not been confirmed.

Renin-Angiotensin-Aldosterone System

GS usually presents with secondary hyperaldosteronism due to salt wasting. In this patient, hyperreninism with a high concentration of Ang I (two to five fold greater than normal) was observed, but hyperaldosteronism and high Ang II concentration were absent. These symptoms persisted for 10 years. In another Japanese case report of GS (serum potassium: 2.6 mmol/l), plasma Ang I and Ang II concentrations were extremely elevated (11,737 pmol/l and 228 pmol/l, respectively) but hyperaldosteronism was mild (0.66 nmol/l) (*16*). Gibbs *et al.* showed that plasma aldosterone may not be as high as expected for the degree of hyperreninemia in BS and GS because of the low total body potassium (*17*). Hypokalemia may also explain the relatively mild hyperaldosteronism in GS. Since high Ang I concentration was not associated with high Ang II concentration in our case, we speculated that an impairment of the Ang II–generating system may have been responsible for the lack of hyperaldosteronism. Serum ACE activity was normal. Administration of captopril suppressed plasma Ang II and plasma aldosteron concentration (PAC), and led to marked elevation of plasma renin activity (PRA) and plasma Ang I concentration, suggesting that ACE effectively induced the generation of Ang II in this patient. We suspect that, in the present case, GS may have primarily impaired the activity of other Ang II–generating enzymes (*i.e.*, chymase or cathepsin G).

Ang II–Generating Enzymes

In addition to ACE, there are other enzymes, including chymase or cathepsin G, with the ability to generate Ang II *in vivo* (*18*). Chymase has high specificity for Ang I and its Ang II–generating ability is higher than that of ACE *in vitro* (*19*). We examined the activities of these three Ang II–generating enzymes (ACE, chymase and cathepsin G) in lymphocytes of the present case. Chymase-dependent AIIFA was not detected and the other enzyme activities were extremely high. Thus, we speculated that a decrease in the activity of chymase was responsible for the absence of hyperaldosteronism in the present case. Moreover, the lack of hyperaldosteronism led to mild hypokalemia in this case. It is not clear that the absence of chymase-dependent AIIFA in lymphocytes can be extrapolated to other tissues. For this reason, it will be important to determine the activity and expression of chymase in the renal tissue of this patient in a future work. An analysis of the chymase gene would also be useful. Ono *et al.* sequenced the human mast cell chymase gene (CMA1) and found 13 single-nucleotide polymorphisms, two of which were loss-of-function mutations (*20*). Since we have not sequenced the chymase gene, we are unable to exclude the possibility that the nonsense mutation of the chymase gene resulted in the lack of chymase activity in this patient. Since the activities of ACE and cathepsin G were extremely elevated, it is not clear whether a suppression of chymase activities could explain the lack of elevated Ang II and aldosterone. In 1981, Bergstein *et al.* reported three patients with a disease that resembled BS; these patients had low plasma concentrations of aldosterone despite their elevated renin activity (*21*). Umeki *et al.* also reported a patient who had hyperreninism associated with Ang I elevation but who lacked Ang II elevation or hyperaldosteronism (*22*). He concluded that the decreased affinity of ACE to the substrate Ang I (so-called ACE dysfunction syndrome) was the cause of this phenomenon. We speculate that chymase-dependent AIIFA were impaired in these cases. There are no reports that show the activity of Ang II–generating enzymes in GS. The severity of clinical findings is variable in BS or GS. Chymase activity in BS or GS may be responsible for the clinically severe forms of these diseases. Moreover, the novel mutation of TSC (R261C) found in this patient may have played a role in his mild hypokalemia and hypomagnesemia.

GS with Glomerulonephritis

Our patient had histologically diagnosed mesangial proliferative glomerulonephritis. To our knowledge, GS associated with glomerulonephritis has not been reported. Because his renal function is preserved and his hypokalemia and proteinuria are mild, we are currently treating him by recommending potassium rich foods.

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研究論文

高血圧と認知機能
―単語記憶テストの有用性についての検討―

大田祐子* 土橋卓也* 尾中宇蘭* 江藤仁香* 上野道雄*
三輪宜一** 鬼木秀幸**

*国立病院機構九州医療センター高血圧内科，**九州大学大学院病態機能内科学

はじめに

高血圧は，血管障害という脳の構造的異常を引き起こすのみならず，高血圧の持続が脳白質病変を招き，痴呆の前段階あるいは初期症状ともいわれている認知機能障害という機能的異常も惹起する[1)~4)]．また血圧管理が脳血管障害の発症のみならず，白質病変の進展を抑制し，認知障害を改善させることが多数報告されている[5)~7)]．Syst-Eur（Systolic Hypertension in Europe trial）試験において，Ca 拮抗薬投与群はプラセボ群にくらべ脳卒中の抑制効果とともにアルツハイマー型を含む痴呆の発症も有意に抑制した[7)]．またSCOPE（Study on Cognition and Prognosis in the Elderly）試験では，ARB 投与群において非致死的脳卒中の発症が有意に低下し，試験前の認知機能が軽度低下していた群でのMMSE（mini-mental state examination）の更なる低下も有意に抑制した[5)]．さらにPROGRESS（Perindopril Protection Against Recurrent Stroke Study）試験では，脳卒中再発例においてACE 阻害薬を基礎薬とした降圧治療により認知機能の低下を有意に抑制した[6)]．しかしながら，高血圧でみられる認知機能障害は，脳血管障害に移行する過程なのか，臨床的に脳血管障害をきたす前に代謝を低下させ脳全体を救う代償機転なのか，血圧値と認知機能との直接的関係はあるか否かなどについては明らかにされていない．

知的機能には，記憶・理解・学習・思考・判断・自発性・意欲などさまざまな側面があり，加齢によって衰えやすい機能とそうでない機能がある．前述の大規模臨床試験では，いずれも認知機能の評価としてMMSEが用いられているが，最近，高血圧を対象とした認知機能の評価として，簡易な単語記憶テストの有用性が報告された[8)9)]．

そこで今回，高血圧診療における認知機能評価の指標としての単語記憶テストの妥当性と有用性について検討した．

対象と方法

健常者，外来高血圧患者，老人保健施設入所者を対象とした．(1) 健常者は24例で，男性12例，女性12例，年齢32.8±9.6歳(20～53歳)，高血圧や糖尿病，脳卒中などの疾患のない者とした．(2) 当院外来通院中の高血圧患者は70例で，男性23例，女性47例，年齢67.5±8.0歳（52～84歳），いずれも最低6ヵ月以上ARB ロサルタンまたはCa 拮抗薬アムロジピンを単独投与されていた．ロサルタン群は33例で，年齢66.5±7.7歳，平均投与量40.2 mg，アムロジピン群は37例で，年齢67.5±8.3歳，平均投与量3.7 mg であった．(3) 老人保健施設入所者は32例で，男性4例，女性28例，年齢83.8±7.0歳(67～97歳)，そのうち高血圧者は23人(72%)，脳卒中既往者は18人（56%）含まれていた．互いに関連のない平易な日常使用されている10個の単語をコンピュータの画面に2秒ごとに示し，直後と10分後における記憶単語数を評価した．テスト画面において対象者が文字を認識できることを確認したうえで試験をおこなった．老人保健施設入所者および一部の外来高血圧患者に対しては，MMSE も施行し，単語記憶テストとの関係について検討した．また外来高血圧患者においては脈波伝播速度（PWV）も測定した．

