terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling, as described previously.[8]

IMMUNOREACTIVITY OF ADIPONECTIN RECEPTORS IN MYOCYTES

To examine the immunoreactivity of adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2), immunohistochemical staining using a streptavidin biotin complex method (K0675 and E0353, DAKO Cytomation Co. Ltd, Kyoto, Japan) was performed on serial sections of transverse ventricular myocardium from different mice on day 4 or day 8 after viral inoculation. The immunoreactivity of AdipoR1 and AdipoR2 in vessels and macrophages from a normal wild-type mouse that had not received viral inoculation or adiponectin administration was used as a positive control.

Rabbit polyclonal anti-mouse AdipoR1 and AdipoR2 antibodies (ADIPOR11-A and ADIPOR21-A. Alpha Diagnostic International Inc., San Antonio, TX, USA) were applied at a dilution of 1:50. Control slides were treated with normal diluted rabbit serum.

The slides were blindly reviewed by the same pathologist. The degree of adiponectin receptor reactivity was assessed in 30 randomly selected myocytes corresponding to surviving cells found on the respective H&E and myosin-stained slides, and was semi-quantitatively graded according to the degree of immunoreactivity: 0, absence of staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining.[9] The slides were also compared with the respective control slides to exclude non-specific staining.

STATISTICAL ANALYSIS

Data were expressed as the mean ± SD. Analysis of variance was used to evaluate differences in body and cardiac weights, cardiac histological scores, numbers of infiltrating or apoptotic cells in the myocardium and immunoreactivity of adiponectin receptors in myocytes between the groups. A P-value < 0.05 was considered to be statistically significant.

Results

Eight wild-type and 28 leptin-deficient mice were inoculated with EMC virus. Eighteen of the leptin-deficient mice did not receive interventional therapy (OB group); the remaining 10 leptin-deficient mice received daily injections of adiponectin (OB + Adipo group).

Eight of the mice in the OB group died from viral myocarditis during the study protocol; there were no deaths in the wild-type (WT) or OB + Adipo groups over the same period. The numbers of mice killed on day 4 and day 8 after viral inoculation were four and four, respectively, in the WT group, six and four, respectively, in the OB group, and five and five, respectively, in the OB + Adipo group.

BODY AND CARDIAC WEIGHTS

Body weights on days 0, 4 and 8 after viral inoculation were significantly higher ($P < 0.05$) in the OB and OB + Adipo groups than in the WT group (Table 1). Cardiac weights in the OB mice on day 8 after viral inoculation were significantly increased ($P < 0.05$) compared with those in the WT mice (Table 1). There was no significant difference in cardiac weight between the OB + Adipo group and the WT group.

HISTOLOGICAL FINDINGS

The histological scores of myocardial necrosis, and the numbers of infiltrating and apoptotic cells per HPF, in hearts from the different types of mice on day 4 and day 8 after viral inoculation are shown in Fig. 1. Hearts from the OB group showed severe myocardial necrosis and mononuclear cell

TABLE 1:
Body weight and cardiac weight after viral inoculation in various types of mice

	Body weight (g)			Cardiac weight (mg)	
Type	Day 0	Day 4	Day 8	Day 4	Day 8
WT	18.3 ± 1.5	18.7 ± 1.8	19.1 ± 1.5	96 ± 8	102 ± 10
OB	37.5 ± 2.4*	37.8 ± 2.9*	38.4 ± 3.1*	103 ± 7	121 ± 9*
OB + Adipo	37.8 ± 2.2*	37.3 ± 2.8*	35.6 ± 3.9*	94 ± 6	98 ± 12

WT, wild-type mice; OB, leptin-deficient ob/ob mice; OB + Adipo, OB mice receiving adiponectin.
Data are the mean ± SD.
*$P < 0.05$ compared with WT mice.

infiltration. The histological scores, numbers of infiltrating cells and numbers of apoptotic cells were significantly higher ($P < 0.05$ for all) in the OB group than in the WT group on day 8 (Fig. 1). There were no significant differences in the histological scores or the number of infiltrating or apoptotic cells between the OB + Adipo group and the WT group.

IMMUNOREACTIVITY OF ADIPONECTIN RECEPTORS

In the normal WT group, immunoreactivity for AdipoR1 was found in the arterial walls and immunoreactivity for AdipoR2 was found in macrophages. The degrees of immunoreactivity for AdipoR1 and AdipoR2 in myocytes from different mice on day 4 and day 8 are shown in Fig. 2. In the OB group, the AdipoR1 reactivity in myocytes was significantly reduced ($P < 0.05$) compared with reactivity observed in the WT group on day 4 and day 8 (Fig. 2A), but the AdipoR1 reactivity was similar in the OB + Adipo group and the WT group. There were no significant differences in the immunoreactivity for AdipoR2 among the groups (Fig. 2B).

Discussion

Circulating levels of leptin and adiponectin are either undetectable or decreased in OB mice.[10] The protective role of adiponectin against fatty liver diseases has recently been demonstrated in non-alcoholic OB mice with insulin resistance and dyslipidaemia.[11] Replacement therapy with adiponectin could in part compensate for the absence of leptin in terms of ameliorating hepatomegaly and steatosis and decreasing serum alanine aminotransferase levels,[11] although such therapy would not alter the primary aetiology. Injection of adiponectin has also been reported to elevate insulin sensitivity and alleviate hyperlipidaemia.[11] Consistent with these findings, adiponectin administration to OB mice receiving EMC viral inoculation in the present study was found to protect the OB mice from inflammatory myocardial damage.

Complementary DNA encoding the two adiponectin receptors AdipoR1 and AdipoR2, which are distantly related to the family of seven-transmembrane spanning G protein-coupled receptors, has been cloned.[12] AdipoR1 and AdipoR2 are expressed ubiquitously in most organs, with AdipoR1 being especially expressed in skeletal muscle and AdipoR2 in liver.[12] Pancreatic β-cells have also been shown to express adiponectin receptors in a cell culture system.[13] These receptors have seven transmembrane domains and activate signalling molecules

