Original Article

Cellular Physiology of Rat Cardiac Myocytes in Cardiac Fibrosis: *In Vitro* Simulation Using the Cardiac Myocyte/Cardiac Non-Myocyte Co-Culture System

Keiichi IKEDA[1)], Katsuyoshi TOJO[2)], Takashi UDAGAWA[1)], Chikara OTSUBO[1)], Masahiro ISHIKAWA[3)], Goro TOKUDOME[3)], Tatsuo HOSOYA[3)], Naoko TAJIMA[2)], Kazuwa NAKAO[4)], and Masahiro KAWAMURA[1)]

An understanding of the cellular physiology of cardiac myocytes (MCs) and non-myocytes (NMCs) may help to explain the mechanisms underlying cardiac hypertrophy. Despite numerous studies using MC/NMC co-culture systems, it is difficult to precisely evaluate the influence of each cell type because of the inherent cellular heterogeneity of such a system. Here we developed a co-culture system using Wistar rat neonatal MCs and NMCs isolated by discontinuous Percoll gradient and adhesion separation methods and cultured on either side of insert well membranes. Co-culture of MCs and NMCs resulted in significant increases in [^{3}H]-leucine incorporation by MCs, in the amount of protein synthesized by MCs, and in the secretion of natriuretic peptides, while the addition of MCs to NMC cultures significantly reduced [^{3}H]-thymidine incorporation by NMCs. Interestingly, the percentage of the brain natriuretic peptide (BNP) component of total natriuretic peptide secreted (atrial natriuretic peptide+BNP) increased as the number of NMCs placed in the MC/NMC co-culture system increased. However, MCs did not affect production of angiotensin II (Ang II) by NMCs or secretion of endothelin-1 and transforming growth factor-β1 into the MC/NMC co-culture system. This finding was supported by the anti-hypertrophic and anti-fibrotic actions of RNH6270, an active form of olmesartan, on MCs in the MC/NMC co-culture system and on NMCs that may synthesize Ang II in the heart. The present data indicate that cardiac fibrosis may not only facilitate MC hypertrophy (possibly through the local angiotensin system) but may also change particular pathophysiological properties of MCs, such as the secretory pattern of natriuretic peptides. (*Hypertens Res* 2008; 31: 693–706)

***Key Words*: cardiac hypertrophy, cardiac fibrosis, cardiac myocyte, cardiac non-myocyte, olmesartan**

Introduction

A range of humoral factors, such as atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), endothelin-1 (ET-1), transforming growth factor-β (TGF-β), and adrenomedullin, have been reported to be secreted from cardiac myocytes (MCs) and/or cardiac non-myocytes (NMCs) under both normal and pathological conditions (*1*–*7*). NMCs in the cardiac interstitium are also known to be a major source of growth

From the [1)]Department of Pharmacology and [2)]Division of Diabetes and Endocrinology and [3)]Division of Nephrology and Hypertension, Department of Internal Medicine, Jikei University School of Medicine, Tokyo, Japan; and [4)]Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto, Japan.

This research was supported by the Jikei University Research Fund.

Address for Reprints: Keiichi Ikeda, M.D., Ph.D., Department of Pharmacology, Jikei University School of Medicine, 3–25–8, Nishishinbashi, Minato-ku, Tokyo 105–8461, Japan. E-mail: ikedak@jikei.ac.jp

Received September 3, 2007; Accepted in revised form October 16, 2007.

factors, such as ET-1 and TGF-β, that can act in an autocrine or paracrine fashion to cause both MC hypertrophy and the proliferation of NMCs (*1*, *2*, *4*, *5*, *7*, *8*). Such cellular processes may represent an important determinant of pathological left ventricular hypertrophy, which is the major risk factor associated with myocardial failure (*1–4*). In previous reports, the effects of a range of cardiovascular factors and agents on MCs and NMCs have been studied by culturing these cell types in both primary, single culture, and co-culture conditions (*2*, *4*, *8*). While the MC/NMC co-culture system may be useful to determine the effects of cross talk between MCs and NMCs, the inherent cellular heterogeneity of these systems complicates these assessments. The preparation of a structured, two-dimensional MC/NMC co-culture system using a photolithographic technique has been reported (*9*), and recent advances in cell biology have also provided techniques for isolating individual cells (*10*, *11*). However, these methods are too complex or require expensive equipment to distinguish each cell's phenotype in a mixed MC/NMC co-culture. Moreover, with the reported methods, such as single-cell sampling techniques, it may be difficult to obtain sufficient sample amounts to evaluate altered gene expression, especially, quantitatively. On the basis of these limitations, we used the insert well MC/NMC co-culture system to examine the nature of cross talk between MCs and NMCs. We previously evaluated the effects of urocortin (Ucn) on MCs in an MC/NMC co-culture system, which was separated by a Transwell insert (*7*), but we did not evaluate the overall properties of the two cell types in the MC/NMC co-culture system containing various ratios of MCs and NMCs. Because a high ratio of NMC to MC, for example, is indicative of cardiac fibrosis, the present study was designed to examine the effects of different ratios of MCs and NMCs on cardiocyte physiology in co-culture. Our aim was to develop MC/NMC co-culture models suitable for the accurate study of the pathologies of cardiac hypertrophy and cardiac fibrosis and to characterize the prepared MC/NMC co-culture system by evaluating protein synthesis levels in MCs, levels of humoral factors, including ANP and BNP secretion by MCs and ET-1 secretion and angiotensin II (Ang II) production by NMCs, cell size of MCs, and the proliferation of NMCs.

Methods

Cell Cultures

All the experiments were performed in accordance with the Guidelines on Animal Experimentation of the Jikei University School of Medicine. MCs and NMCs were prepared from neonatal Wistar rat cardiac tissue by discontinuous Percoll gradient separation and adhesion methods, as previously described, with a slight modification (*4–7*). Although the phenotype and cellular physiology of primary cultured neonatal MCs and adult MCs are not identical, primary cultured neonatal cardiocytes have been widely used to investigate the generalized physiology of cardiac cells. After the separation, MCs were suspended in Iscove's modified Dulbecco's medium (IMDM, Invitrogen, Carlsbad, USA) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, USA), nonessential amino-acid solution (1 ml/L, Invitrogen), and antibiotics (100 unit/mL penicillin G and 10 μg/mL streptomycin). Cells were cultured at 37°C in a 5% CO_2 atmosphere.

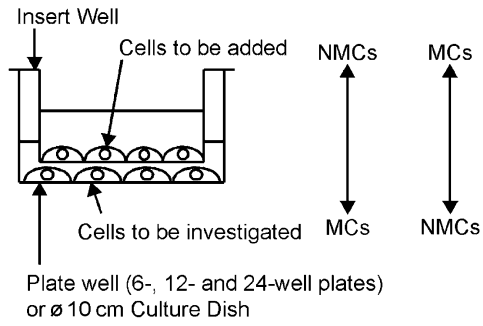

Fig. 1. *Design of insert well MC/NMC co-culture system, allowing MCs to be plated in cluster wells and NMCs to be plated in insert wells and vice versa.*

NMCs were suspended in IMDM containing 10% FBS and plated in ø 10 cm culture dishes. Seeded cells were washed four times with phosphate-buffered balanced salt solution after a 30 min incubation (37°C, 5% CO_2 atmosphere) to remove endothelial cells, and then cultured under the same conditions in IMDM containing 10% FBS. When NMCs reached sub-confluence, they were removed by trypsin treatment and again cultured under the same conditions as described above, in ø 10 cm culture dishes with IMDM containing 10% FBS and antibiotics (first passage).