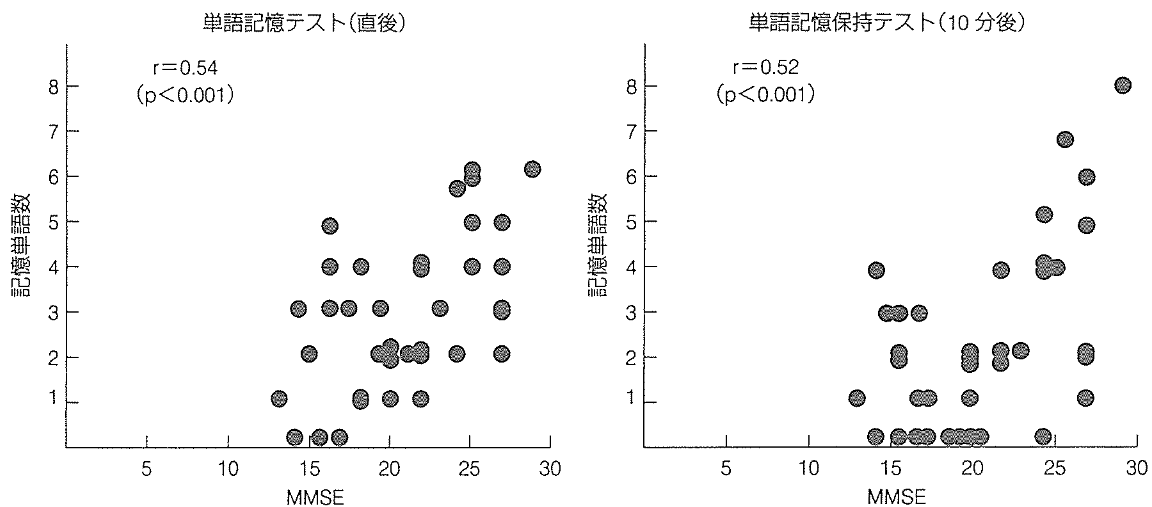

図 1. MMSEと単語記憶テストとの関係(老人保健施設入所者および外来高血圧患者)
年齢:81.6±8.6(64〜97)歳, MMSE:平均 20.7±4.2.

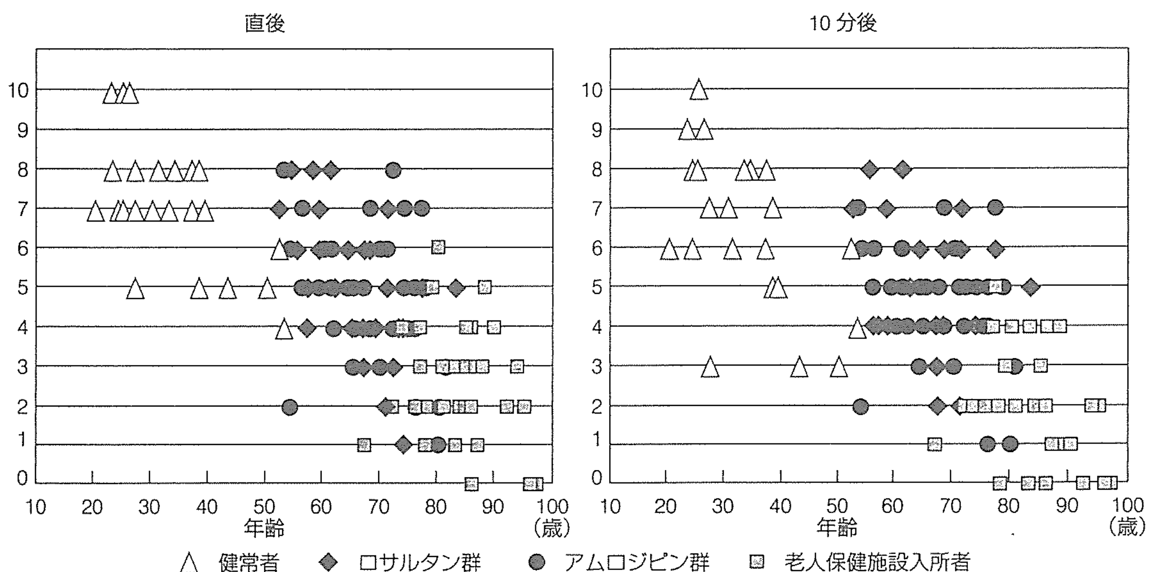

図 2. 単語記憶テストと年齢との関係(全対象者)

結 果

単語記憶テストは直後, 10分後ともにMMSEスコアと有意な相関を認めた(n=39, 直後r=0.54, 10分後r=0.52, 各々p<0.001)が, MMSEスコアが25点以上の比較的高い集団であっても単語記憶テストのスコアにはばらつきを認めた(**図1**). すべての対象者における年齢と単語記憶テストとの関係を**図2**に示す. 外来高血圧患者群におけるスコアは, 直後, 10分後ともに, 年齢と負の相関を示した(直後r=−0.45, p<0.001, 10分後r=−0.36, p<0.005). 同様に健常者, 高齢者においてもスコアと年齢に負の相関を認めた. 記憶単語数は, 健常者で直後7.13±1.60, 10分後6.58±2.00, 外来高血圧患者で, 直後4.90±1.63, 10分後4.69±1.51, 老人保健施設入所者で直後2.50±1.50, 10分後1.91±1.44といずれの群においても有意に低下していた(それぞれp<0.01, p<0.05, p<0.01, **図3**). 外来高血圧患者については, ロサルタン群, アムロジピン群に分けて検討をおこなったが, 両群の患者背景には明らかな差異を認めなかった(**表1**). ロサルタン群における単語記憶テストの

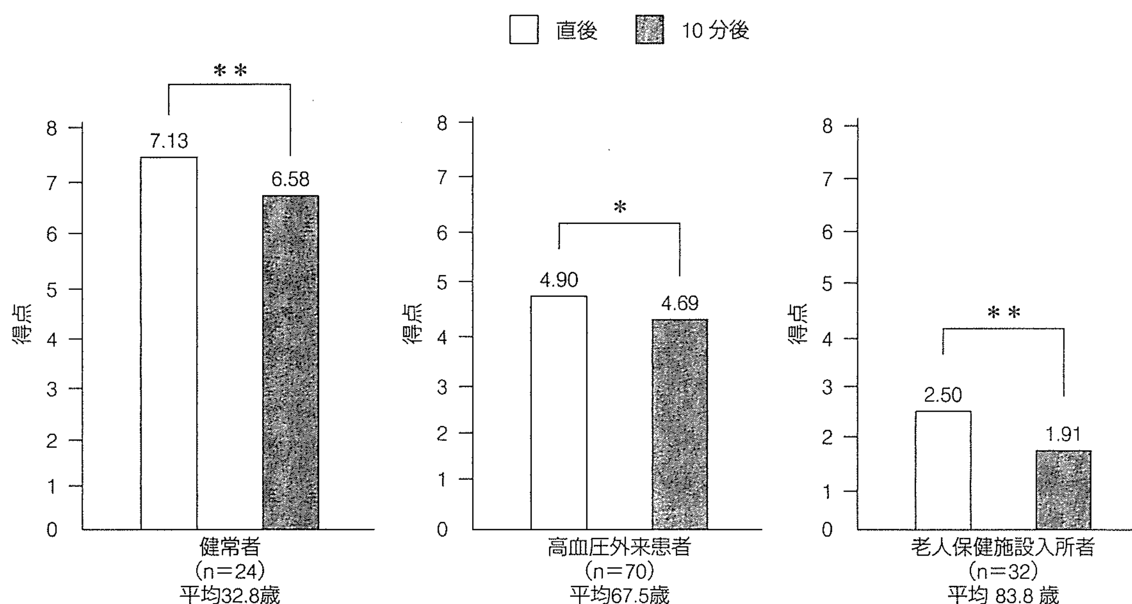

図 3．健常者・高血圧患者・老人保健施設入所者における単語記憶テストのスコア
＊＊p<0.01，＊p<0.05．

表 1．高血圧患者の背景因子

	全対象	ロサルタン群	アムロジピン群
症例数	70	33	37
年齢（歳）	68±8	67±8	68±8
性別（男性/女性）	23/47	12/21	11/26
収縮期血圧（mmHg）	147±18	142±22	148±17
拡張期血圧（mmHg）	86±11	82±14	86±10
脈圧（mmHg）	61±13	60±18	61±12
総コレステロール値（mg/d*l*）	208±32	208±33	208±32
血糖値（mg/d*l*）	107±23	113±29	100±12
body mass index（kg/m²）	23±3	24±3	23±2
ヘマトクリット（%）	41±4	40±4	42±4
baPWV（cm/s）	1,801±333	1,802±333	1,800±338
尿中食塩排泄量（g/日）	9.0±3.3	9.6±3.2	8.3±3.4