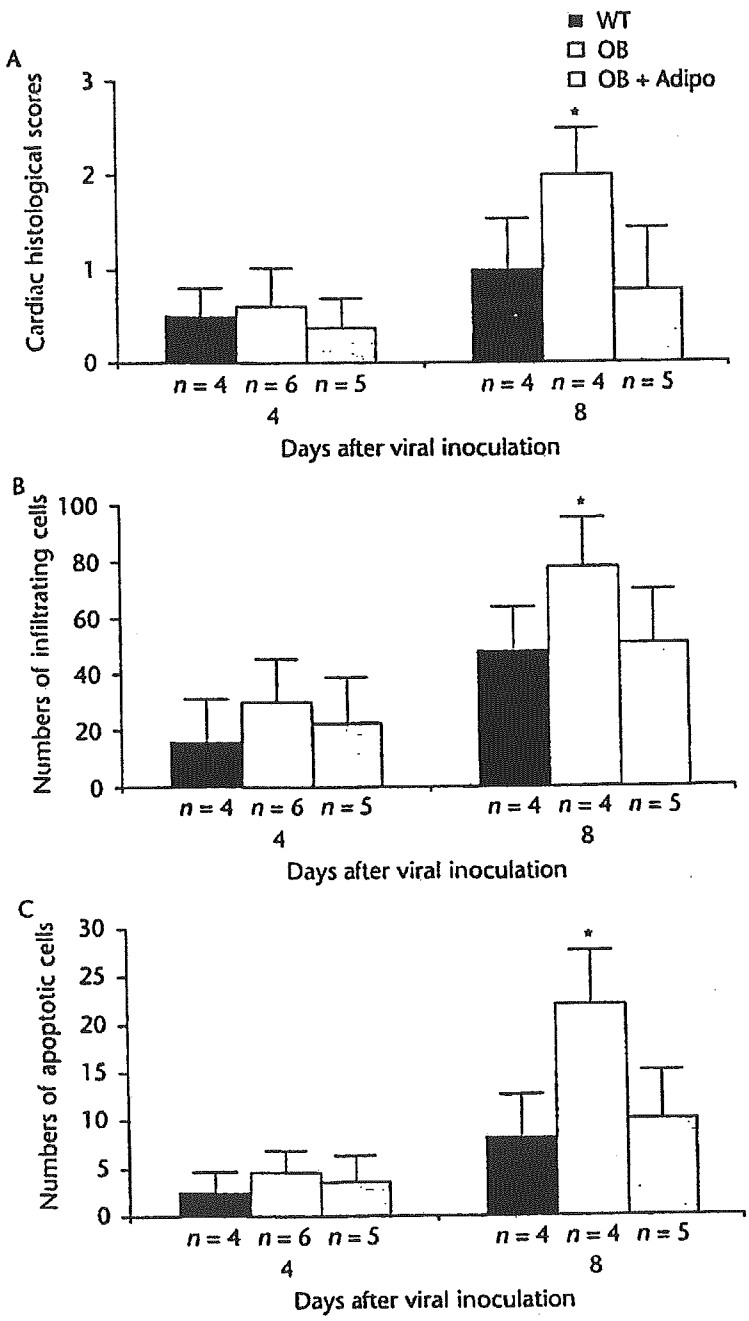

FIGURE 1: Results of histological examination of myocardial sections. (A) Histological scores, (B) numbers of infiltrating cells and (C) numbers of apoptotic cells in different types of mice on day 4 and day 8 after viral inoculation. Data are expressed as the mean ± SD. WT, wild-type mice; OB, leptin-deficient ob/ob mice; OB + Adipo, OB mice receiving subcutaneous administration of mouse adiponectin (30 μg/g daily, starting simultaneously with viral inoculation). $^*P < 0.05$ compared with WT group

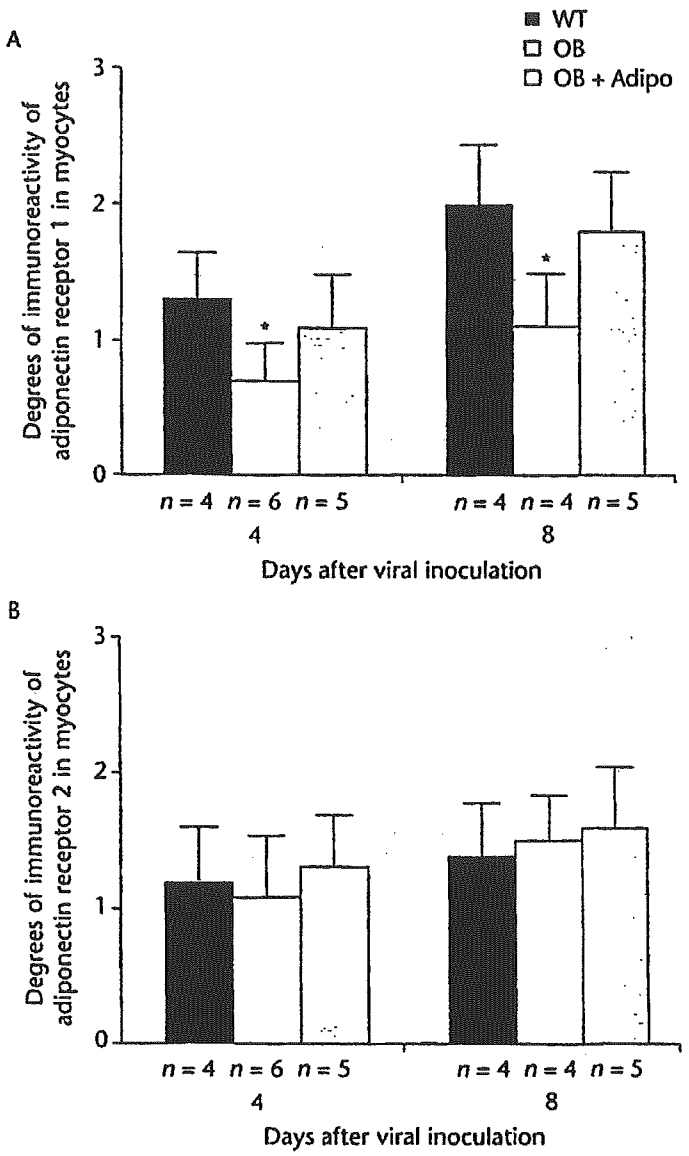

FIGURE 2: Degrees of immunoreactivity of adiponectin receptor 1 (A) or adiponectin receptor 2 (B) in myocytes from different types of mice on day 4 and day 8 after viral inoculation. Data are expressed as the mean ± SD. WT, wild-type mice; OB, leptin-deficient *ob/ob* mice; OB + Adipo, OB mice receiving subcutaneous administration of mouse adiponectin (30 μg/g daily, starting simultaneously with viral inoculation). *$P < 0.05$ compared with WT group

such as peroxisome proliferator-activated receptor-α, adenosine monophosphate-activated protein kinase, and mitogen-activated protein kinase.[12] Possible alterations to adiponectin utilization in the coronary artery and/or heart have been described in type 2 diabetic patients compared with non-diabetic patients based on the transcardiac gradient of adiponectin levels from aortic root to coronary sinus.[14]

One mechanism of impaired transcardiac utilization of adiponectin in subjects with diabetes seems to be a decreased receptor-binding ability of adiponectin in the cardiac myocytes.[14] Interestingly, in the present study we found decreased AdipoR1 immunoreactivity in damaged myocytes from OB mice with viral myocarditis, and adiponectin replacement therapy in OB mice led to recovery of the suppressed AdipoR1 reactivity. These results indicate that adiponectin may act through binding to the AdipoR1, leading to protection against the progression of myocardial inflammation.

In summary, we determined the effects of adiponectin replacement therapy on myocardial damage in OB mice with viral myocarditis. There was significantly decreased reactivity of AdipoR1 in damaged myocytes from OB mice on day 4 and day 8 after viral inoculation compared with that in myocytes from WT mice, together with elevated cardiac weights and severe inflammatory myocardial damage. Replacement of adiponectin in the OB mice inhibited the development of severe myocarditis through augmentation of the AdipoR1 reactivity in the injured myocytes. Our data suggest that exogenously administered adiponectin may inhibit the progression of viral myocarditis through binding to the AdipoR1 in leptin-deficient conditions.

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No conflicts of interest were declared in relation to this article.

