from various mice on day 8. Data are expressed as mean±S.D. WT, wild-type mice; *COX-2*$^{+/-}$, mice with heterozygous deficiency of cyclooxygenase-2 gene. *$P<0.05$ compared with the WT group.

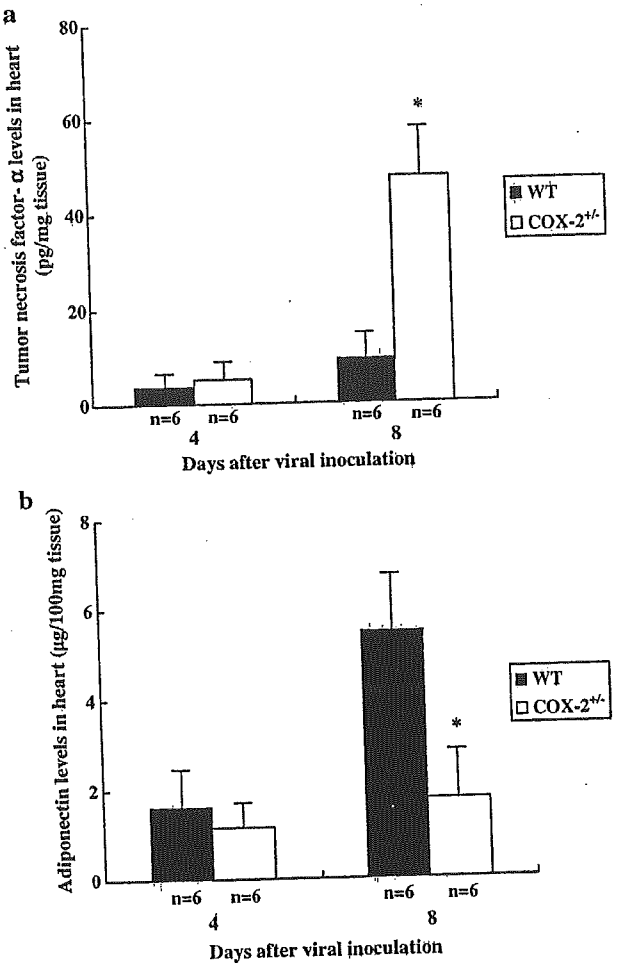

Fig. 4. Results of tumor necrosis factor-α (a) and adiponectin (b) levels in hearts from different mice on days 4 and 8 after viral inoculation. Data are expressed as mean±S.D. WT, wild-type mice; *COX-2*$^{+/-}$, mice with heterozygous deficiency of cyclooxygenase-2 gene. *$P<0.05$ compared with the WT group.

different mice on days 4 and 8 after viral infection are shown in Fig. 2a, b, and c, respectively. COX-2 mRNA levels in hearts on day 8 were significantly lower in the *COX-2*$^{+/-}$ group than in the WT group ($P<0.05$, Fig. 2a). On the other hand, we observed the significantly increased levels of TNF-α mRNA in cardiac tissues from the *COX-2*$^{+/-}$ mice at the same time as compared with those from the WT mice ($P<0.05$, Fig. 2b). Adiponectin mRNA levels in hearts on day 8 were significantly suppressed in the *COX-2*$^{+/-}$ group than in the WT group ($P<0.05$, Fig. 2c). There was no difference in cardiac levels of COX-2, TNF-α, and adiponectin mRNA between the WT mice and the *COX-2*$^{+/-}$ mice on day 4.

Immunoreactivity of COX-2, TNF-α, and adiponectin in cardiomyocytes

Immunoreactivity of COX-2, TNF-α, and adiponectin was not observed in heart from a normal WT mouse. The degrees of COX-2, TNF-α, and adiponectin reactivity in damaged myocytes from different mice on days 4 and 8 after viral inoculation are shown in Fig. 3a, c, and e, respectively. Photographic demonstration of immunoreactivity of COX-2, TNF-α, and adiponectin in cardiac tissues from various mice on day 8 is indicated in Fig. 3b, d, and f, respectively. We found the significantly suppressed reactivity of COX-2 in myocytes from the *COX-2*$^{+/-}$ group on day 8 as compared with that from the WT group ($P<0.05$, Fig. 3a and b). On the other hand, the degrees of TNF-α reactivity in myocytes at the same time were significantly higher in the *COX-2*$^{+/-}$ mice than in the WT mice ($P<0.05$, Fig. 3c and d). The degrees of adiponectin reactivity in myocytes on day 8 were significantly decreased in the *COX-2*$^{+/-}$ group than in the WT group ($P<0.05$, Fig. 3e and f). There were no differences in degrees of COX-2, TNF-α, and adiponectin reactivity between the WT mice and the *COX-2*$^{+/-}$ mice on day 4.