To verify the purity of MCs, cells were plated on a chamber slide (Iwaki Glass, Funabashi, Japan) and cultured overnight. Cells were then fixed with 10% neutral formalin and immunostained with anti-sarcomeric actin antibody (ZMSA-5 (180177), Zymed Laboratory, South San Francisco, USA) and the Histostain-SP Kit (Zymed Laboratory).

Determination of [^{3}H]-Leucine and [^{3}H]-Thymidine Incorporation by MCs and of [^{3}H]-Thymidine Incorporation by NMCs

MCs were plated on 12-well plates at a density of 0.5×10^5 cells/well and incubated in the presence or absence of different densities of NMCs (second passage) by adding them to Transwell inserts (12-well, pore size 0.4 μm, Corning Costar, Corning, USA) as previously reported (*7*) (Fig. 1). The densities of supplemented NMCs examined were 0.5×10^5, 1.0×10^5, 1.5×10^5, 2.5×10^5, and 4.5×10^5 cells/well, so that MCs in the MC/NMC co-culture system might account for 50%, 33%, 25%, 17%, or 10%, respectively, of the total cells present (Table 1). MC percentages of 25% and 16.7% are

Table 1. Summary of Insert Well MC/NMC Co-Culture System for Evaluation of MC-Specific Parameters

Percentage of MCs (%)	12-well plate		ø 10 cm culture dish	
	MCs (cells/well)	NMCs (cells/well)	MCs (cells/well)	NMCs (cells/well)
0 (NMCs alone)				
10	0.5×10^5	4.5×10^5	1.0×10^6	9.0×10^6
17	0.5×10^5	2.5×10^5	1.0×10^6	5.0×10^6
25	0.5×10^5	1.5×10^5	1.0×10^6	3.0×10^6
33			1.0×10^6	2.0×10^6
50	0.5×10^5	0.5×10^5	1.0×10^6	1.0×10^6
100 (MCs alone)	0.5×10^5	0	1.0×10^6	0

MC, cardiac myocyte; NMC, cardiac non-myocyte.

Table 2. Summary of Insert Well MC/NMC Co-Culture System for Evaluation of NMC-Specific Parameters

Percentage of MCs (%)	24-well plate		6-well plate	
	MCs (cells/well)	NMCs (cells/well)	MCs (cells/well)	NMCs (cells/well)
0 (NMCs alone)	0	1.0×10^5	0	6.0×10^5
10	0.11×10^5	1.0×10^5	0.67×10^5	6.0×10^5
17	0.20×10^5	1.0×10^5	1.2×10^5	6.0×10^5
25	0.33×10^5	1.0×10^5	2.0×10^5	6.0×10^5
33	0.50×10^5	1.0×10^5		
50			6.0×10^5	6.0×10^5

MC, cardiac myocyte; NMC, cardiac non-myocyte.

consistent with those reported for normal and diseased rat hearts, respectively, in an animal model in which the diseased state is obtained by constricting the ascending aorta with a silver ring for 7 d (*12*). In addition, co-culture containing 10% MCs was designed to simulate the conditions of more advanced cardiac fibrosis. After culturing cells for 30 to 40 h in IMDM containing 10% FBS followed by 10 h in FBS-free IMDM, [^{3}H]-leucine incorporation by MCs was measured as previously described (*4*). In the MC/NMC co-culture systems containing 17% and 25% MCs, the effects of RNH6270 (generously provided by Daiichi-Sankyo, Tokyo, Japan), an active form of olmesartan medoxomil, Ang II type 1 receptor antagonist (*13*), as well of Ang II and ET-1 (Peptide Institute, Osaka, Japan) on [^{3}H]-leucine incorporation by MCs were also evaluated. [^{3}H]-thymidine incorporation by MCs was measured to evaluate cell survivability in each culture condition. After the deprivation of FBS, the medium was changed to IMDM containing 0.1% bovine serum albumin (BSA) and [^{3}H]-thymidine, and the cells were further incubated for 18 h. The specific activity of [^{3}H]-thymidine in MCs was then measured

NMCs (second passage) were plated on 24-well plates at a density of 1.0×10^5 cells/well with or without MCs added to the inserts (0.11×10^5, 0.20×10^5, 0.33×10^5, 0.5×10^5 cells/well) to obtain final MC percentages of 10%, 17%, 25%, and 33% for each insert well. Transwell inserts (24-well, pore size 0.4 μm, Corning Costar) or Thin Cert inserts (24-well, pore size 0.4 μm, Greiner Bio-One, Frickenhausen, Germany) (Table 2) were used as previously described (*7*). After 48-h culture in IMDM containing 10% FBS followed by 48 h in FBS-free IMDM at 37°C in a 5% CO_2 atmosphere, the specific activity of [^{3}H]-thymidine incorporated by NMCs was measured as previously described (*4*). We also examined the effects of RNH6270 (10^{-6} mol/L) and temocaprilat (10^{-6} mol/L, generously provided by Daiichi-Sankyo), an active form of temocapril, an angiotensin-converting enzyme (ACE) inhibitor, on [^{3}H]-thymidine incorporation by NMCs.

To evaluate changes in the number of NMCs in MC/NMC co-culture systems, these systems were first prepared on 24-well plates, as described above (NMCs alone and 10%, 17%, and 25% MCs, Table 2). To evaluate the effects of MCs on NMC cell numbers, [^{3}H]-thymidine incorporation by NMCs in the MC/NMC co-culture systems was measured. Briefly, after the initial culture with IMDM containing 10% FBS and antibiotics with or without the indicated percentage of MCs for 48 h, NMCs were further cultured without MCs in FBS-free IMDM for 48 h to deprive cells of FBS. The specific activity of [^{3}H]-thymidine incorporated by NMCs without MCs was then measured as previously described (*4*).