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Adiponectin, T-cadherin and Tumour Necrosis Factor-α in Damaged Cardiomyocytes from Autopsy Specimens

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This study determined the presence of adiponectin, T-cadherin (an adiponectin receptor) and tumour necrosis factor-α (TNF-α) in damaged myocytes from autopsied patients with acute or old myocardial infarction (MI) or dilated cardiomyopathy (DCM), using immunohistochemical staining. The enrolled patients included eight with acute MI, six with old MI and seven with DCM. Four autopsied individuals with no cardiac lesions were also enrolled as controls. Adiponectin and TNF-α were not observed in normal myocytes from control subjects, but T-cadherin was weakly detected. Immunoreactivity for adiponectin and T-cadherin was observed at the periphery of damaged myocytes from MI and DCM patients; intracellular reactivity for TNF-α was also seen. There were no statistically significant differences in the degree of reactivity for each molecule in the myocytes between the MI and DCM patients. These results suggest that the presence of adiponectin and TNF-α in damaged myocytes may contribute to the processes of myocardial injury occurring in MI and DCM.

KEY WORDS: MYOCARDIAL INFARCTION; DILATED CARDIOMYOPATHY; ADIPONECTIN; T-CADHERIN; TUMOUR NECROSIS FACTOR-α

Introduction

Adiponectin, which is also known as adipocyte complement-related protein of 30 kDa,[1] is a hormone secreted by adipocytes that acts as an anti-diabetic and anti-atherogenic cytokine.[2] It has structural homology to the protein C1q, and is found in the serum as three distinct oligomers: a trimer, a hexamer and a high molecular weight (HMW) species.[3] Concentrations of adiponectin in blood are decreased in obesity, insulin resistance and type II diabetes.[1] Adiponectin administration has been reported to lower glucose and improve insulin resistance in mice,[4] whereas adiponectin-deficient mice develop insulin resistance and diabetes.[5] This effect of adiponectin appears to be mediated by an elevation in fatty acid oxidation through activation of adenosine monophosphate-activated protein kinase[6] and peroxisome proliferator-activated receptor-α.[2]

Cadherins comprise a large family of cell-surface proteins involved in calcium-mediated cell–cell interactions and signalling. T-cadherin was initially described in the central nervous system, but its tissue distribution is more widespread; the highest expression is found in the cardiovascular system, with low levels in muscle. In the vasculature, T-cadherin is localized to the intima and media and is expressed on endothelial and smooth muscle cells. Expression was shown to be upregulated in the neointima of mouse carotid artery after injury caused by a balloon catheter.[7] Interestingly, T-cadherin has recently been reported to be a receptor for the hexameric and HMW forms of adiponectin; this was demonstrated using a series of expression-cloning studies with panned infected cells on recombinant adiponectin linked to magnetic beads.[8]

Heart failure is generally considered to begin with myocyte damage caused by a variety of pathological conditions, including ischaemia, toxins and myocardial infection. The heart compensates by dilatation and cellular hypertrophy, and eventually decompensates, leading to heart failure. The pro-inflammatory cytokine tumour necrosis factor-α (TNF-α) has been postulated to be one of the pathogenetic factors responsible for the progression from compensated to decompensated heart failure.[9] Yokoyama and colleagues[10] demonstrated that the non-failing human heart does not express TNF-α, whereas the failing human heart expresses significant amounts of this cytokine. Moreover, TNF-α immediately inhibits contractility of isolated cardiac myocytes in a dose-dependent manner; this negative inotropic action is completely reversible upon removal of TNF-α.[10]

In the light of these different findings, we hypothesized that adiponectin, its receptor T-cadherin and TNF-α may contribute to the processes of myocardial injury. In this study, the presence or absence of adiponectin, T-cadherin and TNF-α in damaged myocytes obtained from autopsied patients with acute or old myocardial infarction (MI) or dilated cardiomyopathy (DCM) was determined using immunohistochemical staining. In addition, we analysed differences in the degree of reactivity for each molecule in the myocardium between the two groups.

Patients and methods

PATIENTS

Patients with a confirmed histopathological diagnosis of acute or old MI or a diagnosis of DCM, in whom autopsy examinations were performed in the Department of Clinical Pathology, Kanazawa Medical University Hospital, Ishikawa, Japan, between 1984 and 2004, were randomly selected for inclusion in the study. Autopsied cases from the same period with no cardiac lesions of any kind were also enrolled as normal controls. All individuals were autopsied within 6 h of death. Ethical approval from our institution was not needed, since written consent for each autopsy examination was obtained from each patient's family members.

PREPARATION OF SPECIMENS

In the controls, normal myocardial tissue and surrounding pericardial tissue were dissected from the left ventricle and ventricular septum. In individuals with MI or DCM, the myocardial lesion and surrounding pericardium were dissected in the same manner. Specimens were fixed with 10% neutral buffered formaldehyde and embedded in paraffin, and thin sections were treated with haematoxylin and eosin and Azan–Mallory staining. Based on the histopathological findings, each MI lesion was staged as follows: stage I, early MI; stage II, established myocardial necrosis; stage III, macrophage infiltration; stage IV, granulation formation; stage V, scar formation.[11]

IMMUNOHISTOCHEMICAL STAINING

Immunohistochemical staining was performed on subserial transverse ventricular myocardium and pericardium paraffin sections, using a streptavidin biotin complex method (K0675 or E0466, Dako Cytomation Co. Ltd, Kyoto, Japan). The following primary antibodies were used: rabbit polyclonal anti-human adiponectin antibody at a dilution of 1:500 (AB3784P, Chemicon International Inc., Temecula, CA, USA); rabbit polyclonal anti-human T-cadherin antibody at a dilution of 1:200 (sc-7940, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); and goat polyclonal anti-human TNF-α antibody at a dilution of 1:500 (RC210, Dako Cytomation). The immunostaining was visualized by treating the slides with 3,3'-diaminobezidine tetrahydrochloride and counterstaining with haematoxylin. Negative control slides were treated with normal diluted rabbit or goat serum. For each slide, an area containing approximately 50 myocytes corresponding to the damaged areas found on haematoxylin and eosin and Azan–Mallory staining was blindly reviewed by a pathologist and semiquantitatively graded according to the degree of immunoreactivity for adiponectin, T-cadherin and TNF-α: 0, no staining; 1+, focal staining; 2+, diffuse weak staining; 3+, diffuse moderate staining; 4+, diffuse strong staining.[12] The slides were also compared with the respective negative control slides to exclude non-specific staining.

STATISTICAL ANALYSIS

Differences in the degree of immunoreactivity for each molecule in the damaged myocytes between the MI and the DCM group were analysed using the Mann–Whitney *U*-test. A *P*-value of < 0.05 was considered to be statistically significant.

Results

PATIENT CHARACTERISTICS

Fourteen patients with a confirmed histopathological diagnosis of acute ($n = 8$) or old ($n = 6$) MI, seven patients with DCM and four controls were included in the study. Of the 14 patients with MI, nine were male and five were female. On histopathological examination, two were stage I, three were stage II, three were stage III, four were stage IV and two were stage V. The mean age of the MI patients was 74.9 ± 14.1 years (range 36 – 88 years). Of the seven patients with DCM, five were male and two were female, and the mean age was 51.4 ± 24.5 years (range 17 – 76 years). Of the four control patients without cardiac lesions, three were male and one was female, and the mean age was 55.0 ± 21.0 years (range 33 – 78 years). The main histopathological diagnoses at autopsy for the control subjects were subarachnoid haemorrhage, acute leukaemia, liver cirrhosis and pancreatic cancer, respectively.

IMMUNOREACTIVITY IN NORMAL CARDIOMYOCYTES

Adiponectin and TNF-α were not seen in non-damaged myocytes obtained from the four control subjects, but positive reactivity for adiponectin was observed in pericardial adipocytes. T-cadherin was weakly detected in normal myocytes and the surrounding vessel walls.