Cardiac levels of TNF-α and adiponectin

Specimen of heart from a normal WT mouse showed undetectable values for each targeted molecule. TNF-α and adiponectin levels in hearts from different mice on days 4 and 8 after viral infection are shown in Fig. 4a and b, respectively. Cardiac concentrations of TNF-α on day 8 were significantly higher in the *COX-2*$^{+/-}$ mice than in the WT mice ($P<0.05$, Fig. 4a). On the other hand, there were significantly reduced adiponectin levels in hearts from the *COX-2*$^{+/-}$ group at the same time as compared with those from the WT group ($P<0.05$, Fig. 4b). There were no differences in cardiac concentrations of TNF-α and adiponectin between the *COX-2*$^{+/-}$ mice and WT mice on day 4.

Concentrations of PGE2 in hearts

Tissue specimen of heart obtained from a normal WT mouse showed 0.96 pg/mg protein. Cardiac PGE_2 concentrations from different mice on day 8 after viral infection are shown in Fig. 5. The PGE_2 tissue levels on day 8 were significantly lower in the *COX-2*$^{+/-}$ mice than in the WT mice ($P<0.05$, Fig. 5).

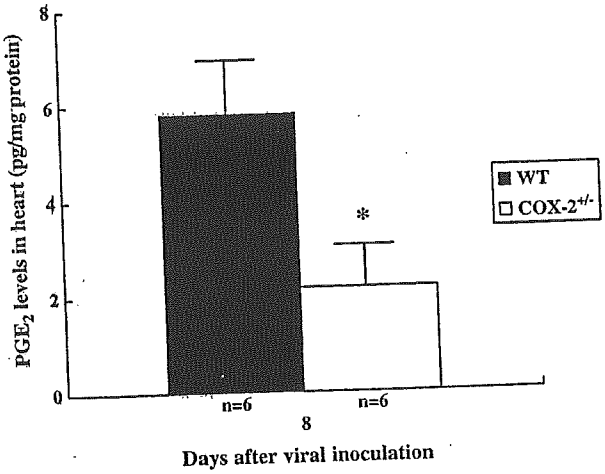

Fig. 5. Results of cardiac prostaglandin E_2 concentrations from different mice on day 8 after viral inoculation. Data are expressed as mean±S.D. WT, wild-type mice; *COX-2*$^{+/-}$, mice with heterozygous deficiency of cyclooxygenase-2 gene. *$P<0.05$ compared with the WT group.

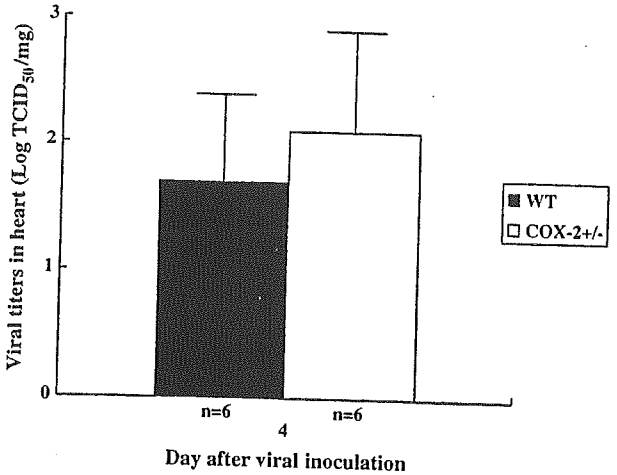

Fig. 6. Results of encephalomyocarditis viral titer in hearts from different mice on day 4 after viral infection. Data are expressed as mean±S.D. WT, wild-type mice; *COX-2*$^{+/-}$, mice with heterozygous deficiency of cyclooxygenase-2 gene; $TCID_{50}$, tissue culture mean infectious dose 50%.