Determination of the Protein Content of MCs

The protein content of MCs (1.0×10^6 cells/dish) cultured with or without NMCs (25% MCs: 3.0×10^6; 17% MCs: 5.0×10^6; 10% MCs: 9.0×10^6, Table 1) on Transwell inserts for ø 10 cm culture dishes (pore size 0.4 μm, Corning Costar)

Table 3. The Specific Primers and Annealing Temperatures for RT-PCR

Name of primer		Sequence of primer	Annealing temperature (°C)
Angiotensinogen	Forward	AATTCGGGGATCCTACAACC	62.9
	Reverse	ACACCACATTTTGGGGGTTA	
Renin	Forward	TGGATCAGGGAAGGTCAAAG	62.6
	Reverse	CCCTCCTCACACAACAAGGT	
ACE	Forward	GAGCCATCCTTCCCTTTTTC	62.6
	Reverse	AGGGTGCCACCAAGTCATAG	
Ang II type 1a receptor	Forward	CGTCATCCATGACTGTAAAATTTC	64.0
	Reverse	GGGCATTACATTGCCAGTGTG	
Ang II type 1b receptor	Forward	CATTATCCGTGACTGTGAAATTG	62.6
	Reverse	GCTGCTTAGCCCAAATGGTCC	
Ang II type 2 receptor	Forward	GGAGCGAGCACAGAATTGAAAGC	62.6
	Reverse	TGCCCAGAGAGGAAGGGTTGCC	
TGF-β1	Forward	AATACGTCAGACATTCGGGAAGCA	64.0
	Reverse	GTCAATGTACAGCTGCCGTACACA	
β-Actin	Forward	GACTACCTCATGAAGATCCT	54.4
	Reverse	CCACATCTGCTGGAAGGTGG	

RT-PCR, reverse transcription–polymerase chain reaction; ACE, angiotensin-converting enzyme; Ang II, angiotensin II; TGF-β1, transforming growth factor β1.

was measured after 24-h culture in IMDM containing 0.1% BSA followed by 36-h co-culture with IMDM containing 10% FBS and then 10 h in FBS-free IMDM. The culture medium was collected for the measurement of ANP and BNP, following which MCs in ice-cold phosphate-buffered saline (PBS) were collected by scraping cells into a 50 mL centrifuge tube. After centrifugation at 4°C, the supernatant was discarded and collected cells were suspended in ice-cold PBS (1.0 mL/tube) and transferred into 1.5 mL tubes. The 50 mL centrifuge tubes were again washed with ice-cold PBS (0.5 mL), and this wash was transferred into 1.5 mL tubes. After centrifugation at 12,000×*g* at 4°C, the supernatant was discarded. Pelleted cells were immediately stored at −80°C until used for the measurement of protein or genomic DNA. Protein levels in MC samples were measured using the bicinchoninic acid (BCA) method quantifying total protein (*14*) with the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, USA) after cell pellets had been lysed with Mammalian Protein Extraction Reagent (M-PER, Pierce Biotechnology) according to the manufacturer's protocol. Protein content data were normalized to the amount of genomic DNA of MCs isolated, as previously described (*6*).

Determination of ANP and BNP Produced by MC Cultures and by MC/NMC Co-Cultures

Culture media from MC cultures (ø 10 cm culture dishes) and from co-culture insert wells were mixed in 50 mL centrifuge tubes on ice and stored in 1.5 mL tubes at −80°C until assayed for ANP- and BNP-like immunoreactivity (ANP-LI and BNP-LI) using ANP- and BNP-specific enzyme immunoassay (EIA) kits (Peninsula Laboratories, San Carlos, USA). Data were normalized to the amount of genomic DNA of MCs present. ANP and BNP secretion by NMCs (90×10^5 cells in 20 mL culture medium) into culture medium was investigated, as was the secretion by MCs because of previous reports of perturbed expression of ANP and BNP mRNAs and ANP- and BNP-LI in the culture medium of cardiac fibroblasts (*8*, *15*, *16*).

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) for Angiotensinogen, Renin, ACE, and Ang II Receptor mRNAs in MCs and NMCs

Total RNA extraction from MCs, NMCs, and tissues as positive controls (angiotensinogen: neonatal Wistar rat liver; renin: neonatal Wistar rat kidney; ACE: neonatal Wistar rat lung; Ang II receptors: neonatal Wistar rat kidney), synthesis of cDNA, and RT-PCR analyses were performed as previously reported. There were 35 PCR cycles except for β-actin (25 cycles) with the specific primers and annealing temperatures noted in Table 3; angiotensinogen (GenBank No. NM 13443 [1128–1432]), estimated amplicon size: 307 bp; renin (GenBank No. X07033 [473–874] and J02941 [656–1057]), estimated amplicon size: 404 bp; ACE (GenBank No. U03734 [2428–2733]), estimated amplicon size: 308 bp; Ang II type 1a receptor (*17*), estimated amplicon size: 306 bp; Ang II type 1b receptor (*15*), estimated amplicon size: 344 bp; Ang II type 2 receptor (*17*), estimated amplicon size: 445 bp; TGF-β1 (*18*), estimated amplicon size: 500 bp; β-actin, estimated amplicon size: 512 bp.

MCs in the MC/NMC co-culture system were cultured, and culture medium samples were taken out for the measurement of ANP and BNP before the treatment of cells for mRNA

extraction and cDNA synthesis. Primers for angiotensinogen, renin, and ACE mRNAs were designed using the primer3 web site (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). PCR products were separated using 2% agarose gel electrophoresis, and the intensity of ethidium bromide staining was detected using ultraviolet transillumination.

Ang II, TGF-β1, and ET-1 Secretion by NMCs in the MC/NMC Co-Culture System

NMCs were plated onto 6-well plates at a density of 6.0×10^5 cells/well to prepare the MC/NMC co-culture system for determination of NMC TGF-β1 secretion and Ang II production. MCs were added to the insert wells (Transwell or ThinCert for 6-well plates, pore size 0.4 μm) at the densities of 0 cells/well (vehicle-only control), 0.67×10^5 cells/well (10% MCs), 1.2×10^5 cells/well (17% MCs), and 2.0×10^5 cells/well (25% MCs) (Table 2). After 48-h co-culture in IMDM containing 10% FBS and antibiotics followed by 48-h culture in FBS-free IMDM, cells were cultured in 4 mL (1.5 mL on the insert well and 2.5 mL in the lower compartment of the culture system) of IMDM containing 0.1% BSA and antibiotics followed by culture medium sample collection. Samples for the measurement of genomic DNA were also obtained as described in the previous section. Cell samples were quickly frozen and stored at −80°C until used for the measurement of protein or genomic DNA. TGF-β1-, Ang II-, and ET-1-LI levels were measured using the Quantikine TGF-β1 immunoassay kit (R&D Systems, Minneapolis, USA) or specific radioimmunoassay (RIA) kits for Ang II and ET-1 (Peninsula Laboratories), according to the manufacturers' protocols. Total secretion of Ang II- and ET-1-LI was normalized to the amount of genomic DNA of NMCs, because ET-1 mRNA is altered by culture alone, *i.e.*, it increases even without the addition of MCs and is not detected in MCs (*2*).

Evaluation of Paracellullar Permeability of D-[1-^{3}H(N)]-Mannitol through the High-Density NMC-Plated Insert Well

Radioisotope-labeled mannitol was used to evaluate the paracellular permeability of substances through the NMC-plated insert well, as previously described, with slight modification (*19*). Briefly, in order to evaluate the paracellular permeability of D-[1-^{3}H(N)]-mannitol through NMC-plated inserts, NMCs (passage 2 to passage 3) were plated at the density of 4.5×10^5 cells/well on Transwell inserts in 12-well plates (4.02×10^5 cells/cm^2) and 90×10^5 cells/well on Transwell inserts for ø 10 cm culture dishes (2.05×10^5 cells/cm^2). After the initial culture with IMDM containing 10% FBS and the deprivation of FBS (as described above), the culture medium was replaced with IMDM containing 0.1% BSA. Insert well systems containing the same volume of culture medium and not plated with NMCs were also prepared as vehicle controls. Then, 37 kBq and 370 kBq of D-[1-^{3}H(N)]-mannitol (Perkin-Elmer Life and Analytical Sciences, Waltham, USA) were added into the culture medium on the Transwell inserts in the 12-well plate and ø 10 cm culture dishes, respectively (the final concentration in the culture medium of the lower component was up to 18.5 kBq/mL). Then, 50 μL/well of culture medium in each culture system was sampled from the lower compartment of the culture wells or dishes at the indicated time points, and radioactivity was measured by liquid scintillation counting (cpm of each samples is represented as "B"). Data were normalized for the total vehicle control count at the same concentration of D-[1-^{3}H(N)]-mannitol diluted by IMDM containing 0.1% BSA (18.5 kBq/mL, 50 μL, cpm indicated by "B0") and represented as %B/B0.