baPWV：brachial-ankle pulse wave velocity.
（平均±標準偏差）

スコアは，直後 5.03±1.69，10 分後 4.82±1.59 であった（p=0.23）．一方，アムロジピン群における単語記憶テストのスコアは，直後 4.78±1.67，10 分後 4.57±1.44 であり（p=0.05），ロサルタン群と有意差を認めなかったが，ロサルタン群では記憶保持がよい傾向にあった（図 4）．外来高血圧患者における単語記憶テストの関連因子として，直後の単語記憶数は年齢，脈圧，PWV と負の相関を，10 分後の単語記憶数は年齢と負の相関を，直後の単語記憶数と 10 分後の単語記憶数の差は拡張期血圧と正の相関を認めた（**表 2**）．外来高血圧患者において単語記憶テストのスコアを目的変数とした多変量回帰分析では，年齢が負の規定因子として検出された（**表 3**）．

考　察

本研究において，単語記憶テストが MMSE スコアと有意な相関を認めたことから，単語記憶テストは記銘力障害を簡易に評価できる方法として有用であると考えられる．しかしながら，MMSE スコアが比較的高い集団においても単語記憶テストのスコアにばらつきを認めたことから，単語記憶テストは MMSE とは異なる機能を評価しているのかもしれない．MMSE は，見当識，即時・

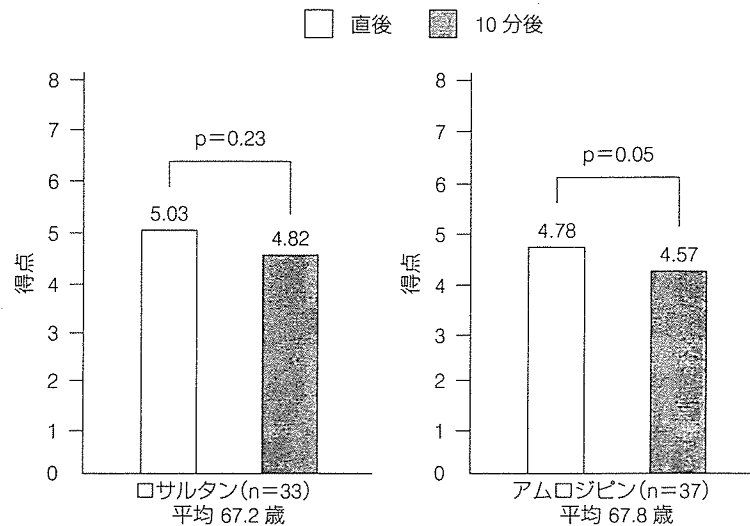

図 4. ロサルタン投与群とアムロジピン投与群における単語記憶テストの比較

表 2. 高血圧患者における単語記憶テストの関連因子(単相関)

	直後		10分後		直後−10分後	
	相関係数	p	相関係数	p	相関係数	p
年齢(歳)	−0.451	<0.001	−0.360	0.002	−0.228	0.058
収縮期血圧(mmHg)	−0.055	0.652	−0.044	0.715	−0.027	0.827
拡張期血圧(mmHg)	0.208	0.084	0.093	0.442	0.237	0.048
脈圧(mmHg)	−0.243	0.042	−0.136	0.261	−0.228	0.057
baPWV (m/s)	−0.307	0.010	−0.228	0.059	−0.187	0.124
BMI (kg/m²)	0.005	0.971	−0.096	0.429	0.183	0.129

baPWV : brachial-ankle pulse wave velocity, BMI : body mass index.

表 3. 高血圧患者における単語記憶テストに対する規定因子(多変量解析)

	直後			10分後			直後−10分後		
	β	p	R^2	β	p	R^2	β	p	R^2
年齢(歳)	−0.103	<0.001	0.244	−	−	−	−0.035	0.046	0.071
BMI (kg/m²)	−	−	−	−	−	−	0.102	0.037	0.073

BMI : body mass index.

遅延記憶，注意，計算，呼称，復唱，口頭命令動作，読字，書字，図形模写の検査からなる．一方，単語記憶テストは，言語記憶検査における直後自由再生，遅延自由再生検査であり記銘-保持-想起という言語再生機能を反映している．認知機能検査にはいろいろあるが，それぞれ短所，長所がある．痴呆の評価としてはWAIS-R (Wechsler adult intelligence scale-revised：改訂版)が広く使われており，記憶，抽象思考，言語概念形成，注意，集中，推理，判断，精神的敏速性，計画能力，視覚・運動の協応など多岐にわたる心理機能を測定しているが，WAIS-Rは施行にかなりの時間を必要とし，老人には困難な場合も少なくない．また，わが国で最もよく用いられている長谷川式簡易知能検査改訂版には，前頭葉機能を反映する語想起の評価項目はあるが，MMSEの評価項目にはない．一方，MMSEは空間・位置的関係の認知や図形構成など動作性知能を評価できるが，短時間でできる長谷川式簡易知能検査改訂版では評価できない．このように認知機能検査では用いられる検査法の特性によって結果が変わる可能性がある．本研究で用いた単語記憶テストは短時間でできるが，これのみで認知機能全般を評価したわけではない．しかしながら認知機能の一部の簡易評価法，とくに記銘力の評価としては有用

であると思われる．

Ca拮抗薬，ACE阻害薬，ARBを用いた大規模臨床試験[5)~7)]において，認知機能低下あるいは痴呆発症が抑制されることが報告されているが，これらの薬剤はいずれも降圧とは独立した脳保護作用がある可能性が示唆されている．すなわち，Ca拮抗薬には脳虚血時の神経細胞死における脳血管拡張作用が指摘されており，ACE阻害薬やARBには脳血管リモデリングの改善，脳血流自動能の低下抑制，頸動脈などの粥状硬化の抑制，内皮機能改善，ラジカルストレスの抑制などの関与が報告されている．一方，利尿薬はSHEP（Systolic Hypertension in the Elderly Program）試験において認知機能障害の予防はできなかったと報告されているが[10)]，PROGRESS試験のようにACE阻害薬と併用すれば，認知機能の低下を抑制すると推測される．

今回われわれが用いた単語記憶テストと同様の方法を用いて，Fogariら[9)]はACE阻害薬とARBを4ヵ月内服させた後の単語記憶数はARB投与群で有意に多かったと報告している．脳内のアンジオテンシンⅣが海馬など記憶や認知に関与している部位に存在するAT_4受容体を介して認知機能の保持に作用する可能性が示唆されている[11)]．このように降圧薬自体の認知機能障害の抑制度に違いがある可能性があると考えられるが，一方ではβ遮断薬，利尿薬，中枢性作動性降圧薬，ACE阻害薬，Ca拮抗薬を含んだ多種降圧薬間での認知機能への影響はみられなかったとの報告もみられる[12)]．本研究では，ロサルタンまたはアムロジピンを最低6ヵ月以上内服後の患者を対象に検討をおこなった結果，ロサルタン群で記憶の保持がよい傾向にあったが，記憶単語数および記憶保持能力に有意差を認めなかった．

本研究では，降圧薬投与前の血圧値や単語記憶テストの結果を評価しておらず，今後，降圧薬や血圧値との関係を明らかにするための前向き研究が必要であると思われる．

おわりに

単語記憶テストはMMSEと有意な相関を認めたが，MMSEスコアが比較的高い集団であっても単語記憶テストのスコアにはばらつきを認めた．単語記憶テストのスコアには年齢の影響が最も強く認められ，ロサルタン服用者とアムロジピン服用者のあいだに明らかな差異は認めなかった．単語記憶テストは，認知機能の簡易評価法として有用である可能性があり，今後，降圧薬や血圧値との関係について前向き試験での評価が望まれる．