Viral titers in hearts

EMC viral titers in hearts from various mice on day 4 after viral inoculation are indicated in Fig. 6. There was no difference in local viral titers between the *COX-2*$^{+/-}$ mice and the WT mice on day 4 after viral inoculation.

Reassessment study by a selective COX-2 inhibitor

We observed two dead WT mice treated with NS-398 on day 8 after viral infection. The numbers of mice from which hearts were extracted on days 4 and 8 were six and four, respectively. Cardiac weights and ratios of heart weight to body weight in the NS-398 group on day 8 were significantly increased compared to those in the WT group ($P<0.05$, Table 1). The histological scores and numbers of infiltrating or apoptotic cells in myocardium on day 8 were significantly higher in the WT mice treated with NS-398 than in the WT mice without intervention ($P<0.05$, Fig. 1a, b, and c). The augmented myocardial damage in the WT mice treated with NS-398 is shown in Fig. 1d. There were no differences in the histological scores and numbers of infiltrating or apoptotic cells between the NS-398 group and the WT group on day 4.

Discussion

The fold-increase of COX-2 mRNA expression in *COX-2*$^{+/-}$ mice compared to that in sham-infected animal was "one-seventh fold" less than the fold-increase in WT mice on day 8 after viral inoculation. Cardiac PGE_2 levels on day 8 were about one-third suppressed in the *COX-2*$^{+/-}$ group compared to those in the WT group. EMC virus infection has recently been reported to stimulate COX-2 expression in macrophages in in vitro experiment (Steer et al., 2003). Another study showed no expression of COX-2 mRNA in *COX-2*$^{-/-}$ primary embryonic fibroblasts and less expression of mRNA in *COX-2*$^{+/-}$ fibroblasts under stimulation (Dinchuk et al., 1995). Thus, decreased expression of COX-2 mRNA in hearts from *COX-2*$^{+/-}$ mice with viral infection seems to be due to the disruption of one COX-2 allele.

COX-2 products can exhibit both beneficial and deleterious effects on inflammatory process in the same organ such as the kidney (Wang et al., 2000). COX-2 expression is also described to protect against apoptosis in several types of cells, particularly in colon cancer cells (Kinoshita et al., 1999). Myocardial COX-2 expression is demonstrated in rat heart during cardiac transplant rejection (Yang et al., 2000), and COX activity induced by doxorubicin in rat neonatal cardiomyocytes is due to COX-2 gene expression (Hemler and Lands, 1976). It has recently been reported that COX-2 expression may provide some degrees of protective effects against the myocardial damage in the rat model of doxorubicin-induced cardiotoxicity (Dowd et al., 2001). On the other hand, COX-2 is indicated to play a deleterious role in heart after myocardial infarction caused by chronic occlusion of left anterior descending coronary artery (LaPointe et al., 2004). COX-2 inhibition partly reverses both the increase in cardiac collagen content and hypertrophy and the decrease in cardiac function in the mouse model of myocardial infarction. Similarly to the findings in the former manuscript, we observed impaired expression of cardiac COX-2 resulted in augmented myocardial injury in *COX-2*$^{+/-}$ mice with EMC virus-induced myocarditis as compared with that in WT mice. Since the inoculation of EMC virus promotes COX-2 expression and PGE_2 generation (Steer et al., 2003), the up-regulation of COX-2 expression is considered to play a crucial role for compensatory mechanism in the mouse model of heart failure induced by EMC virus infection.

TNF-α mRNA is shown to be detectable in myocardium obtained from subjects with ischemic heart disease (IHD) and dilated cardiomyopathy (DCM) using northern blot analysis, while there is no evidence for TNF-α gene expression in non-failing human hearts (Torre-Amione et al., 1996). Immunohistochemical examinations also indicated that there was obvious TNF-α immunostaining of cardiomyocytes in the myocardium from patients with IHD and DCM, whereas the TNF-α immunoreactivity was not detectable in the non-failing hearts (Torre-Amione et al., 1996). Natriuretic peptides including atrial and B-type peptides are synthesized in and secreted from hearts, and play a critical role in cardiovascular homeostasis (Grepin et al., 1994). Similarly to the previous demonstration, identification of TNF-α expression in hearts from mice with viral myocarditis in our study is also a good example of contribution made by molecular biology to understanding of mechanisms for heart failure compensation.