IMMUNOREACTIVITY IN DAMAGED CARDIOMYOCYTES

Moderate to strong immunoreactivity for adiponectin was seen at the periphery of injured myocytes from MI and DCM patients (Fig. 1). There was also weak to moderate reactivity for T-cadherin at the periphery of damaged myocytes (Fig. 2). In addition, moderate to strong intracellular reactivity for TNF-α was seen in the myocytes (Fig. 3).

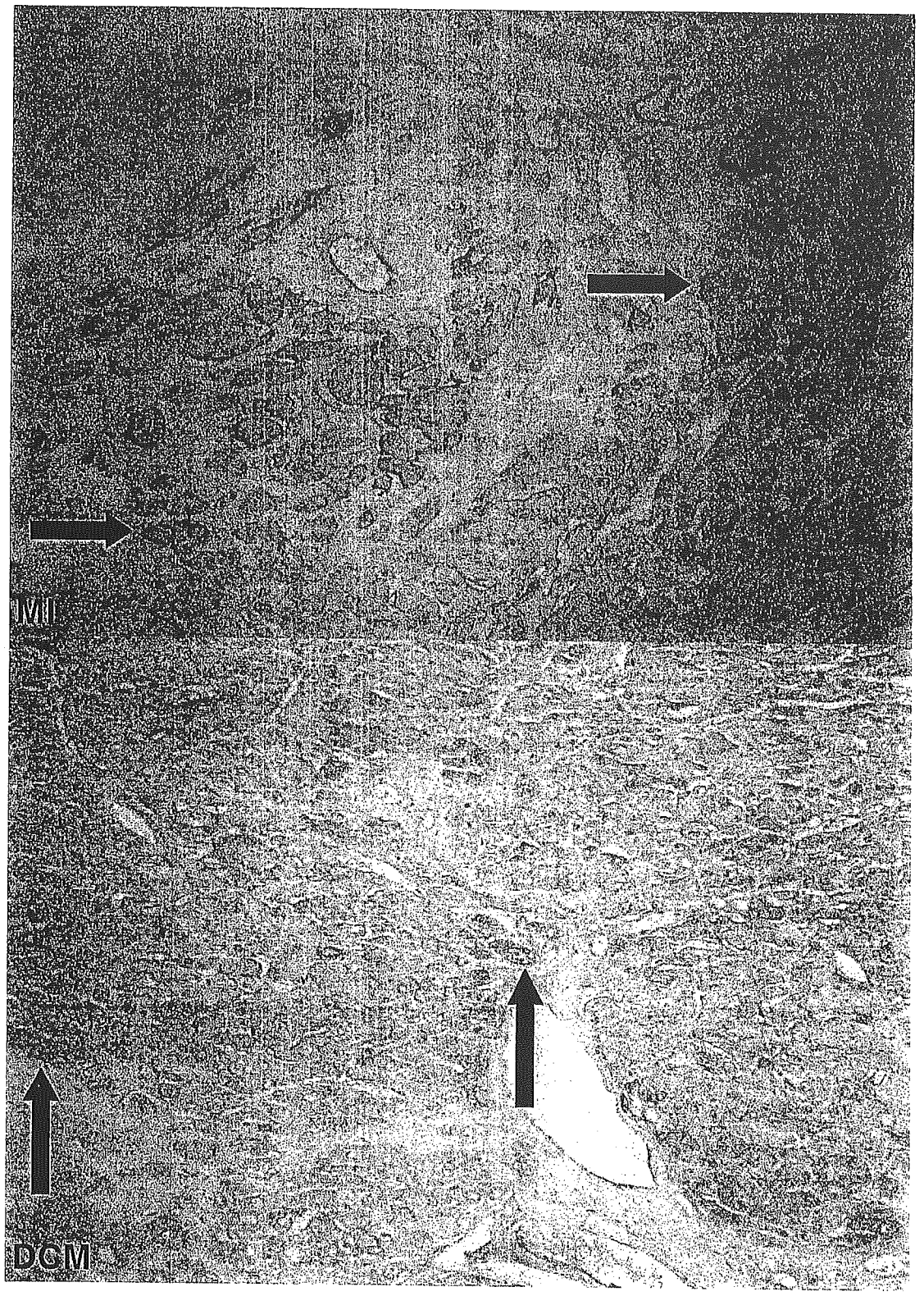

FIGURE 1: Photomicrograph showing moderate to strong adiponectin reactivity (arrows) in autopsy myocardial specimens from patients with myocardial infarction (MI) or dilated cardiomyopathy (DCM). × 75