Determination of MC Cell Size in the Presence and Absence of NMCs

After the preparation of isolated MC cultures and of co-cultures containing 25% MCs, 17% MCs, and 10% MCs (12-well plate culture system), cells were co-cultured for 30 h in IMDM containing 10% FBS followed by 10 h in FBS-free IMDM. Thereafter, cells were cultured in IMDM containing 0.1% BSA for 24 h. Phase-contrast microscope images of MCs were photographed, scanned, and stored in our personal computer. The perimeters of 100 MCs in each culture were traced, and the cell area was calculated (CANVAS 9J Pro Scientific Imaging Edition, ACD Systems, Victoria, Canada).

Data Analysis and Statistics

BCA protein assays and EIA data were analyzed using Prism 4 for Windows version 4.02 (GraphPad Software, San Diego, USA). Statistical analyses were performed using Bonferroni/Dunn post-hoc tests (StatView 5.0, SAS Institute, Cary, USA); values of p less than 0.05 were considered to indicate statistical significance. All data were expressed as means±SD.

Results

[^{3}H]-Leucine and [^{3}H]-Thymidine Incorporation by MCs and [^{3}H]-Thymidine Incorporation by NMCs

Immunostaining of cultured MCs revealed that approximately 97% of cells were positive for anti-sarcomeric actin, thus verifying their MC phenotype. [^{3}H]-Thymidine incorporation by NMCs cultured with MCs (10%, 17%, and 25% MCs) or without MCs during the deprivation of FBS did not change significantly compared with that seen for NMCs alone (data not shown), indicating that differences in NMC proliferation rates between the conditions of NMCs alone and of either of the MC/NMC co-culture systems were not significant, at least within the first 48 h of culture.

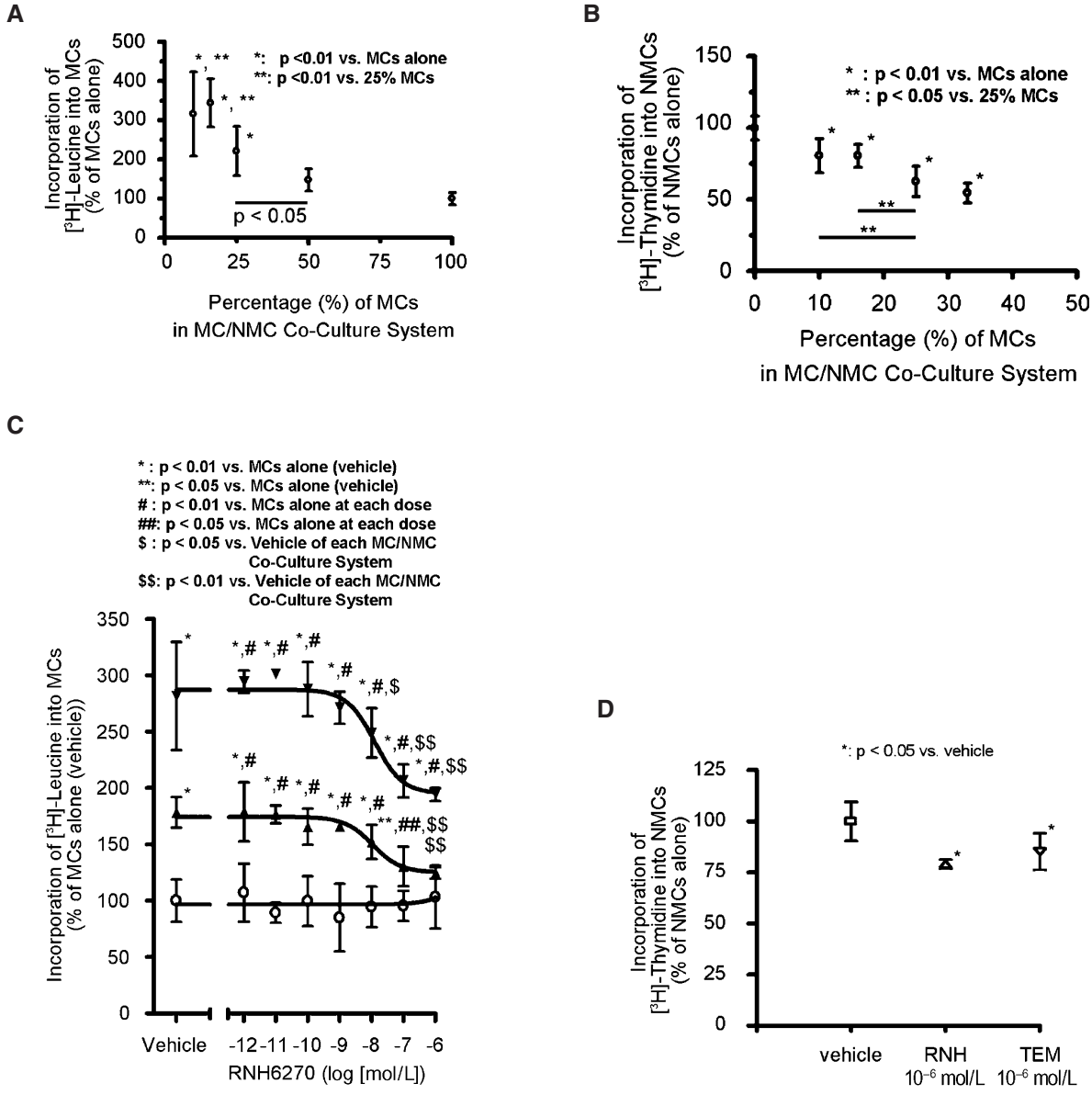

Fig. 2. *[^{3}H]-Leucine incorporation by MCs and [^{3}H]-thymidine incorporation by NMCs. A: MCs were seeded at a density of 0.5×10^5 cells/well in 12-well plates for measurement of [^{3}H]-leucine incorporation by MCs. NMCs were seeded in insert wells at the densities indicated in Table 1. B: NMCs were seeded at a density of 1.0×10^5 cells/well in 24-well plates for measurement of [^{3}H]-thymidine incorporation by NMCs. The seeding densities of MCs in insert wells are indicated in Table 1. C: Effects of RNH6270 on [^{3}H]-leucine incorporation by MCs in MCs cultured alone (○) and MCs in the MC/NMC co-culture systems (17% MCs [▼] and 25% MCs [▲]). The seeding densities of MCs in insert wells are indicated in Table 1. D: Effects of RNH6270 (10^{-6} mol/L) and temocaprilat (10^{-6} mol/L) on [^{3}H]-thymidine incorporation by NMCs. Data are represented as means±SD. RNH, RNH6270 (10^{-6} mol/L); TEM, temocaprilat (10^{-6} mol/L).*