Prostaglandins acts on 2 classes of receptors consisting of surface transmembrane-spanning, G-protein-coupled receptors and peroxisome proliferator-activated receptors (PPARs), which are nuclear membrane receptors. Prostaglandins and their precursors are ligands for several PPARs including PPARα, PPARδ, and PPARγ (Bishop-Bailey and Hla, 1999). PPARγ activates transcription level of adiponectin with liver receptor homologue-1 by binding the promoter region of

adiponectin gene (Ouchi et al., 2004). Cardiac expression of adiponectin was impaired together with decreased PGE_2 concentrations in hearts from *COX-2*$^{+/-}$ mice with viral myocarditis. Reactivity of adiponectin for immunostaining has recently been reported to be observed at periphery of surviving cardiomyocytes around the lesions at granulative stage in myocardial tissues obtained from 47 autopsied hearts with infarction (Ishikawa et al., 2003). In another immunohistochemical analysis, boundary of mouse hepatocytes showed positive signals for adiponectin after 3–6 h of carbon tetrachloride treatment, and their cytoplasma was intensely stained after 18 h of the treatment (Yoda-Murakami et al., 2001). Adiponectin was considered to be produced by the liver in mice, where it underwent tissue damage-induced transcriptional regulation (Yoda-Murakami et al., 2001). Our data regarding adiponectin expression in damaged myocytes suggest that this adipocyte-specific cytokine might have important implications for acute phase of viral myocarditis.

Adiponectin is described to be involved in ending inflammatory responses through its inhibitory functions (Yokota et al., 2000). This cytokine increases mRNA expression of anti-inflammatory molecule, interleukin-10 (IL-10), at the transcriptional level, and elevates IL-10 protein secretion in in vitro experiment of human monocyte-derived macrophages (Kumada et al., 2004). In addition, reciprocal relationship between adiponectin and high-sensitive C-reactive protein is shown in both human plasma and adipose tissue from subjects with coronary artery disease (Ouchi et al., 2003). In our experiment, suppressed expression of cardiac adiponectin mRNA and immunoreactivity in *COX-2*$^{+/-}$ mice was associated with the development of severe myocarditis, whereas increased expression of adiponectin in hearts from WT mice resulted in the inhibition of cardiac inflammatory process. Therefore, we speculate that local expression of adiponectin in the damaged myocardium might be a compensatory phenomenon against the severe inflammatory conditions of viral myocarditis.

A COX-2 inhibitor, Dup-697, suppressed the biological action of adiponectin on differentiation of cloned stromal preadipocytes (Yokota et al., 2002). Adiponectin was described to inhibit TNF-α production in macrophages (Yokota et al., 2000). Adiponectin-knockout mice revealed high levels of TNF-α mRNA in adipose tissue and high plasma TNF-α concentrations (Maeda et al., 2002). Thus, impaired COX-2 functions seem to link to increased cardiac expression of TNF-α mRNA, immunoreactivity, and protein through the reduced expression of adiponectin mRNA, reactivity, and protein in our experiment.

In conclusion, decreased expression of COX-2 mRNA and immunoreactivity in myocardium from *COX-2*$^{+/-}$ mice after viral infection were observed together with increased heart weights, severe myocardial inflammation, and suppressed cardiac PGE_2 concentrations in *COX-2*$^{+/-}$ mice. There was also an enhanced expression of TNF-α mRNA, reactivity, and protein in hearts, while attenuated cardiac expression of adiponectin mRNA, reactivity, and protein was found in *COX-2*$^{+/-}$ mice with viral myocarditis. Moreover, infected WT mice treated with the selective COX-2 inhibitor showed the augmented myocardial damage on day 8. Our observations suggest that inhibition of COX-2 and subsequent reduced PGE_2 production in heart may contribute to enhanced myocardial injury through reciprocal cardiac expression of TNF-α and adiponectin in a mouse model of viral myocarditis.