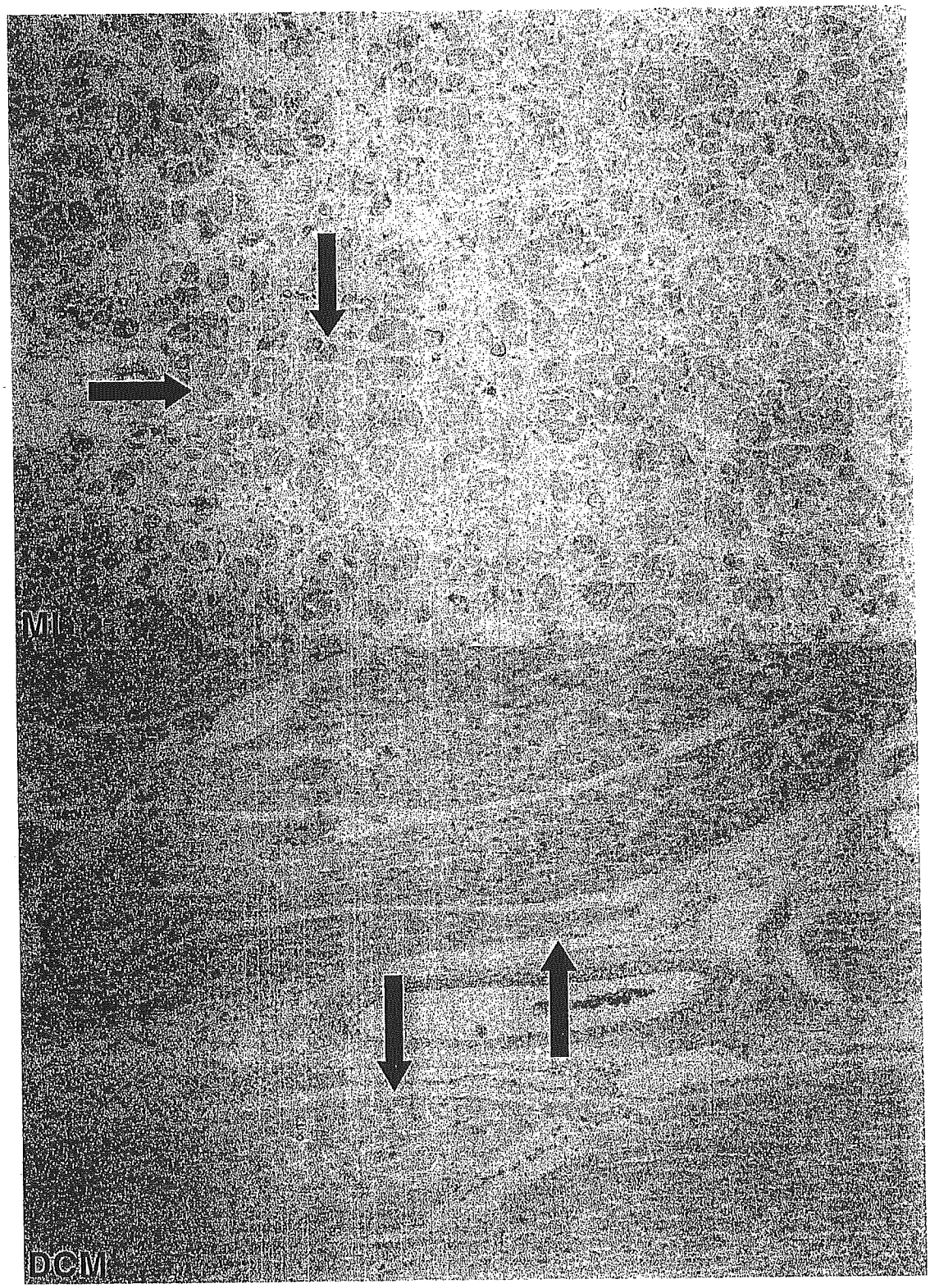

FIGURE 2: Photomicrograph showing weak to moderate T-cadherin reactivity (arrows) in autopsy myocardial specimens from patients with myocardial infarction (MI) or dilated cardiomyopathy (DCM). × 75

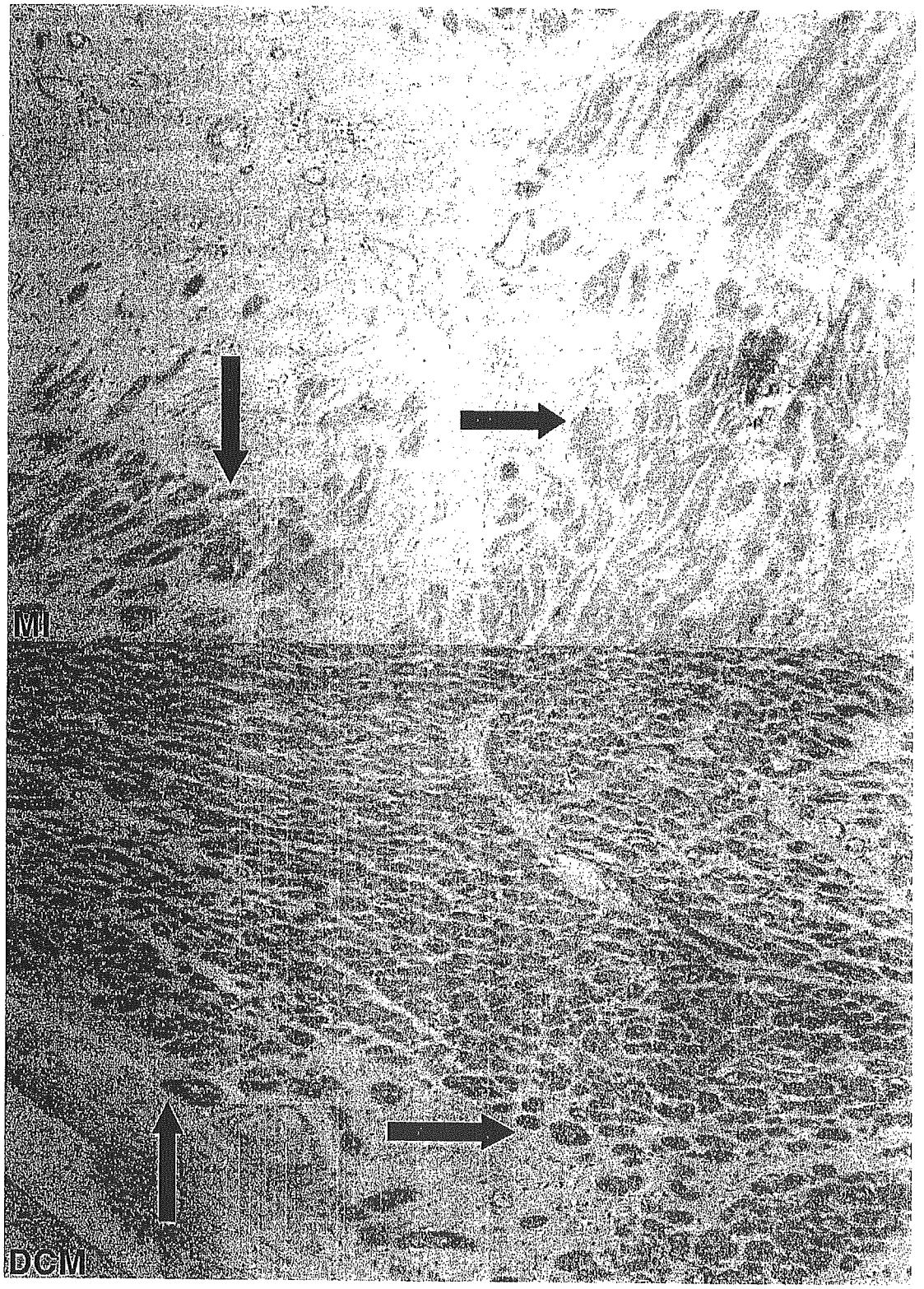

FIGURE 3: Photomicrograph showing moderate to strong intracellular tumour necrosis factor-α reactivity (arrows) in autopsy myocardial specimens from patients with myocardial infarction (MI) or dilated cardiomyopathy (DCM). × 75

The degrees of reactivity for each molecule in the damaged myocytes from individuals with MI or DCM are shown in Fig. 4. There were no statistically significant differences between the two patient groups.

Discussion

High plasma adiponectin concentrations were associated with a lower risk of MI during 6 years of follow-up in a nested case–control study among 18 225 male participants aged 40 – 75 years.[13] Possible utilization of adiponectin in the coronary artery and/or heart has been described for non-diabetic patients based on a trans-cardiac gradient of adiponectin levels from aortic root to coronary sinus.[14] It is interesting that immunostaining for adiponectin was observed at the periphery of damaged cardiomyocytes in lesions at the granulative stage obtained from autopsied hearts with infarction.[15] In another immunohistochemical analysis, the boundaries of mouse hepatocytes were positive for adiponectin after 3 – 6 h of carbon tetrachloride treatment, and the cytoplasm was intensely stained after 18 h of treatment.[16] The authors suggested that adiponectin was produced by the damaged hepatocytes, and undergoes tissue damage-induced transcriptional regulation.[16] In the present study, adiponectin was seen in damaged myocytes from both DCM and MI patients, suggesting that the adipose tissue-specific cytokine adiponectin may have important implications for the processes of myocardial damage.