[^{3}H]-Leucine incorporation by MCs increased significantly as the percentage of NMCs in the co-culture system rose (10% MCs: 315.9±107.1*,**, 17% MCs: 344.2±61.7*,**, 25% MCs: 221.5±62.5*, 50% MCs: 147.6±28.4% of MCs alone, *$p<0.01$ *vs*. MCs alone, **$p<0.01$ *vs*. 25% MCs, Fig. 2A). In contrast, [^{3}H]-thymidine incorporation by MCs co-cultured with NMCs did not change significantly as the percentage of NMCs increased (data not shown). However, [^{3}H]-thymidine incorporation by NMCs decreased significantly as the percentage of MCs in the MC/NMC co-culture system increased (10% MCs: 80.7±11.9*,#, 17% MCs: 73.7±14.2**, 25% MCs: 62.7±10.6**, 33% MCs: 54.6±6.8%** of NMCs alone, *$p<0.05$ *vs*. NMCs alone, **$p<0.01$ *vs*. NMCs alone, #$p<0.05$ *vs*. 25% MCs, Fig. 2B). [^{3}H]-Leucine incorporation

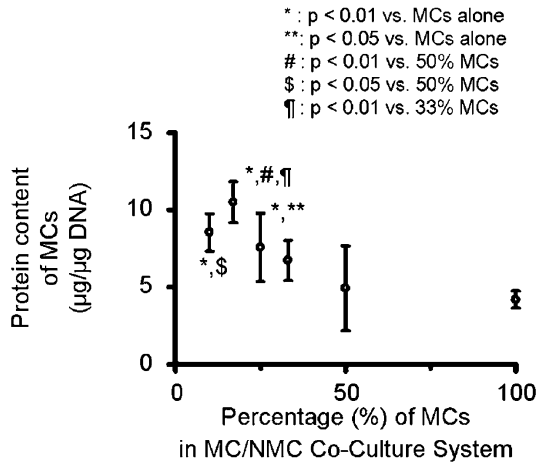

Fig. 3. *MC protein content as a function of MC percentages in the co-culture system. Data are represented as means±SD.*

by MCs in the MC/NMC co-culture systems containing 17% MCs and 25% MCs was significantly decreased by RNH6270 in a dose-dependent manner (17% MCs: 281.5±48.0 (vehicle) to 248.9±21.8$^{\$}$ (10^{-8} mol/L), 206.3±14.6$^{\$\$}$ (10^{-7} mol/L), and 194.3±5.7%$^{\$\$}$ (10^{-6} mol/L) of MCs alone (vehicle), 25% MCs: 178.3±13.6 (vehicle) to 130.5±17.6$^{\$\$}$ (10^{-7} mol/L) and 124.6±5.0%$^{\$\$}$ (10^{-6} mol/L) of MCs alone (vehicle), $^{\$}p<0.05$ and $^{\$\$}p<0.01$ *vs*. vehicle of each MC/NMC co-culture system, Fig. 2C). RNH6270 did not affect [^{3}H]-leucine incorporation by MCs cultured alone (Fig. 2C). In addition, [^{3}H]-leucine incorporation by MCs in the MC/NMC co-culture system containing 17% MCs cultured with or without RNH6270 (less than or equal to 10^{-8} mol/L) was significantly higher than that in the co-culture system containing 25% MCs cultured without RNH6270 ($p<0.01$). However, differences in MC [^{3}H]-leucine incorporation in the MC/NMC co-culture systems containing 17% MCs cultured with RNH6270 (10^{-7} mol/L and 10^{-6} mol/L) were not statistically significant compared with that containing 25% MCs cultured without RNH6270 ($p=0.0586$ and $p=0.3167$, respectively), which simulated the normal condition of the heart. Ang II increased [^{3}H]-leucine incorporation by MCs (MCs alone) to approximately 1.2-fold that of vehicle at 10^{-6} mol/L ($p<0.05$ *vs*. vehicle) compared with an approximately 1.9-fold increase by ET-1 at 10^{-8} mol/L ($p<0.01$ *vs*. vehicle), and RNH6270 (10^{-6} mol/L) completely inhibited the Ang II–induced increase in [^{3}H]-leucine incorporation by MCs. The addition of Ang II (10^{-6} mol/L) into the MC/NMC co-culture system (25% MCs) also markedly increased [^{3}H]-leucine incorporation by MCs (144.1±16.4 to 190.6±5.0% of MCs alone, $p<0.01$). [^{3}H]-thymidine incorporation by NMCs was significantly decreased by RNH6270 and temocaprilat (RNH6270 10^{-6} mol/L: 79.0±2.2*; temocaprilat 10^{-6} mol/L: 85.2±9.0% of vehicle**, $*p<0.01$, $**p<0.05$ *vs*. vehicle, respectively, Fig. 2D).

MC Protein Content

The protein content of MCs was significantly higher in co-cultures containing 10% MCs and 17% MCs than in those of MCs alone or those containing 25% MCs (10% MCs: 8.55±1.22$^{*,\$}$, 17% MCs: 10.50±1.32$^{*,\#,\P}$, 25% MCs: 7.58±2.22**, 33% MCs: 6.74±1.30, 50% MCs: 4.94±2.74, MCs alone: 4.18±0.56 μg/μg DNA, $*p<0.01$ *vs*. MCs alone, $**p<0.05$ *vs*. MCs alone, $^{\#}p<0.01$ *vs*. 50% MCs, $^{\$}p<0.05$ *vs*. 50% MCs, $^{\P}p<0.01$ *vs*. 33% MCs, Fig. 3).