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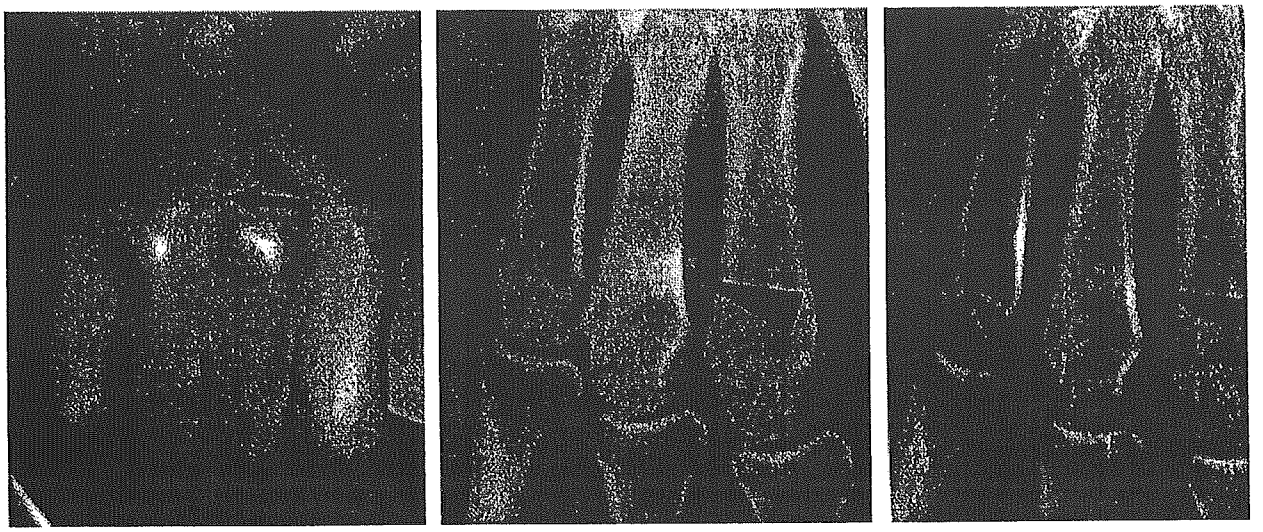

Figure 1. Left panel, subcutaneous nodule with pain (arrow). Radiographic examination before (middle panel) and after 3 months on etidronate (right panel). Arrowheads indicate the positions of nodular calcification.

Our present clinical experience demonstrates a novel therapeutic option for an otherwise incurable complication of Werner syndrome. Moreover, it rediscovers the usefulness of bisphosphonate for ectopic calcification.

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HYPOADIPONECTINEMIA IN BEDRIDDEN FEMALE PATIENTS YOUNGER THAN 75

To the Editor: Older people have several hormonal alterations, but the effect on the endocrine function of adipose tissue in older bedridden patients has not been fully elucidated. Adiponectin is a newly discovered antiinflammatory protein, secreted exclusively by adipocytes, that plays a protective role against atherosclerosis.[1] Hypoadiponectinemia plays a crucial role in atherosclerosis in men, but there have been no studies of plasma adiponectin in bedridden women. The aim of the present study was to estimate plasma adiponectin concentration in bedridden elderly female patients in comparison with age-matched healthy volunteers.

Seventy-four bedridden female patients admitted to geriatric wards and nursing homes in Osaka, Japan, and age-matched volunteers were studied. Clinical diagnoses were defined using detailed physical examination and routine biochemical analyses of blood and urine, as well as clinical tools including computed tomography. Their mean bedridden period ± standard deviation was 49.4 ± 37.4 months. All plasma analyses were performed on samples from fasting subjects. Adiponectin was measured using high-sensitive radioimmunoassay (Linco Research, St. Louis, MO). Bedridden subjects and healthy volunteers were divided into two groups: younger than 75 and aged 75 and older. All statistical analyses were performed using the SPSS (SPSS Inc., Chicago, IL). The statistical differences in the variables were compared using the Mann-Whitney *U* test, and the association between any two parameters was assessed using Spearman correlation.

Table 1. Clinical Characteristics of 74 Bedridden Female Patients and 42 Healthy Age-Matched Volunteers

Characteristic	Healthy Female Volunteers		Bedridden Patients	
	Age 60–74 (n = 22)	Age 75–98 (n = 20)	Age 60–74 (n = 18)	Age 75–98 (n = 56)
Body mass index, mean ± SD	24.1 ± 2.3	23.8 ± 2.7	23.6 ± 2.6	24.9 ± 3.8
Systolic blood pressure, mmHg, mean ± SD	138 ± 22	140 ± 33	154 ± 19*	144 ± 20
Diastolic blood pressure, mmHg, mean ± SD	73 ± 10	77 ± 13	82 ± 14	84 ± 13
Plasma adiponectin, μg/mL, mean ± SD	15.9 ± 7.2	16.2 ± 7.7	11.8 ± 4.8*	14.3 ± 6.9
Serum albumin, g/dL, mean ± SD	4.2 ± 0.3	4.1 ± 0.3	3.6 ± 0.2*	3.5 ± 0.3*
Total cholesterol, mg/dL, mean ± SD	228 ± 24	211 ± 19	183 ± 15*	178 ± 16*
Cerebrovascular accident as cause of bedridden state, %	NA	NA	76	68