The adiponectin receptors AdipoR1 and AdipoR2 are expressed ubiquitously in most organs, but in particular AdipoR1 is found in skeletal muscle and AdipoR2 in liver.[17] Complementary DNA for these receptors has been cloned, and they have been shown to be distantly related to the family of seven-

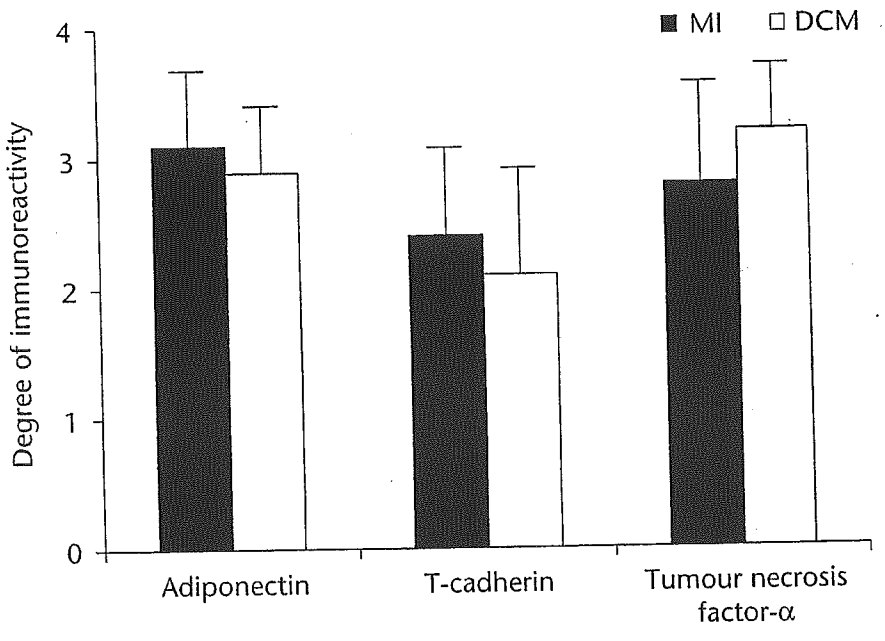

FIGURE 4: Degrees of immunoreactivity for adiponectin, T-cadherin and tumour necrosis factor-α in damaged myocytes obtained from 14 autopsied patients with myocardial infarction (MI) or dilated cardiomyopathy (DCM)

transmembrane-spanning G protein-coupled receptors.[17] However, these receptors have an inverted topology with an intracellular N terminus, unlike other seven-transmembrane spanning receptors.[8] In addition, the extracellular portion of these molecules is small, which is distinct from the members of this class of receptors that bind peptide hormone.[8] T-cadherin, a glycosylphosphatidylinositol-anchored extracellular protein, has been shown to be a novel receptor for the hexameric and HMW forms of adiponectin.[8] In the present study, both T-cadherin and adiponectin were seen in damaged myocardial cells from autopsied MI or DCM patients. This indicates that damaged cardiac cells may possess an adiponectin autocrine system, which leads to protection against the progression of myocardial injury. TNF-α expression was also observed in the damaged myocytes from subjects with DCM and MI using immunohistochemical staining as previously demonstrated.[9] We found cytoplasmic or perinuclear distribution of TNF-α expression in the damaged myocytes, and peripheral distribution of adiponectin expression in the injured cells.

In conclusion, the results of the present study suggest that the presence of adiponectin and TNF-α in damaged myocytes may contribute to the processes of myocardial injury occurring in MI and DCM.

Acknowledgements

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Conflicts of interest

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Osteoclast-like cells express receptor activity modifying protein 2: application of laser capture microdissection

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Abstract

Receptor activity modifying proteins (RAMPs) act as receptor modulators that determine the ligand specificity of receptors for the calcitonin (CT) family. The purpose of this study was to analyze the expression of RAMPs in osteoclast-like cells using the laser capture microdissection (LCM) technique. Mouse bone marrow and spleen cells were co-cultured on a film designed for LCM. After 10 days, 250 osteoclast-like cells were captured using the LCM system. Total RNA from these cells was used to synthesize cDNA and RT-PCR analysis was performed. Osteoclast-like cells expressed CT receptor (CTR), CT receptor-like receptor (CRLR) and RAMP2, but did not express RAMP1 or RAMP3. These results indicated (1) that a pure population of osteoclast-like cells can be prepared by LCM and gene expression of this population can be analyzed by RT-PCR and (2) that RT-PCR shows that osteoclast-like cells express RAMP2, CTR and CRLR, suggesting the potential for adrenomedullin binding to osteoclast-like cells. This is the first report that osteoclast-like cells express RAMP2.

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Introduction

The calcitonin (CT) family of peptides comprises five known members: CT, amylin (AMY), two CT gene-related peptides (CGRP1 and CGRP2) and adrenomedullin (ADM). Receptor activity modifying proteins (RAMPs) comprise a family of accessory proteins for G protein-coupled receptors, three of which act as receptor modulators that determine the ligand specificity of receptors for CT family members. CT receptor-like receptor (CRLR) has been shown to form a high affinity receptor for CGRP, when associated with RAMP1, or, when associated with RAMP2 or RAMP3, to specifically bind ADM (McLatchie *et al.* 1998, Sexton *et al.* 2001). RAMPs are type I transmembrane proteins that share ~30% amino acid identity and a common predicted topology, with short cytoplasmic C termini, one transmembrane domain and large extracellular N termini that are responsible for the specificity (McLatchie *et al.* 1998, Fraser *et al.* 1999). More recently, CT receptor (CTR) was demonstrated to form heterodimeric complexes with RAMP. CTR/RAMP1 and CTR/RAMP3 heterodimers exhibited the pharmacological profiles of receptors specific for AMY (Christopoulos *et al.* 1999, Muff *et al.* 1999, Sexton *et al.* 2001).

There is significant interest in analyzing gene expression of distinct cell populations. Heterogeneous populations of cells within tissues of various types possess correspondingly different patterns of gene expression, and these cell types must be separated from one another for accurate assessment of gene expression. Tong *et al.* (1994) has reported that a microisolation system using a micromanupilator tool was applied for mRNA phenotyping of a blood cell lineage. Laser capture microdissection (LCM) is a particularly useful tool for recovering small cell samples and even enables the collection of individual cells from tissue sections (Emmert-Buck *et al.* 1996). This method facilitates the separation of histologically distinct cells so that proteins, DNA or RNA from these cells can be analyzed in isolation from the surrounding cells (Bonner *et al.* 1997). Osteoclasts act centrally in the remodeling of bone in normal and diseased states. Nonetheless, because of their low numbers within bone, cell culture model systems have been increasingly used to investigate the biochemical functions of osteoclasts (Udagawa *et al.*1989, Nakamura *et al.* 1998). However, because of their heterogeneity and adherence to the plate in such systems, there has been difficulty and controversy in analyzing these cell types. Thus, a more sensitive isolation method for osteoclasts is needed.

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To address this problem, we used LCM techniques to isolate a pure population of osteoclast-like cells. We then analyzed RAMP gene expression in microdissected osteoclast-like cells using RT-PCR.

Materials and methods

An *in vitro* osteoclast model

Osteoclast differentiation *in vitro* was induced using the technique described by Udagawa *et al.* (1989). Both bone marrow cells and spleen cells were obtained from 10- to 14-week-old male C57BL/6 mice (Charles River, Sagamihara, Japan). The bone marrow cells were collected from tibiae and femora. Splenic tissue was cut with scissors and dispersed by pipetting, then the spleen cells were collected by centrifugation at 1000 r.p.m. for 5 min at 4 °C. Bone marrow cells were co-cultured with spleen cells (2×10^6 cells/ml for each cell type) on a film produced for use in LCM (Matsunami Glass Co., Osaka, Japan) for 10 days at 37 °C in a humidified atmosphere of 5% CO_2. Cultures were fed with α-modified Eagle's medium supplemented with penicillin and streptomycin, 10% fetal calf serum (Hyclone, Logan, UT, USA) and 10^{-8} M 1,25$(OH)_2$ D_3 (Calbiochem-Novabiochem Co., San Diego, CA, USA). Multinucleated osteoclast-like cells were then isolated using LCM. All the animal experimental procedures were approved by the Animal Care and Use Committee of Wakayama Medical University (Wakayama, Japan).