ANP and BNP Secretion from MC Cultures and MC/NMC Co-Cultures

Compared with MC cultures alone, ANP-LI was markedly increased in the culture medium of MC/NMC co-cultures, particularly in co-cultures containing 25% MCs (10% MCs: 4.77±1.05$^{*,\#,\P,\S}$, 17% MCs: 5.02±0.87$^{*,\#,\P}$, 25% MCs: 8.37±1.20$^{*,\#,\$}$, 33% MCs: 5.27±1.33$^{*,\#}$, 50% MCs: 1.49±0.44, MCs alone: 1.29±0.05 pmol/μg DNA, $*p<0.01$ *vs*. MCs alone, $^{\#}p<0.01$ *vs*. 50% MCs, $^{\$}p<0.01$ *vs*. 33% MCs, $^{\P}p<0.01$ *vs*. 25% MCs, $^{\S}p<0.05$ *vs*. 17% MCs, Fig. 4A). BNP-LI was also increased in the culture medium of MC/NMC co-cultures compared with MC cultures alone (10% MCs: 2.85±0.59$^{*,\#,\$,\S}$, 17% MCs: 2.10±0.23$^{*,\#,\$,\P}$, 25% MCs: 3.79±0.63$^{*,\#,\$}$, 33% MCs: 1.37±0.08$^{*,\#}$, 50% MCs: 0.41±0.09, MCs alone: 0.37±0.04 pmol/μg DNA, $*p<0.01$ *vs*. MCs alone, $^{\#}p<0.01$ *vs*. 50% MCs, $^{\$}p<0.01$ *vs*. 33% MCs, $^{\P}p<0.01$ *vs*. 25% MCs, $^{\S}p<0.01$ *vs*. 17% MCs, Fig. 4B). To evaluate changes in secretory patterns of natriuretic peptide from MCs co-cultured with different densities of NMCs, the total amount of BNP was divided by the sum of ANP and BNP, these being the two principle natriuretic peptides produced by MCs (MCs do not synthesize a third cardiac natriuretic peptide, C-type natriuretic peptide (*8*)). In MC cultures alone, the level of BNP-LI was lower than that of ANP-LI. However, the BNP/(ANP+BNP) ratio was significantly increased in MC/NMC co-cultures containing 25%, 17%, and 10% MCs (10% MCs: 43.0±6.0$^{*,\#,\$,\P,\S}$, 17% MCs: 29.8±3.3$^{**,\$}$, 25% MCs: 31.2±4.5$^{\#\#,\$}$, 33% MCs: 21.5±5.4, 50% MCs: 24.6±5.7, MCs alone: 22.1±2.2%, $*p<0.01$ *vs*. MCs alone, $^{\#}p<0.01$ *vs*. 50% MCs, $^{\$}p<0.01$ *vs*. 33% MCs, $^{\P}p<0.01$ *vs*. 25% MCs, $^{\S}p<0.01$ *vs*. 17% MCs, $**p<0.05$ *vs*. MCs alone, $^{\#\#}p<0.01$ *vs*. 50% MCs, Fig. 4C). This change in the patterns of ANP and BNP production suggests that the synthesis of BNP by MCs may be upregulated in the presence of NMCs. Neither ANP- nor BNP-LI was detectable in the culture medium of NMCs in the culture system (data not shown).

Angiotensinogen, Renin, ACE, and Ang II Receptor Expression in MCs and NMCs

Angiotensinogen was expressed by NMCs, but not MCs, while ACE mRNA was detectable in both MCs and NMCs.

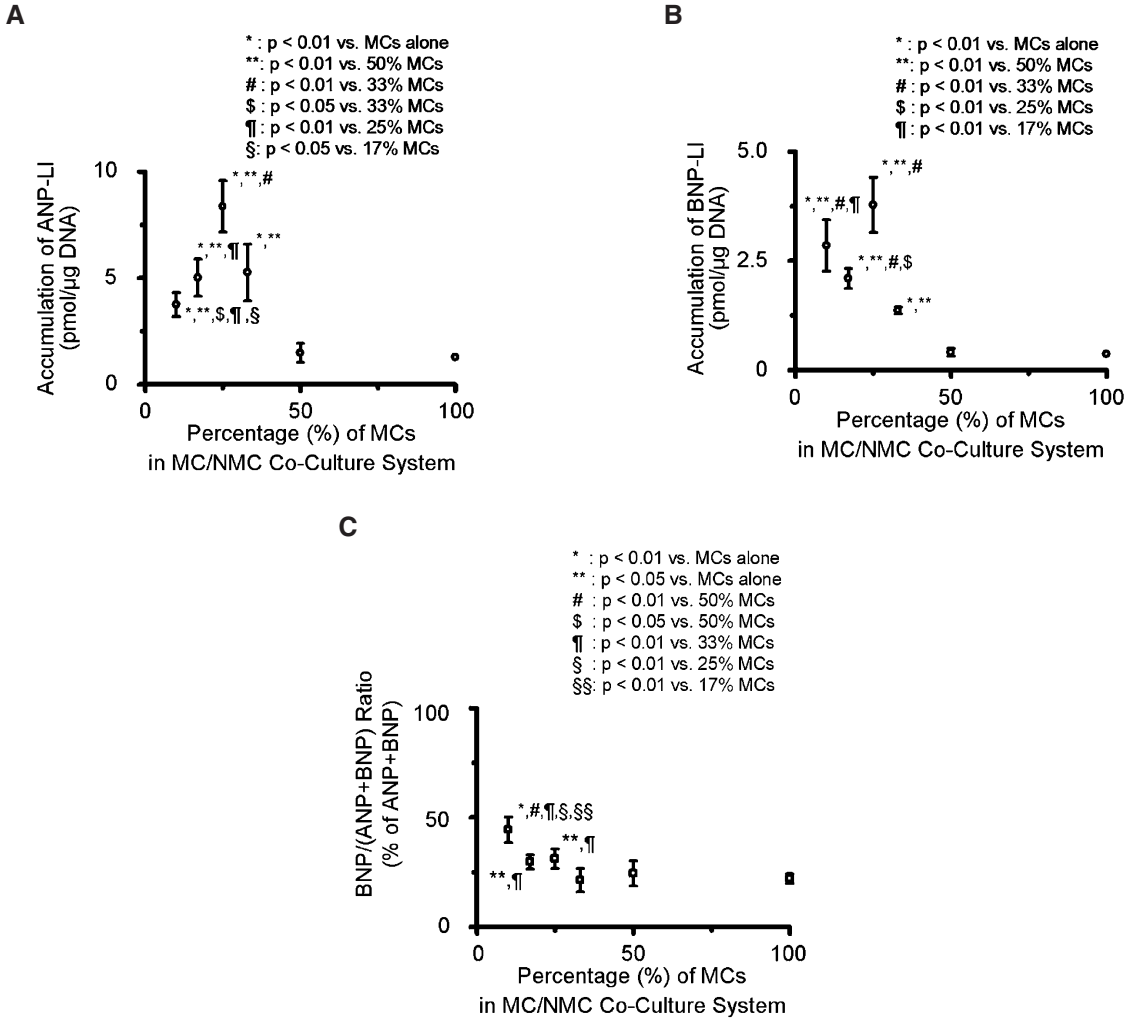

Fig. 4. *Production of ANP (A) and BNP (B) from MCs and relative ratios of BNP/ANP (C) in each culture system. Data are represented as means±SD. ANP-LI, ANP-like immunoreactivity; BNP-LI, BNP-like immunoreactivity.*

Renin mRNA was not detectable in MCs or NMCs (Fig. 5A).

Expression of these components by MCs cultured in the MC/NMC co-culture system was also evaluated. ACE mRNA was readily detected in co-cultured MCs, but these MCs barely expressed angiotensinogen and renin (Fig. 5B).

Ang II type 1a receptor was clearly expressed by both NMCs and MCs. Ang II type 1b and type 2 receptors were not detected in either MCs or NMCs by RT-PCR (Fig. 5C). In MCs isolated from the MC/NMC co-culture system, Ang II type 1a receptor mRNA was detected, but Ang II type 1b and type 2 receptor mRNAs were not (Fig. 5D). TGF-β1 was strongly expressed by both MCs and NMCs (Fig. 5E).