*$P < .05$ vs healthy age-matched volunteer.
SD = standard deviation; NA = not assessed.

The clinical characteristics of the subjects are shown in Table 1. Of 74 bedridden patients, the main cause of being bedridden was cerebrovascular accident (CVA, 70%); others were bone fracture, infection, and cardiovascular disease. There was no statistical difference in body mass index between the four groups. Bedridden women aged 60 to 74 were characterized by significantly lower plasma adiponectin (11.8 ± 4.8 μg/mL) concentration than healthy women of the same age (15.9 ± 7.2 μg/mL, $P < .01$). In the entire studied group, a weak, positive, but insignificant, correlation was found between plasma adiponectin concentration and age ($r = 0.18$, $P = .12$). There was no correlation between serum adiponectin and albumin or total cholesterol levels. Additionally, significant correlation was found between plasma adiponectin concentration and the length of time patients had been bedridden when analyzed separately ($r = 0.27$, $P = .04$).

The relationship between aging and plasma adiponectin concentration has recently been described in some manuscripts. Men aged 70 and older have significantly higher plasma adiponectin concentrations than younger men, whereas plasma adiponectin concentration in women does not change significantly with age.[2] The results of the current study in female patients aged 60 and older were consistent with these reports. Sex differences of adiponectin levels are also reported in older diabetic patients.[3]

The main cause of being bedridden was CVA in female patients in this study. The relationship between CVA and lower adiponectin level is controversial. A recent report reported that CVA was associated with hypoadiponectinemia with or without diabetes mellitus,[4] but in a Swedish study, adiponectin levels were not associated with CVA in men.[5] The current results concerning hypoadiponectinemia in younger bedridden female patients mainly caused by cerebrovascular disease would indicate a relationship between hypoadiponectinemia and occurrence of CVA. Hypoadiponectinemia may be due to the severity of cerebrovascular atherosclerosis in bedridden patients.

Precise mechanisms underlying recovery of adiponectin level in long-term bedridden patients are not known. First, being bedridden may affect the decrease in adiposity caused by multiple factors including immobilization, malnutrition, and disease, although several causes could be responsible, including aspiration pneumonia, urinary tract infection, and pressure ulcers. Indeed, the current results showed that albumin and total cholesterol levels in bedridden female patients were significantly lower than in healthy controls. Nutritional restriction due to controlled feeding increases the survival of mammalians by delaying the aging process.[6] Decrease in adiposity due to being bedridden longer may induce higher levels of circulating adiponectin in elderly female patients. A second possibility may be the effects of exercise in bedridden patients. Lack of skeletal muscle exercise is apt to induce adiposity by decreasing the release of cytokine interleukin-6, which stimulates lypolysis.[7] Third, that hypoadiponectinemia was observed mainly in those who had been bedridden for a shorter length of time may reflect greater endothelial dysfunction. A vascular study showed that severity of endothelial dysfunction was closely associated with plasma adiponectin levels in Japanese subjects according to the measurement of forearm blood flow.[8] Elevated adiponectin level would be related to longevity even in bedridden women. Longitudinal studies of serum adiponectin levels and prognosis of bedridden state should be conducted to elucidate the mechanism of lipid metabolism in bedridden patients to help with their future health care.[9]

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SPONTANEOUS FRACTURES OF LONG BONES ASSOCIATED WITH JOINT CONTRACTURES IN BEDRIDDEN ELDERLY INPATIENTS: CLINICAL FEATURES AND OUTCOME

To the Editor: Reports on the occurrence of fractures without any apparent external force in completely bedridden elderly patients under care are limited. One study[1] described six individuals with "spontaneous fractures of long bones" in nursing home patients. Another elderly nonweight-bearing woman with "transfer" and "turning" fracture was also reported.[2] A survey of 11 nursing homes identified 16 subjects with "minimal trauma fractures."[3] Fifty-five "spontaneous long-bone insufficiency fractures" in 53 extremely elderly residents in long-term nursing homes, including 38 bedridden subjects, were recently described.[4]