謝　辞

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Impaired Expression of Cardiac Adiponectin in Leptin-Deficient Mice With Viral Myocarditis

Takashi TAKAHASHI,[1] MD, Fei YU,[1] MD, Seiichiro SAEGUSA,[1] MD,
Hiroyuki SUMINO,[3] MD, Takeshi NAKAHASHI,[2] MD, Kunimitsu IWAI,[2] MD,
Shigeto MORIMOTO,[2] MD, Masahiko KURABAYASHI,[3] MD,
and Tsugiyasu KANDA,[1] MD

SUMMARY

A mouse model of encephalomyocarditis (EMC) virus-induced myocarditis was used to investigate the expression of adiponectin in damaged cardiomyocytes. We intraperitoneally injected EMC virus into leptin-deficient *ob/ob* (OB) mice and wild-type (WT) mice. OB mice were divided into two subgroups consisting of mice with no intervention and mice receiving leptin replacement starting simultaneously with viral inoculation. We determined differences in heart weight, cardiac histological score, numbers of infiltrating and apoptotic cells in the myocardium, expression levels of adiponectin and TNF-α mRNA in the heart, adiponectin immunoreactivity in myocytes, adiponectin and TNF-α concentrations in the heart, and immunoreactivity of adiponectin receptors in myocytes between OB mice and WT mice. There was significantly decreased adiponectin mRNA expression, immunoreactivity, and protein level in the heart, and reduced immunoreactivity of adiponectin receptor 1 in myocytes from OB mice on days 4 and 8 after viral inoculation as compared with those in WT mice, together with increased cardiac weight, severe inflammatory myocardial damage, and increased levels of cardiac TNF-α mRNA and protein. Replacement of leptin in OB mice inhibited the development of severe myocarditis through augmentation of adiponectin mRNA, immunoreactivity, and protein level, increased adiponectin receptor 1 immunoreactivity in myocytes, and suppressed levels of TNF-α mRNA and protein. These results suggest that impaired expression of cardiac adiponectin may contribute to the progression of viral myocarditis through enhanced expression of TNF-α under a leptin-deficient condition. (Int Heart J 2006; 47: 107-123)

Key words: Adiponectin, Leptin deficiency, Viral myocarditis, Cardiomyocyte

HEART failure is generally considered to begin with myocyte damage caused by a variety of pathological conditions that include ischemia, toxins, and myocardial infection. The heart compensates by dilatation and cellular hypertrophy, and eventually decompensates, resulting in heart failure. A proinflammatory cytok-

From the Departments of [1] General Medicine and [2] Geriatric Medicine, Kanazawa Medical University, Ishikawa and [3] Department of Medicine and Biological Science, Gunma University Graduate School of Medicine, Gunma, Japan.
Address for correspondence: Tsugiyasu Kanda, MD, Department of General Medicine, Kanazawa Medical University, 1-1 Daigaku, Uchinada-machi, Kahoku-gun, Ishikawa 920-0293, Japan.
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ine, tumor necrosis factor-α (TNF-α), has recently been postulated to be one of the pathogenetic factors responsible for the progression from compensated to decompensated heart failure. Mann and colleagues in 1996 demonstrated that the nonfailing human heart does not express, whereas the failing human heart expresses a significant amount of TNF-α.[1] Moreover, TNF-α immediately inhibits the contractility of isolated cardiac myocytes in a dose-dependent manner, and this negative inotropic action is completely reversible upon the removal of TNF-α.[2]

Identification of leptin is a good example of a contribution made by molecular biology to understanding the mechanisms initially hypothesized from classic physiological studies.[3] Several papers have focused on the association between leptin and cardiovascular disease, such as hypertension and cachexia, in chronic heart failure.[4-6] A reduced leptin concentration may diminish the degree of cardiac adaptation to heart failure.[7] It has also been shown that plasma leptin levels are inappropriately low in patients with cachectic chronic heart failure.[5] We have recently found that leptin deficiency enhances myocardial necrosis and lethality in a mouse model of viral myocarditis, suggesting a protective action of leptin against myocyte damage.[8] However, it is still unclear how severe inflammatory myocardial injury induced by viral infection develops under leptin deficiency.

Adiponectin, also known as 30-kDa adipocyte complement-related protein,[9] is a hormone secreted by adipocytes that acts as an antidiabetic and antiatherogenic cytokine.[10] The concentration of adiponectin in blood is diminished under conditions of obesity, insulin resistance, and type II diabetes.[9] Adiponectin administration is reported to have glucose-lowering effects and to improve insulin resistance in mice.[11] On the other hand, adiponectin-deficient mice develop insulin resistance and diabetes.[12] This effect of adiponectin appears to be mediated by elevation of fatty acid oxidation through activation of AMP-activated protein kinase[13] and peroxisome proliferator-activated receptor (PPAR)-α.[10] In clinical research, plasma adiponectin concentrations in subjects with both diabetes mellitus and coronary artery disease have been shown to be lower than in patients with coronary artery disease alone.[14] Individuals with very low plasma adiponectin levels may be at increased risk of developing both diabetes mellitus and coronary artery disease.

Comparison of the organization of the adiponectin gene with that of the obese gene, which encodes leptin, shows several striking similarities in humans.[15] These two genes, which are composed of three exons and have a long first intron, are expressed specifically in adipose tissue.[15] Adiponectin and leptin control fuel homeostasis, body weight, and insulin sensitivity. In another recent study, amelioration of insulin resistance, pancreatic β-cell degranulation, and diabetes after crossing leptin-deficient mice with globular domain adiponectin trans-

genic mice has been described, indicating that globular adiponectin and leptin may have overlapping functions.[10] Thus, adiponectin may also possess a function similar to the crucial role of leptin in the development of heart failure.

We hypothesized that cardiac expression of adiponectin could play a protective role against the progression of severe viral myocarditis under a leptin-deficient status. Therefore, we examined cardiac adaptation to heart failure through adiponectin expression in leptin-deficient mice with acute viral myocarditis, and the influence of leptin replacement therapy on adiponectin expression in the myocardium of leptin-deficient mice.

Materials and Methods

Animals: Six-week-old female leptin-deficient *ob/ob* (OB) mice and C57BL wild-type (WT) mice were obtained from Jackson Laboratory (Bar Harbor, Maine, USA).

Virus: A myocarditic variant of encephalomyocarditis (EMC) virus was obtained from Y. Seto, PhD (Keio University, Tokyo, Japan). Virus preparations were stored at –80°C in Eagle's minimum essential medium 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: WT ($n = 10$) and OB ($n = 40$) mice were injected with EMC virus. In addition, OB mice were randomly assigned to one of two groups. The first group ($n = 20$) received no interventional therapy. The second group ($n = 20$) of OB mice received a daily intraperitoneal injection of recombinant mouse leptin (300 μg per day, starting simultaneously with EMC virus inoculation, OB + Lep group).[8] Cardiac tissues were immediately extracted after sacrifice by cervical dislocation on days 4 and 8 after viral inoculation.

Histological examinations of hearts: Body weight was recorded before sacrifice. The heart was immediately weighed after sacrifice. Half of the cardiac tissue was fixed in 10% buffered formalin and stained with hematoxylin-eosin (H & E), while the other half was immediately frozen in liquid nitrogen and stored at –80°C for cytokine analyses. 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 the 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 staining of myosin to identify myocyte necrosis accurately. In addition, the pathologist randomly selected 5

high power fields (HPF) (× 400 magnification) from each transverse section of the myocardium, and counted the infiltrating cells. The number of apoptotic cells in 5 randomly selected HPF (× 400 magnification) per section in the transverse sections of myocardium was determined by *in situ* TUNEL as previously described.[16)]

Detection of adiponectin mRNA in cardiomyocytes: *In situ* hybridization (ISH) using Digoxigenin (DIG) REMBRANDT for DNA ISH and a detection kit (Code HKD38003, Pan Path Co. Ltd., Amsterdam, Netherlands) was performed on serial sections of the heart from various mice on day 8 after viral inoculation as previously described.[17)] The presence of adiponectin mRNA was also examined in cardiac tissue from a normal wild-type mouse with neither viral inoculation nor leptin administration. We used commercially synthesized DIG-labeled mouse adiponectin sense and antisense RNA probes (Hokkaido System Science Co. Ltd., Hokkaido, Japan). Each section was hybridized with the labeled probes at 37°C for 2 hours. This was followed by several washes in Tris-buffer and RNase A solutions. The sections were then incubated with alkaline phosphatase-conjugated rabbit anti-DIG Fab fragments (#D5105, DAKO Cytomation Co. Ltd., Kyoto, Japan). Signals were visualized with 4-nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The slides were blindly reviewed by the same pathologist, and the hybridization signal of adiponectin mRNA was determined for myocytes.