Ang II, TGF-β1, and ET-1 Secretion by NMCs Isolated from the MC/NMC Co-Culture System

Ang II-LI was detected in all of the culture systems. When compared with vehicle, however, the production of Ang II in each system did not reach statistical significance (NMCs alone: 134.7±14.0, 10% MCs: 132.3±7.5, 17% MCs: 121.2±10.2, 25% MCs: 143.1±4.8* fmol/μg DNA, *$p<0.05$ *vs*. 17% MCs, Fig. 6A).

TGF-β1-LI was detected in NMCs and in the MC/NMC co-culture system, but not in MCs alone. This was an interesting finding despite the obvious expression of TGF-β1 mRNA as determined by RT-PCR (Fig. 5E). Although total TGF-β1-LI tended to increase as the percentage of MCs in the MC/NMC co-culture system increased, the increase was not statistically significant (NMCs alone: 16.3±2.3, 10% MCs: 8.7±3.0, 17% MCs: 11.1±0.8, 25% MCs: 16.3±8.10* pg/culture, *$p<0.05$ *vs*. 10% MCs, Fig. 6B).

ET-1-LI secretion by NMCs in the MC/NMC co-culture containing 25% MCs was significantly greater than that of NMCs alone. ET-1-LI secretion by NMCs in other MC/NMC co-cultures was not significantly increased (NMCs alone: 26.1±1.8, 10% MCs: 26.2±1.8, 17% MCs: 29.5±2.8, 25%

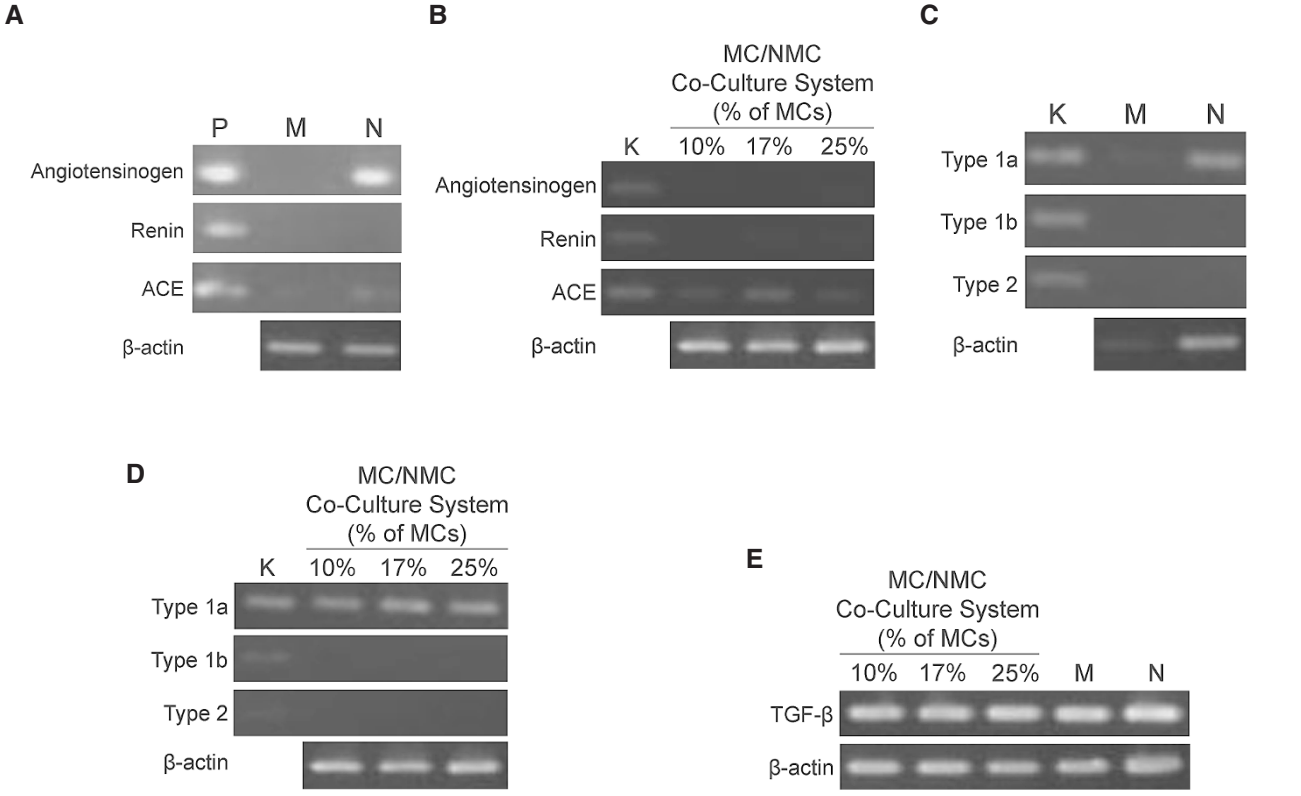

Fig. 5. *Expression of components of the renin-angiotensin system (namely angiotensinogen, renin, and angiotensin-converting enzyme [ACE] mRNAs) in MCs and NMCs cultured alone (A) and in MCs from the MC/NMC co-culture system (B), Ang II receptors in MCs and NMCs (the single culture system) (C), Ang II receptors in MCs of the MC/NMC co-culture system (D), and TGF-β (E) mRNAs in MCs from the MC/NMC co-culture systems. P, positive controls (angiotensinogen: neonatal Wistar rat liver, renin: neonatal Wistar rat kidney, ACE: neonatal Wistar rat lung); K, neonatal Wistar rat kidney; M, MCs (MCs alone); N, NMCs.*

MCs: 26.6±4.1, 50% MCs: 32.2±4.5*,**,# fmol/μg DNA, *$p<0.05$ *vs*. NMCs alone, **$p<0.05$ *vs*. 10% MCs, #$p<0.05$ *vs*. 25% MCs, Fig. 6C).

Paracellular Permeability of D-[1-^{3}H(N)]-Mannitol through the High-Density NMC-Plated Insert Well

As shown in Fig. 7, elevation of D-[1-^{3}H(N)]-mannitol concentrations in the culture medium of the lower compartment of the high-density NMC-plated insert well was slightly delayed compared with that observed in the control vehicle system, but it was preserved well. Thus, MCs and NMCs cultured in this system can communicate with each other only *via* secreted humoral factors; *i.e.*, they cannot make direct contact with each other.

MC Area in the Presence or Absence of NMCs

The average area or cell size of MCs co-cultured with NMCs was significantly larger than that of MCs cultured alone, even though the presence of NMCs did not increase the protein content of MCs in the co-culture containing 25% MCs. This finding indicates that the presence of NMCs may cause morphological changes to MCs (Figs. 8 and 9).