An observational study was conducted in a hospital and long-term care facility with 1,993 beds for older people (male/female ratio approximately 3/10; mean age 79 for men, 85 for women), from 1998 to 2004 in Japan. Reports of accidents and possible abuse in the hospital were constructed from daily observations of nursing staff. Numbers of bedridden patients and those with joint contracture(s) were approximately 500 and 250, respectively, in the hospital during the study period. Spontaneous fractures were defined as fractures occurring in long bones in bedridden older people, without any apparent external force or abuse, during daily care procedures.

Clinical features of spontaneous fractures, as cited in Table 1, and the outcome up to 1 year after fracture(s) were reviewed from the medical records.

Eighteen bedridden inpatients (one man and 17 women, mean age ± standard deviation 88 ± 9) with spontaneous fractures were identified (Table 1). Their mean period of being bedridden was 7 ± 6 years. Their nutritional state just before they sustained fractures was poor, as evaluated using serum albumin level. Spontaneous fractures affected the femur in 12 cases (8 supracondylar fractures, 2 intertrochanteric fractures, 1 shaft fracture, and 1 neck fracture), the humerus in five cases (2 neck fractures, 2 shaft fractures, and 1 supracondylar fracture), and the proximal phalanx in one case. All spontaneous fractures occurred near joint contractures at proximal or distal sites of extremity bones. Ten patients had previously suffered long-bone fractures during nonbedridden periods, and in six of these 10 cases, spontaneous fractures reoccurred in the same bone. Four of five fractures in hemiplegic patients occurred on the paralytic side. Although one patient died due to worsening of pneumonia 1 month after fracture, 17 of 18 subjects were successfully treated with bandage procedures and showed recovery within approximately 2 months after fracture.

One of the characteristic features of spontaneous fractures in bedridden older people in this study was that one-third of the subjects had had previous fractures of the same bone where the spontaneous fractures occurred. More than half of the patients also had a history of fractures of long bones of traumatic or nontraumatic origin, indicating that elderly subjects with previous long-bone fractures during nonbedridden periods are prone to reoccurrence of long-bone fractures, especially in the healed bone, even after the start of their bedridden status.

As additional evidence of absorbing interest in this investigation, joint contractures adjacent to the fractures were found in all individuals. There were no cases of spontaneous fractures in the population of bedridden elderly without joint contractures during the survey. Joint contractures might be one of the risk factors leading to fractures. Of the 18 bedridden patients with fractures, joint contractures were observed at the proximal site in 17, at the distal site in 16, and at the proximal or distal sites of the fractured bones in all 18 subjects. A marked decrease in bone mass and bone quality due to multiple risk factors, including immobilization, disease, and malnutrition, should also be considered to be a fundamental factor in fracture.[3,4]

It has been reported that bone mineral density (BMD) decreased more rapidly on the paretic side than the nonparetic side,[5] and hemiplegic patients showed more-severe joint contractures on the paretic side than the nonparetic side in this study. The fractures seemed to occur at weakest point of the bone, near the contracted articulation. Joint contractures of an extremity fix the limb to the torso so that the contracted joint acts like a supporting point of leverage and any minimal external force or torque maneuver during passive transfer or lifting on the distal part of a long bone might easily make a bone with low BMD reach its fracture threshold. A subtle external force such as changing a diaper, washing, or putting the patient in an ambulatory or sitting position might produce a deforming force strong enough to make the bone reach its fracture threshold.