Comparative expression levels of adiponectin and TNF-α mRNA in cardiac tissues: RNA extraction was performed on half of each frozen cardiac tissue specimen as specified by the manufacturer (RNeasy Mini Kit, QIAGEN Inc., Tokyo). Total RNA concentration was determined by measuring the optical density at 260 nm. Aliquots of 20 μL of RNA from each tissue sample were used for the production of cDNA. Comparative expression levels of adiponectin mRNA in cardiac tissue from different groups were determined using quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously.[18)] The expression levels of TNF-α mRNA were also examined by quantitative real-time RT-PCR. We used a commercially available kit for TNF-α and adiponectin RT-PCR (Mm00443258 mL and Mm00456425 mL, respectively, Applied Biosystems Inc., Foster City, CA, USA). The optimal number of cycles of RT-PCR was examined for the adiponectin mRNA level. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous internal standard, and was amplified with specific primers for the number of cycles. The gradient of the cycle numbers for adiponectin to GAPDH was determined. Adiponectin mRNA levels in the heart were calculated as comparative values, which were normalized to adiponectin mRNA in the heart from a normal wild-type mouse with neither viral inoculation nor leptin administration (assigned value

equal to 1).

Adiponectin immunoreactivity in cardiomyocytes: Immunohistochemical staining using the streptavidin biotin complex method (#K0675 and #E0353, DAKO Cytomation) was performed on serial transverse sections of ventricular myocardium from different mice on days 4 and 8 after viral inoculation. As a normal control, adiponectin immunoreactivity was determined in a heart from a normal wild-type mouse with neither viral inoculation nor leptin administration. We used the following commercially available primary antibody at a dilution of 1:50; rabbit polyclonal anti-mouse adiponectin antibody (#ACRP303-A, Alpha Diagnostic International Inc., San Antonio, TX, USA). Control slides were treated with normal diluted rabbit serum. The slides were blindly reviewed by the same pathologist, and were semiquantitatively graded according to the degree of immunoreactivity: 0 for absence of staining, 1+ for weak, 2+ for moderate, and 3+ for strong staining.[19] They were compared with the respective control slides to exclude nonspecific staining. Adiponectin immunoreactivity was assessed in 30 randomly selected myocytes corresponding to the surviving cells found in respective H&E- and myosin-stained slides.

Concentrations of adiponectin and TNF-α in heart: Adiponectin and TNF-α levels in the homogenate of each tissue were measured using the other half of frozen cardiac tissue samples. An enzyme-linked immunosorbent assay (ELISA), which used a polyclonal antibody specific for mouse TNF-α or adiponectin precoated onto a microtiter plate (ELISA kit for TNF-α: BioSource International Inc., Camarillo, CA, USA; ELISA kit for adiponectin: Otsuka Pharmaceutical Co., Ltd., Tokyo), was performed on tissue samples according to the manufacturers' instructions. As a normal control, cardiac levels of adiponectin and TNF-α were determined in the heart from a normal WT mouse without viral infection. For data processing, we allocated the minimum values detected by the ELISA assay to all samples with concentrations below the detection threshold. The ELISA kit used for TNF-α concentration showed that the limit of sensitivity and the intra- and interassay variations were 3.0 pg/mL, 6.5%, and 8.7%, respectively. The ELISA kit used for adiponectin levels demonstrated sensitivity, intra-assay variance, and cross-reactivity of 0.25 ng/mL, less than 10%, and no response for specimens from other animals including sheep, respectively.

Immunoreactivity of adiponectin receptors in cardiomyocytes: To examine the immunoreactivity of adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) in the heart, immunohistochemical staining by the streptavidin biotin complex method was performed on serial sections of myocardium on days 4 and 8, which had been used to determine the adiponectin immunoreactivity of myocytes. The immunoreactivity of AdipoR1 and AdipoR2 in vessels and macrophages was considered as a positive control. As a normal control, the immunoreactivity of

AdipoR1 or AdipoR2 was determined in the heart from a normal wild-type mouse with neither viral inoculation nor leptin administration. We used the following primary antibodies at a dilution of 1:50: rabbit polyclonal anti-mouse AdipoR1 or AdipoR2 antibodies (#ADIPOR11-A or ADIPOR21-A, Alpha Diagnostic International Inc.). Control slides were treated with normal diluted rabbit serum. The slides were blindly reviewed by the same pathologist, and were semi-quantitatively graded according to the criteria for immunoreactivity as used for adiponectin immunoreactivity. They were compared with the respective control slides, and AdipoR1 or AdipoR2 immunoreactivity was evaluated in 30 myocytes corresponding to cells where adiponectin immunoreactivity was observed.

Statistical analyses: Data are expressed as the mean ± standard deviation. Analysis of variance (ANOVA) was used to evaluate the differences in body and cardiac weights, numbers of infiltrating and apoptotic cells in the myocardium, comparative expression level of adiponectin mRNA in cardiac tissue, and adiponectin concentration in the heart, as compared with those in the WT group. The Kruskal-Wallis test was used for nonparametric analysis to assess the differences in cardiac histological score and immunoreactivity of adiponectin or its receptors in myocytes. A value of $P < 0.05$ was considered to be statistically significant.

Results

Mortality in different mice with viral myocarditis: The numbers of mice with viral myocarditis that died were 1, 9, and 2 in the WT, OB, and OB + Lep groups, respectively, during the period of the treatment protocol. The numbers of mice in different groups from which heart specimens were obtained on days 4 and 8 after viral inoculation were 5 and 4 in the WT group, 6 and 5 in the OB group, and 10 and 8 in the OB + Lep group, respectively.

Body weight and cardiac weight: Body weight on days 0, 4, and 8 after viral infection was significantly greater in the OB and OB + Lep groups than in the WT group ($P < 0.05$, Table). Heart weight in the OB group on day 8 after viral inocu-

Table. Body Weight and Cardiac Weight in Different Mice Groups After Viral Inoculation

	Body weight (g)			Cardiac weight (mg)	
	Day 0	Day 4	Day 8	Day 4	Day 8
WT	18.1 ± 1.2	18.3 ± 1.5	18.9 ± 1.7	97 ± 5	101 ± 8
OB	37.3 ± 2.5*	37.1 ± 2.9*	38.2 ± 3.1*	105 ± 7	118 ± 15*
OB + Lep	36.9 ± 2.8*	37.2 ± 3.5*	35.1 ± 4.1*	99 ± 8	103 ± 11

WT indicates wild-type mice; OB, ob/ob mice; and OB ± Lep, ob/ob mice receiving leptin. Data are expressed as means ± SD. * $P < 0.05$ compared with WT mice group.