LCM of samples

Before LCM, cells were fixed in ethanol for 1 min and stained for 3 min with filtered hematoxylin. They were then washed with sterilized water and air-dried for 10 min. LCM of cultured osteoclast-like cells was performed using the Application Solutions Laser Microdissection System (Leica Microsystems Co., Tokyo, Japan) according to the manufacturer's instructions.

RNA isolation

Total RNA was extracted from 250 LCM-captured cells and 250 spleen cells. The spleen cells used for RNA extraction were from an aliquot of those prepared for the co-culture system. Total RNA extraction was performed using TRIzol LS Reagent (Invitrogen Life Technologies Co., Carlsbad, CA, USA) as described by the manufacturer. Briefly, 170 μl TRIzol reagent was added to a tube containing LCM cells and this was incubated for 5 min at room temperature. Forty microliters of chloroform were then added and the tube was incubated at room temperature for a further 15 min. The samples were then centrifuged at 12 000 ***g*** for 15 min. The aqueous phase was transferred to a ne tube and isopropyl alcohol was added followed l centrifugation at 12 000 ***g*** for 10 min. The RN precipitate was washed with 70% ethanol and dissolve in 20 μl sterilized water.

RT-PCR

The SUPERSCRIP One-Step RT-PCR with PLAT NUM *Taq* (Invitrogen Life Technologies Co.) was use to synthesize cDNA and PCR was performed a described by the manufacturer. The nucleic aci sequences of primers used for RT-PCR are shown i Table 1. RT-PCR reactions were initially performed i a 25 μl reaction volume containing 1 μl of each prime (at 100 ng/μl) and 3 μl RNA as template. The reaction were run at 55 °C for 30 min (cDNA synthesis) an 94 °C for 2 min (pre-denaturation), followed by 45 cycle of 94 °C for 30 s (denaturation), 53 °C for 30 (annealing) and 72 °C for 30 s (extension), followe by 7 min at 72 °C (final extension). To increase th detection capacity, we performed a second round o PCR. The second-round PCR reactions were carried ou using Taq polymerase (Perkin-Elmer-Cetus, Norwalk CT, USA) with 8 μl RT-PCR products as template (fina 25 μl reaction mixture) under the following conditions 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C fo 30 s. In the second-round PCR, CTR was amplifie using 2nd sense and anti-sense primers (Table 1). The primers of CRLR, RAMP1, 2, 3, alkaline phosphatase (ALP) and β-actin for the second-round PCR were the same primers as those used in the initial RT-PCR. The samples were electrophoresed in 3% agarose gels and stained with ethidium bromide.

Results and discussion

We have developed a rapid and precise method for the isolation of pure populations of osteoclast-like cells using LCM. Figure 1 illustrates two osteoclast-like cells before and after LCM. Two hundred and fifty cells with > three nuclei each were isolated. Total RNA was extracted and RT-PCR was used to analyze multiple gene expressions. Figure 2 shows the RT-PCR results for CTR, RAMP1, 2 and 3, CRLR and β-actin. The predicted sizes were clearly visualized by ethidium bromide staining. RT-PCR results showed that the ubiquitous gene, β-actin, was amplified from both spleen and osteoclast-like cells, whereas CTR mRNA was amplified from osteoclast-like cells alone. RAMP1 and RAMP3 mRNAs were amplified from spleen cells alone. RAMP2 and CRLR mRNAs were amplified from both types of cells.

In the present study, we were able to isolate a pure population of osteoclast-like cells and detect a series of gene expressions. Two hundred and fifty cells were

Table 1 Oligonucleotide sequences used for PCR

Target	Sequence	Size
CTR	1st sense, 5′-GTCTTGCAACTACTTCTGGATGC-3′	255 bp
	1st antisense, 5′-AAGAAGAAGTTGACCACCAGAGC-3′	(Inoue *et al.* 1999)
	2nd sense, 5′-GTCTTGCAACTACTTCTGGATGC-3′	104 bp
	2nd antisense, 5′-GAAGATAGTACCAGCGTAGGC-3′	(U18542*)
RAMP1	Sense, 5′-CACCATCTCTTCATGGTCACTG-3′	187 bp
	Antisense, 5′-CAATCGTGTGCGCCACGTGC-3′	(AJ250489)
RAMP2	Sense, 5′-TGGATCTCGGCTTGGTGTGAC-3′	217 bp
	Antisense, 5′-GCAAGGTAGGACATGTGTTTCG-3′	(AJ250490)
RAMP3	Sense, 5′-TTGTGGTGAGTGTGCCCAGG-3′	189 bp
	Antisense, 5′-CCCATGATGTTGGTCTCCATC-3′	(AJ250491)
CRLR	Sense, 5′-TGTAATAACAGCACGCATGAG-3′	225 bp
	Antisense, 5′-GTTATTGGCCACTGCCGTGA-3′	(AF146525)
ALP	Sense, 5′-ATCGGGACTGGTACTCGGATAA-3′	152 bp
	Antisense, 5′-ATCAGTTCTGTTCTTCGGGTAC-3′	(NM007431)
β-Actin	Sense, 5′-GTGGGCCGGTCTAGGCACCA-3′	246 bp
	Antisense, 5′-GGTTGGCCTTAGGGTTCAG-3′	(Flores-Delgado *et al.* 1998)

*Accession number of GeneBank.
ALP, alkaline phosphatase.

used for RNA extraction and cDNA synthesis. Three microliters of the 20 μl cDNA solution was successful for each gene amplification. Approximately 40 cells were therefore used for RT-PCR analysis. Naot *et al.* (2001) have reported that osteoclastic cells such as primary osteoblasts and UMR 106-06 cells expressed all three types of RAMP analyzed using RT-PCR. A very high expression of mRNA for RAMP2 was detected in those cells, compared with those for RAMP1 and RAMP3. Previous studies showed that osteoblast but not osteoclast cells express ALP (Tong *et al.* 1994). To exclude the possibility of osteoblast contamination, we investigated ALP mRNA expression in the microdissected osteoclast-like cells. The result showed that no ALP mRNA was detectable (Fig. 3), which supported the idea that RAMP2 was amplified from osteoclast-like cells. Thus, LCM is a useful technique for isolation of small cell samples, and our strategy might be extended to other procedures, such as quantitative RT-PCR to measure mRNA levels in the osteoclast. The bone marrow macrophages are the precursors of osteoclasts; it will be interesting to compare gene expression between osteoclasts and bone marrow macrophages. Immunostaining of the Fc receptor, C3 receptor or vitamin D receptor will help to distinguish those cell types in our co-culture system. However, in order to perform RNA