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Table 1. Clinical Characteristics of Patients with Spontaneous Fractures of Long Bones

No.	Age	Sex	Bedridden Period (Years)	Main Cause of Bedridden State	Serum Albumin Level (g/L)	Complications	Date of Spontaneous Fracture	Location of Fracture	Episode that Might Have Caused Fracture	History of Fracture of Same Bone	History of Other Long-Bone Fracture	Side of Hemiplegia if Present	Joint Contractures Adjacent to Fractured Bone	Treatment of Fracture	Outcome
1	91	Female	7	Cerebral infarction	29	Aspiration pneumonia, pressure ulcers	27-Oct-98	Shaft of right humerus	Unknown	—	Right hip fracture by falling	Right	Shoulder and elbow	Splint bandage	Died 1 month later from aspiration pneumonia
2	91	Female	2	Multiple cerebral lacunar infarction	33	Pressure ulcers	4-Apr-99	Supracondylar area of left femur	Unknown	—	—	—	Hip and knee	Plaster bandage	Recovered (died 9.5 months later from aspiration pneumonia)
3	91	Female	4	Multiple cerebral lacunar infarction	33	—	25-May-99	Supracondylar area of right femur	"Crack" heard during changing diaper	Left hip fracture by falling	—	—	Hip and knee	Plaster bandage	Recovered
4	100	Female	3	Hip fracture	33	—	31-May-99	Supracondylar area of right femur	Unknown	—	Left hip fracture by falling	—	Hip and knee	Plaster bandage	Recovered (died 3 months later from gastrointestinal bleeding)
5	86	Female	20	Multiple cerebral lacunar infarction	24	Aspiration pneumonia	3-Apr-00	Supracondylar area of left femur	Unknown	—	Left hip fracture by falling	—	Hip and knee	Plaster bandage	Recovered (died 9 months later from aspiration pneumonia)
6	93	Female	3	Senile dementia	27	—	3-Dec-00	Supracondylar area of right femur	Unknown (knee joint found to be swollen and skin reddish when nurse was applying ointment)	Right hip fracture by falling	—	—	Hip and knee	Plaster bandage	Recovered
7	82	Female	5	Intracerebral hemorrhage	31	Aspiration pneumonia	1-Jun-01	1) Shaft of right humerus 2) Shaft of right humerus	Unknown	Fracture of right humerus by falling	—	Right	Shoulder and elbow	Splint bandage	Recovered (died 5 months later from aspiration pneumonia)
8	63	Male	17	Intracerebral hemorrhage	39	—	8-Aug-01	Supracondylar area of left femur	Unknown	—	Left hip fracture by falling	Right	Knee	Plaster bandage	Recovered
9	85	Female	10	Multiple cerebral lacunar infarction	34	Aspiration pneumonia	8-Sep-01	Intertrochanteric area of right femur	Unknown	Fracture of right humerus by falling	—	—	Hip and knee	Splint bandage	Recovered
10	89	Female	5.5	Multiple cerebral lacunar infarction	30	—	15-Nov-01	Neck of left humerus	Unknown	—	—	Left	Shoulder	Splint bandage	Recovered
11	96	Female	3.7	Cerebral infarction	25	—	18-Jan-02	Supracondylar area of left femur	Unknown	Left hip fracture by falling	—	—	Hip and knee	Plaster bandage	Recovered
12	82	Female	22	Multiple cerebral lacunar infarction	35	—	30-Apr-02	Supracondylar area of right humerus	Unknown	—	—	Right	Shoulder and elbow	Adhesive plaster bandage	Recovered
13	94	Female	12	Multiple cerebral lacunar infarction	36	Urinary tract infection	9-May-02	Neck of right humerus	Unknown (rigid contracted joint found to be floppy when changing diaper)	—	—	—	Shoulder and elbow	Splint bandage	Recovered
14	73	Female	2	Parkinson disease	33	Urinary tract infection, pressure ulcers	6-Sep-03	Shaft of right femur	Fracture might have occurred while lifting right leg during treatment of a decubitus ulcer	—	—	—	Hip and knee	Open reduction and internal fixation	Recovered
15	90	Female	4	Senile dementia	37	—	22-Dec-03	Supracondylar area of left femur	Unknown	Left hip fracture by falling	Right hip fracture by falling	—	Hip and knee	Plaster bandage	Recovered
16	89	Female	4	Senile dementia	32	—	30-Dec-03	Proximal phalanx of left second finger	Unknown	—	—	—	Fingers and wrist	Adhesive plaster bandage	Recovered
17	98	Female	2	Multiple cerebral lacunar infarction	32	—	14-Jan-04	Intertrochanteric area of right femur	Unknown	—	—	—	Hip and knee	Traction only	Recovered
18	90	Female	6	Multiple cerebral lacunar infarction	34	—	16-Jul-04	Neck of left femur	Unknown	—	—	—	Hip and knee	Plaster bandage	Recovered

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