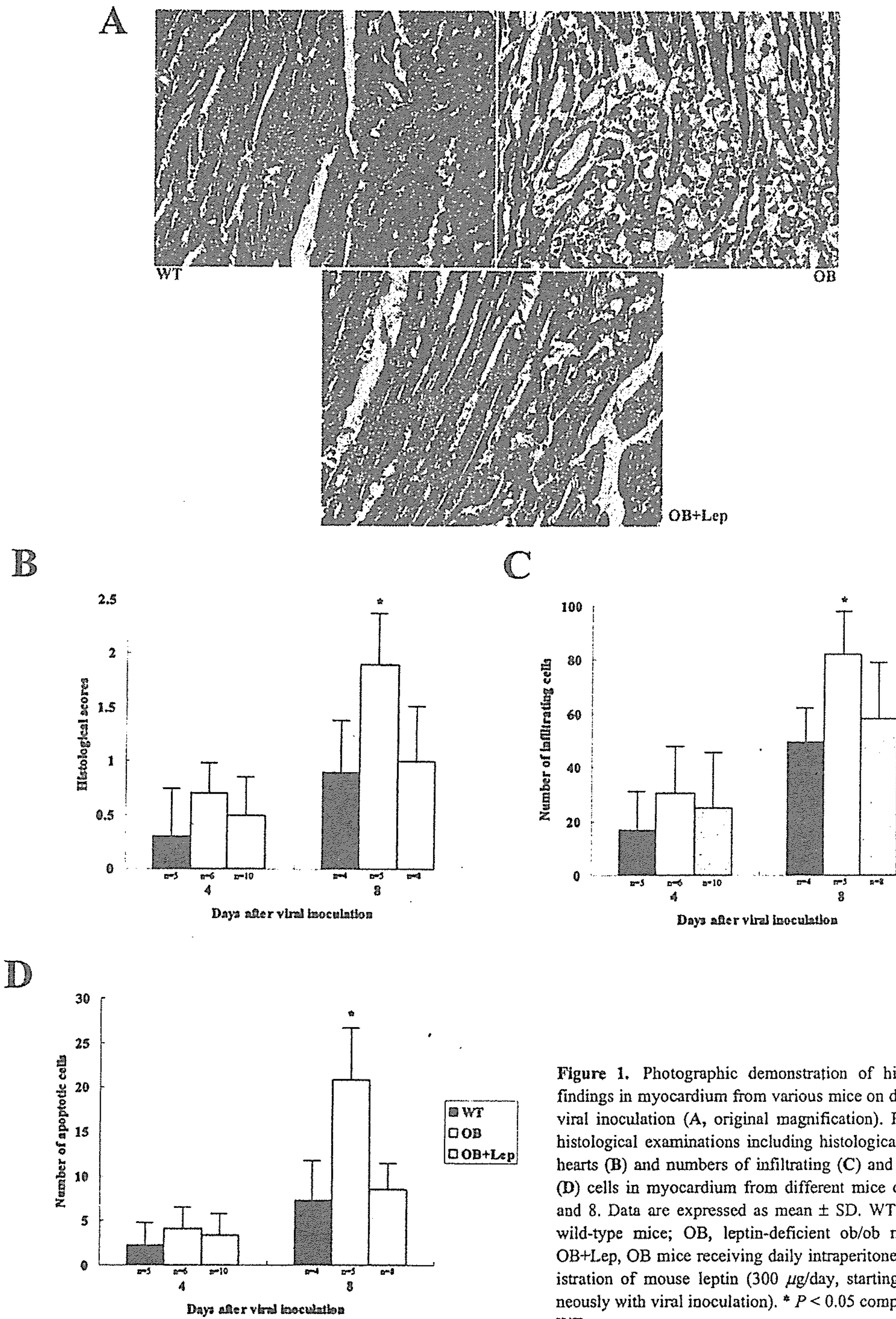

Figure 1. Photographic demonstration of histological findings in myocardium from various mice on day 8 after viral inoculation (A, original magnification). Results of histological examinations including histological score in hearts (B) and numbers of infiltrating (C) and apoptotic (D) cells in myocardium from different mice on days 4 and 8. Data are expressed as mean ± SD. WT indicates wild-type mice; OB, leptin-deficient ob/ob mice; and OB+Lep, OB mice receiving daily intraperitoneal administration of mouse leptin (300 μg/day, starting simultaneously with viral inoculation). * $P < 0.05$ compared with WT group.

lation was significantly increased as compared with that in the WT group ($P < 0.05$, Table). There was no significant difference in cardiac weight between the OB + Lep group and the WT group.

Histological findings in heart: Photographic demonstration of the histological findings in the myocardium from various mice on day 8 after virus infection is shown in Figure 1A. The histological score and numbers of infiltrating and apoptotic cells per field in hearts from different mice on days 4 and 8 are shown in Figures 1B, 1C, and 1D, respectively. The hearts from the OB group showed severe myocardial necrosis and mononuclear cell infiltration. The histological score for myocardial necrosis and cell infiltration on day 8 was significantly higher in the OB group than in the WT group ($P < 0.05$, Figure 1B). The number of infiltrating cells per field in the ventricular myocardium on day 8 in the OB group was significantly elevated as compared with that in the WT group ($P < 0.05$, Figure 1C). The number of apoptotic cells per field in the heart on day 8 was significantly higher in the OB group than in the WT group ($P < 0.05$, Figure 1D). There were no significant differences in histological score and numbers of infiltrating and apoptotic cells between the OB + Lep mice and WT mice.

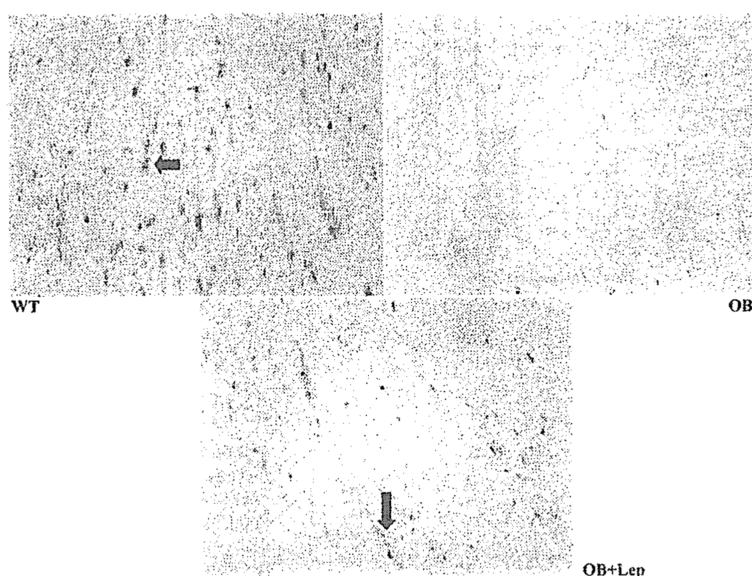

Figure 2. Detection of adiponectin mRNA (arrows) by *in situ* hybridization in myocardium from various mice on day 8 after viral infection. *In situ* hybridization using a digoxigenin-labeled adiponectin antisense riboprobe was performed on myocardial sections from different mice. WT indicates wild-type mice; OB, leptin-deficient ob/ob mice; and OB + Lep, OB mice receiving daily intraperitoneal administration of mouse leptin (300 μg/day, starting simultaneously with viral inoculation).

Detection of adiponectin mRNA in cardiomyocytes: To investigate the localization of adiponectin mRNA in the heart, ISH was performed on myocardial sections using an adiponectin antisense RNA probe. Adiponectin mRNA was not found in myocytes from a normal wild-type mouse. A moderate to strong signal for adiponectin mRNA was detected in myocytes from WT mice and OB + Lep mice on day 8 after viral infection (Figure 2). The adiponectin signal in the myocardium from OB mice at the same time was only very slight (Figure 2). There was no detectable adiponectin signal when ISH was performed on these sections using the sense probe.

Comparative expression levels of adiponectin and TNF-α mRNA in cardiac tissue: Comparative expression levels of adiponectin and TNF-α mRNA in the hearts from different mice on days 4 and 8 after virus infection are shown in Figures 3A and 3B, respectively. Adiponectin mRNA levels in the hearts on days 4 and 8 were significantly lower in OB mice than in WT mice ($P < 0.05$, Figure 3A). There was no difference in the cardiac levels of adiponectin mRNA between OB + Lep mice and WT mice. On the other hand, significantly elevated levels of TNF-α mRNA were observed on days 4 and 8 in the OB group compared with those in the WT group ($P < 0.05$, Figure 3B). There was no significant difference in TNF-α mRNA levels between the OB + Lep group and WT group.