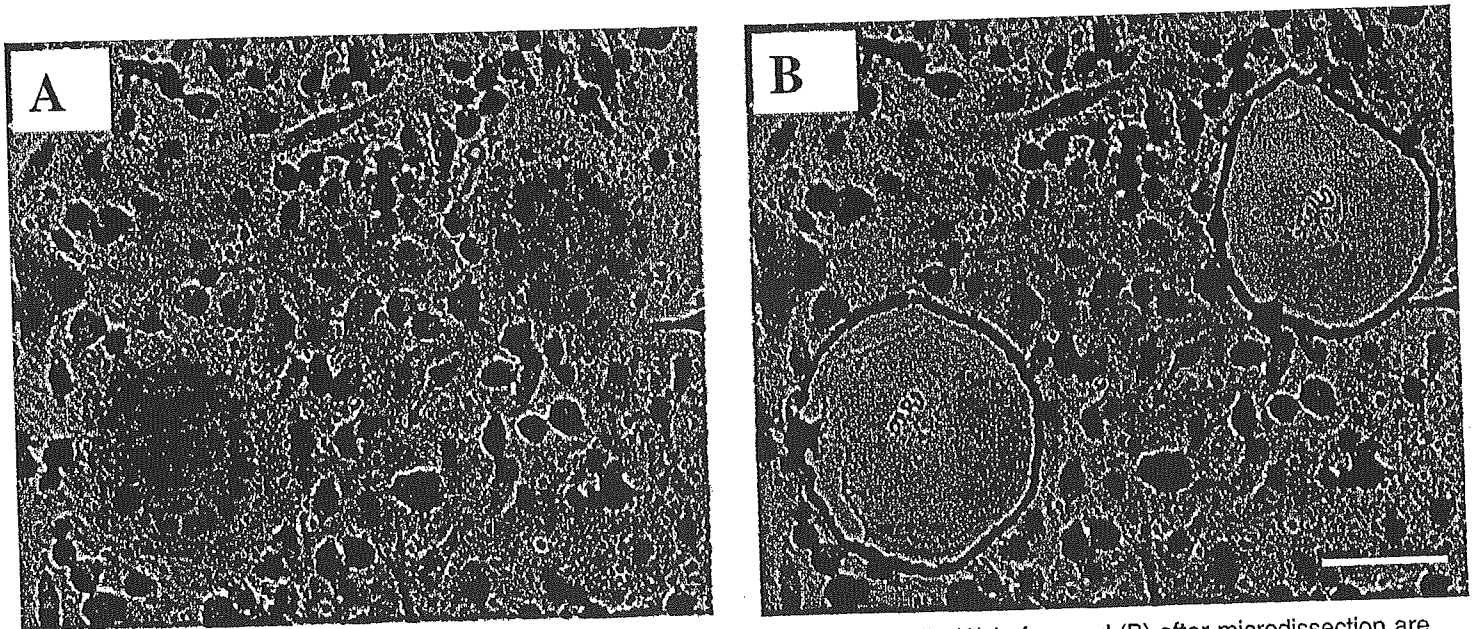

Figure 1 Osteoclast-like cells were isolated by using LCM. Two osteoclast-like cells (A) before and (B) after microdissection are shown. Stained by hematoxylin; bar denotes 50 μm.

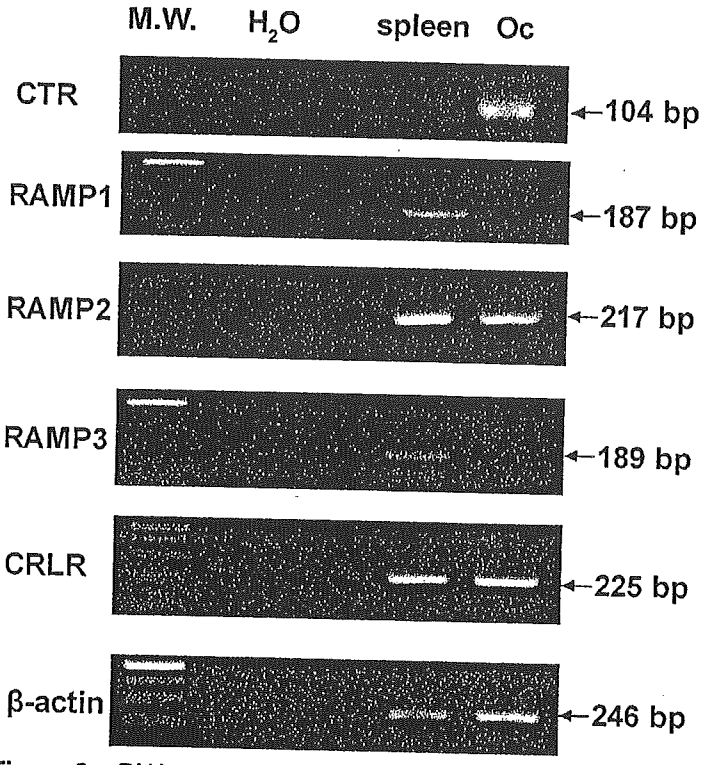

Figure 2 mRNA expression of various genes in spleen and osteoclast-like cells. Total RNA was extracted from microdissected osteoclast-like cells and spleen cells, RT-PCR was carried out to analyze the gene expression. β-actin served as the positive control. The products were electrophoresed in 3% agarose gels and stained with ethidium bromide. RAMP1, RAMP2, RAMP3 and CRLR were detected in spleen cells. M.W., molecular weight markers; H_2O, H_2O as template served as negative control; spleen, using RNA from spleen as template; Oc, using RNA from osteoclast-like cells from LCM as template.

analysis after immunostaining, further efforts should be made to modify the conventional staining protocol to protect RNA from degradation.

Our findings that osteoclast-like cells expressed RAMP2 and CRLR as well as CTR provide the first evidence that osteoclasts express RAMP2. These results suggest that osteoclasts may have the ability to bind

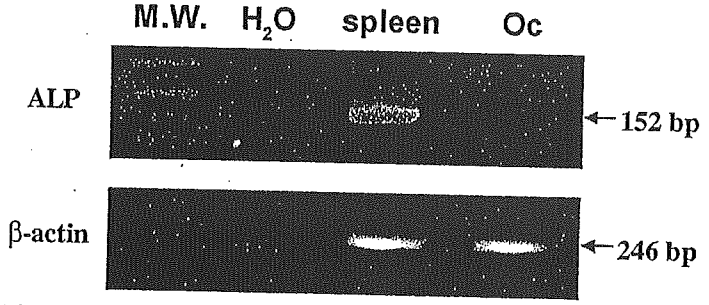

Figure 3 RT-PCR analysis of ALP expression. ALP expression was detected in spleen cells but not in osteoclast-like cells. M.W., molecular weight markers; H_2O, RT-PCR products using H_2O as template; spleen, using RNA from spleen as template; Oc, using RNA from osteoclast-like cells from LCM as template.

ADM through the CRLR/RAMP2 heterodimer. ADM is a 52 amino acid peptide first described in a human phaeochromocytoma but subsequently found to be present in many tissues, including the vascular system and bone tissue (Kitamura *et al.* 1993). Naot *et al.* (200 has suggested that ADM is mitogenic to osteoblasts raising the possiblity that ADM is a local regulator of bone growth; however, the action of ADM or RAMP on the osteoclast is not clear. It has been reported that bone abnormalities were observed in both CTR +/− and AMY +/− mice, thereby ruling out the possibility that AMY uses CTR to inhibit osteoclastogenesis *in vivo* (Dacquin *et al.* 2004).

In summary, we have demonstrated that LCM is a useful solution for osteoclast research. We found that osteoclast-like cells expressed mRNAs for CTR, CRLR and RAMP2 but not RAMP1 or RAMP3; RAMP2 may therefore play an important role in osteoclast function. Further study is needed to elucidate the role of RAMP2 and its relationship to the CT family of receptors.

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