Adiponectin immunoreactivity in cardiomyocytes: Adiponectin immunoreactivity was not observed in the myocardium from a normal wild-type mouse. Photo-

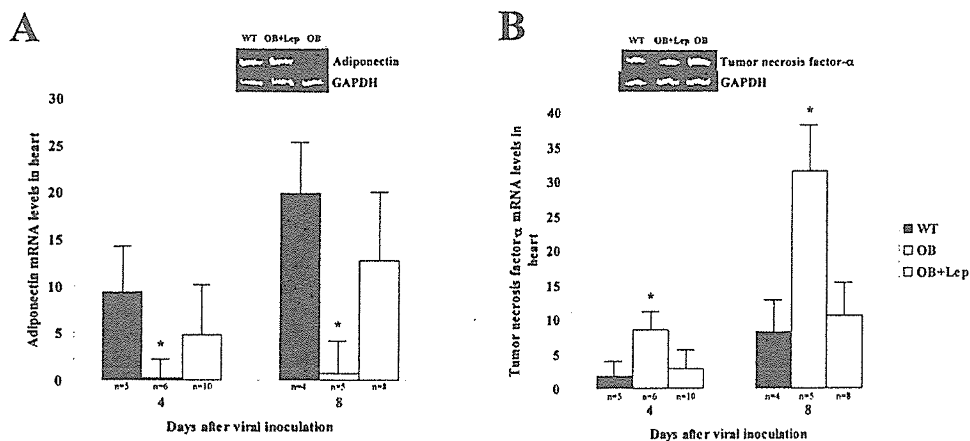

Figure 3. Comparative adiponectin (A) and tumor necrosis factor-α (B) mRNA levels using quantitative real-time reverse transcriptase-polymerase chain reaction with hearts from various mice on days 4 and 8 after viral infection, and demonstration of amplified cardiac adiponectin (A) and tumor necrosis factor-α (B) mRNA and corresponding glyceraldehyde-3-phosphate dehydrogenase gene on agarose gel, which were derived from various mice on day 8 after viral inoculation. Data are expressed as mean ± SD. WT indicates wild-type mice; OB, leptin-deficient ob/ob mice; and OB + Lep, OB mice receiving daily intraperitoneal administration of mouse leptin (300 μg/day, starting simultaneously with viral inoculation). * $P < 0.05$ compared with WT group.

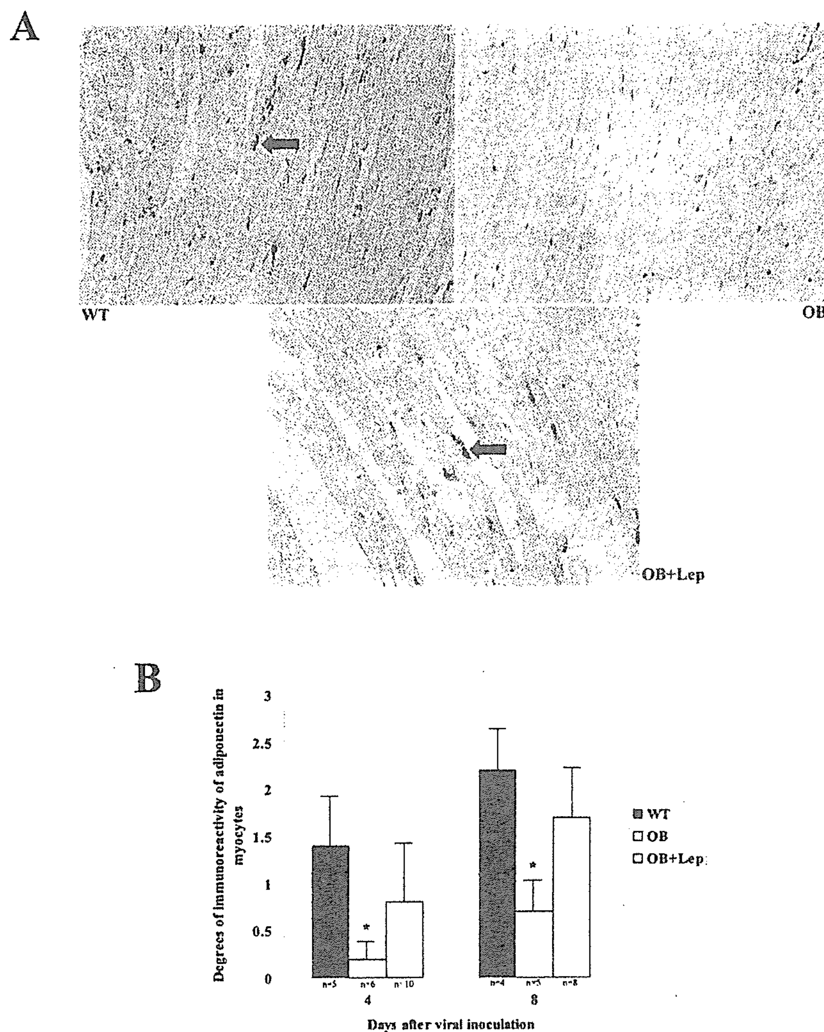

Figure 4. Adiponectin immunoreactivity in cardiomyocytes consisting of photographic demonstration of adiponectin immunoreactivity (arrows) in myocardium from different mice on day 8 after viral inoculation (A, original magnification) and adiponectin immunoreactivity in myocytes (B) from various mice on days 4 and 8. Data are expressed as mean ± SD. WT indicates wild-type mice; OB, leptin-deficient ob/ob mice; and OB + Lep, OB mice receiving daily intraperitoneal administration of mouse leptin (300 μg/day, starting simultaneously with viral inoculation). * $P < 0.05$ compared with WT group.

graphic demonstration of immunoreactivity of adiponectin in myocytes from different mice on day 8 after viral inoculation is shown in Figure 4A. Adiponectin immunoreactivity in myocytes from various mice on days 4 and 8 is shown in Figure 4B. We found significantly suppressed adiponectin immunoreactivity in myocytes from the OB group as compared with those from the WT group at the

same times ($P < 0.05$, Figure 4B). There was expression of adiponectin in myocardial cells from OB + Lep mice, which was similar to the findings of adiponectin immunoreactivity in myocytes from WT mice. The immunoreactive distribution of adiponectin protein in transverse ventricular sections largely overlapped with that of adiponectin mRNA observed using the ISH method.

Concentrations of adiponectin and TNF-α in heart: Specimens of heart from a normal wild-type mouse showed undetectable levels of each molecule. Adiponectin and TNF-α concentrations in the hearts from different mice on days 4 and 8 after viral infection are shown in Figures 5A and 5B, respectively. Cardiac concentrations of adiponectin on days 4 and 8 were significantly lower in the OB group than in the WT group ($P < 0.05$, Figure 5A). There was no difference in cardiac concentrations of adiponectin between the OB + Lep group and WT group. On the other hand, significantly increased concentrations of TNF-α were found on days 4 and 8 in OB mice compared with those in WT mice ($P < 0.05$, Figure 5B). There was no significant difference in TNF-α concentrations between OB + Lep mice and WT mice.

Immunoreactivity of adiponectin receptors in cardiomyocytes: AdipoR1 and AdipoR2 immunoreactivity was found in the arterial wall and macrophages obtained from a normal wild-type mouse, respectively. Photographic demonstration of immunoreactivity of adiponectin receptor 1 in myocardium from various mice on day 8 after viral inoculation is shown in Figure 6A. AdipoR1 and AdipoR2 immunoreactivity in myocardial cells from different mice on days 4 and 8 is shown in Figures 6B and 6C. We observed significantly lower AdipoR1 immunoreactivity in myocytes from OB mice at the same times (Figure 6B). The

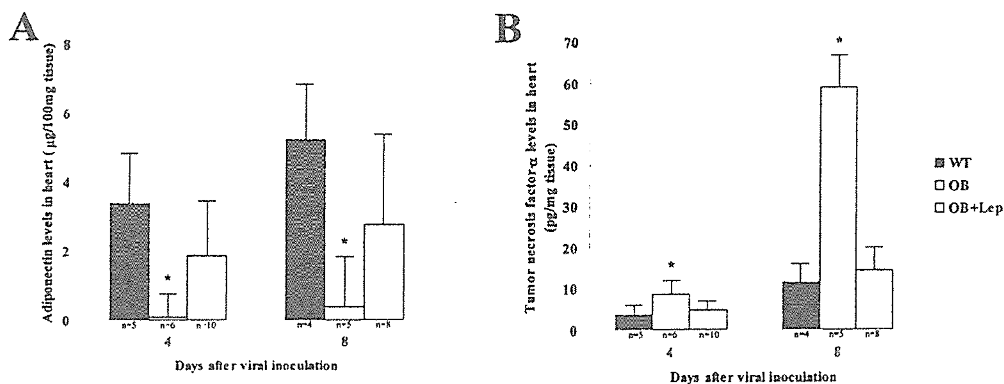

Figure 5. Concentrations of adiponectin (A) and tumor necrosis factor-α (B) in hearts from different mice on days 4 and 8 after viral inoculation. Data are expressed as mean ± SD. WT indicates wild-type mice; OB, leptin-deficient ob/ob mice; and OB + Lep, OB mice receiving daily intraperitoneal administration of mouse leptin (300 μg/day, starting simultaneously with viral inoculation). * $P < 0.05$ compared with WT group.

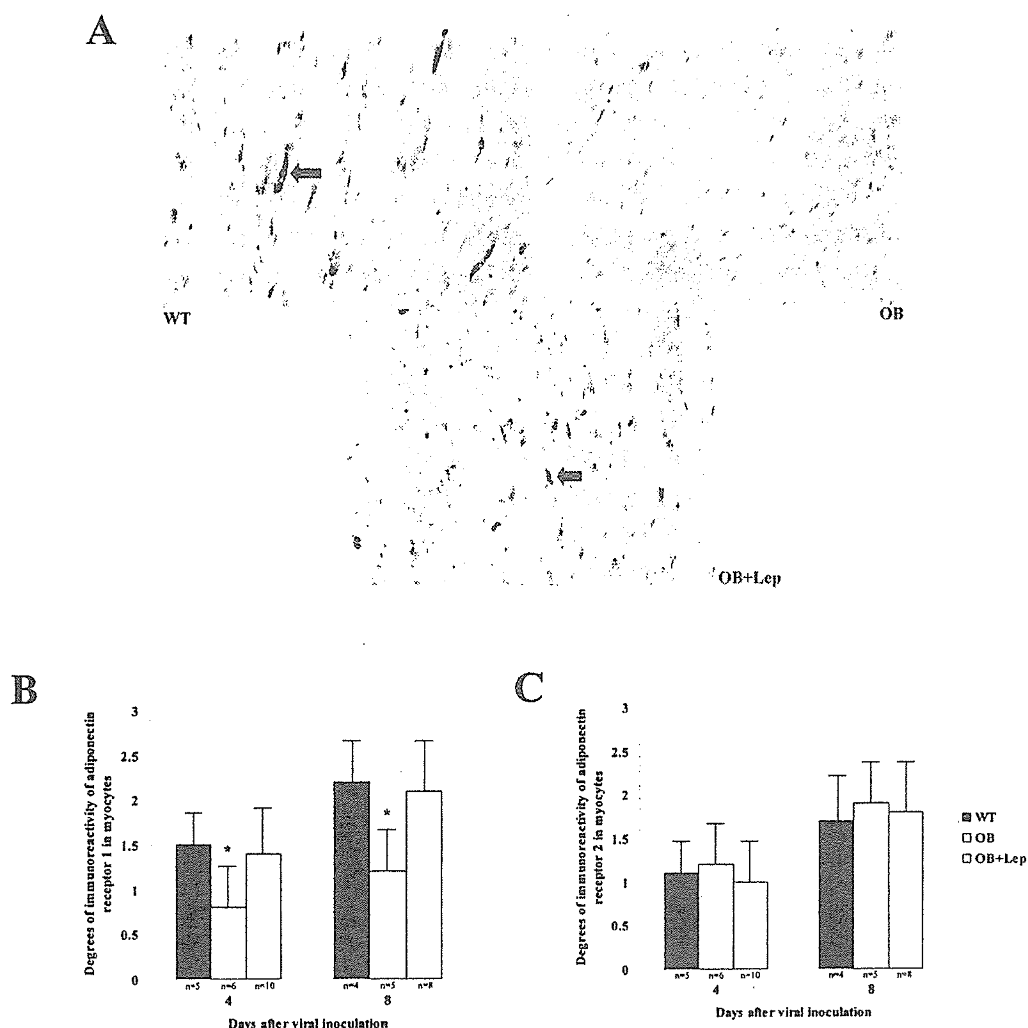

Figure 6. Adiponectin receptor immunoreactivity in cardiomyocytes consisting of photographic demonstration of adiponectin receptor 1 immunoreactivity (arrows) in myocardium from different mice on day 8 after viral inoculation (A, original magnification) and adiponectin receptor 1 (B) or 2 (C) immunoreactivity in myocytes from various mice on days 4 and 8. Data are expressed as mean ± SD. WT indicates wild-type mice; OB, leptin-deficient ob/ob mice; and OB + Lep, OB mice receiving daily intraperitoneal administration of mouse leptin (300 μg/day, starting simultaneously with viral inoculation). * $P < 0.05$ compared with WT group.

degree of AdipoR1 immunoreactivity in myocytes from OB + Lep mice was similar to that in myocytes from WT mice. There was no significant difference in AdipoR2 immunoreactivity in myocytes among all groups.

Discussion

Cardiac expression of adiponectin was impaired in leptin-deficient mice with viral myocarditis. Adiponectin reactivity on immunostaining was recently reported to be observed at the periphery of surviving cardiomyocytes around lesions in the granulation stage in myocardial tissues obtained from 47 autopsied hearts with infarction.[20] In another immunohistochemical analysis, the boundary of mouse hepatocytes showed positive signals for adiponectin after 3-6 hours of carbon tetrachloride treatment, and their cytoplasm was intensely stained after 18 hours of treatment.[17] Adiponectin was suggested to be produced by the liver in mice, where it underwent tissue damage-induced transcriptional regulation.[17] Our data regarding adiponectin expression in surviving myocytes suggest that this adipocytokine may have important implications in the acute phase of viral myocarditis.

Adiponectin is reported to be involved in ending inflammatory responses through its inhibitory actions.[21] This cytokine increases mRNA expression of the anti-inflammatory molecule interleukin-10 (IL-10) at the transcriptional level, and promoted IL-10 protein secretion in an *in vitro* experiment in human monocyte-derived macrophages.[22] In addition, a reciprocal relationship between adiponectin and highly-sensitive C-reactive protein was shown in both human plasma and adipose tissue from subjects with coronary artery disease.[18] In our experiment, suppressed expression of cardiac adiponectin mRNA, immunoreactivity, and protein in OB mice was associated with the development of severe myocarditis, whereas increased expression of adiponectin in the hearts from WT mice and OB + Lep mice led to inhibition of the myocardial inflammatory process. Therefore, we speculate that local expression of adiponectin in the damaged heart may be a compensatory phenomenon against the severe inflammatory condition of viral myocarditis.

TNF-α mRNA was 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 the nonfailing human heart.[1] Immunohistochemical examination also indicated that there was obvious TNF-α immunostaining of cardiomyocytes from patients with IHD and DCM, whereas TNF-α immunoreactivity was not detectable in the nonfailing heart.[1] Natriuretic peptides including atrial and B-type peptides are synthesized and secreted by the heart, and play a critical role in cardiovascular homeostasis.[23] Similarly to the previous demonstration, identification of adiponectin expression in the myocardium is also a good example of a contribution made by molecular biology to understanding the mechanisms for heart failure compensation.