Discussion

While the addition of NMCs (or of NMC-conditioned media) to cultures of MCs results in MC hypertrophy (*2*), it is difficult to define the causative physiological properties of cardiocytes in the mixed MC/NMC co-culture system (*2–4*, *8*). Different ratios of MC to NMC should be used because the presence of NMCs is known to affect MC physiology through the influence of humoral factors secreted by both cell types (*2–8*). Actual cardiac tissue has a drainage system of humoral factors secreted and/or produced by NMCs, but the response of MCs to these factors may be influenced by the number of NMCs around the MCs, due to the paracrine properties of these factors. As a matter of course, an *in vitro* experimental system (*i.e.*, a closed system) lacks several conditions of *in vivo* tissue (*i.e.*, an open system); for example, a drainage system of factors that cells secrete and/or produce, which affects

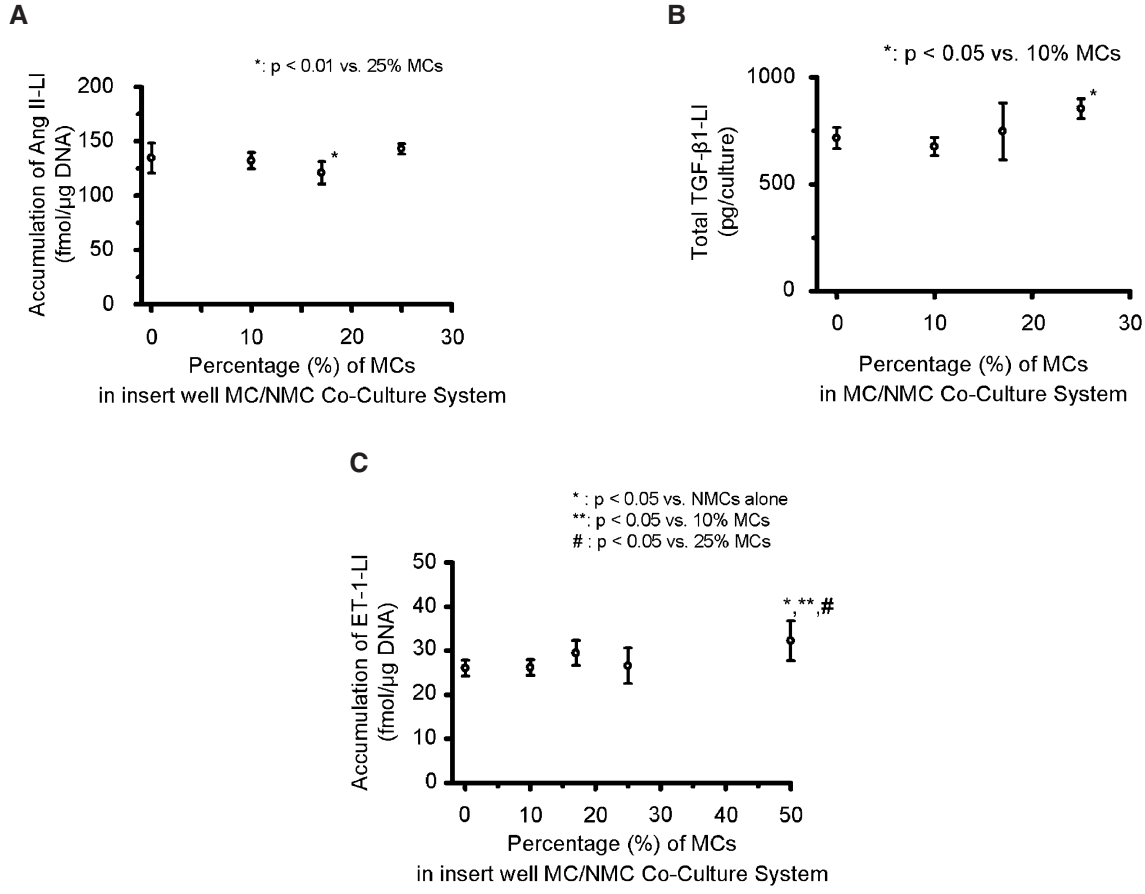

Fig. 6. *Secretion of Ang II (A), TGF-β1 (B), and ET-1 (C) in the MC/NMC co-culture system. Data are represented as means±SD. Ang II-LI, angiotensin II–like immunoreactivity; TGF-β1-LI, transforming growth factor β1–like immunoreactivity; ET-1-LI, endothelin-1–like immunoreactivity.*

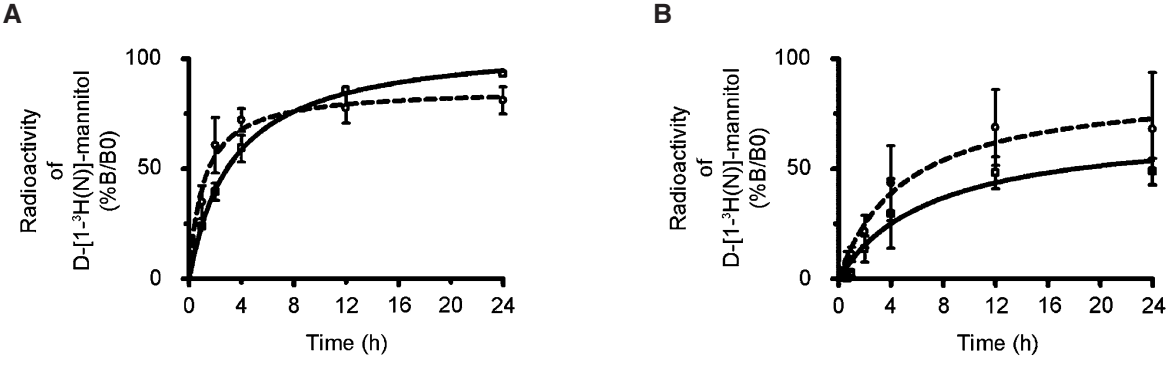

Fig. 7. *Paracellular permeability of D-[1-^{3}H(N)]-mannitol through high-density NMC-plated insert well. A broken line (○) indicates the permeability of the Transwell membrane alone in the control vehicle-only system, and a solid line (□) indicates the paracellular permeability of NMC-plated Transwells for ø 10 cm culture dishes and 12-well plates. A: NMC-plated Transwell for ø 10 cm culture dish (2.05×10^5 cells/cm^2). B: NMC-plated Transwell for 12-well plate (4.02×10^5 cells/cm^2). Three (ø 10 cm dish) or four (12-well plate) NMC culture wells were examined in each experimental group.*

the concentration of testing factors around target cells. However, such a difference is an inherent limitation in *in vitro* studies. Therefore, MCs and NMCs were physically separated in the insert well MC/NMC co-culture system, which

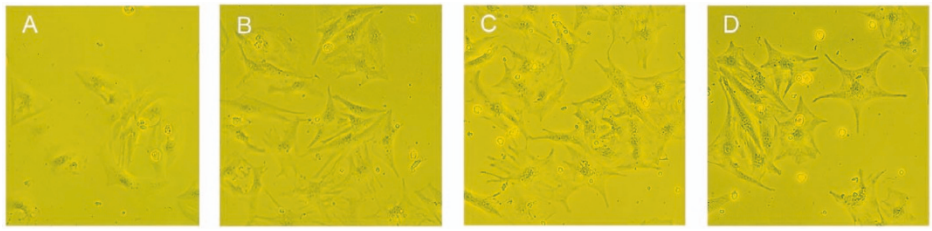

Fig. 8. *Phase-contrast microscope images of MCs in an MC-only culture (A) and in an insert well from the MC/NMC co-culture system (25% [B], 17% [C], and 10% [D]). The areas of MCs co-cultured with NMCs are significantly larger than those for MCs cultured alone.*

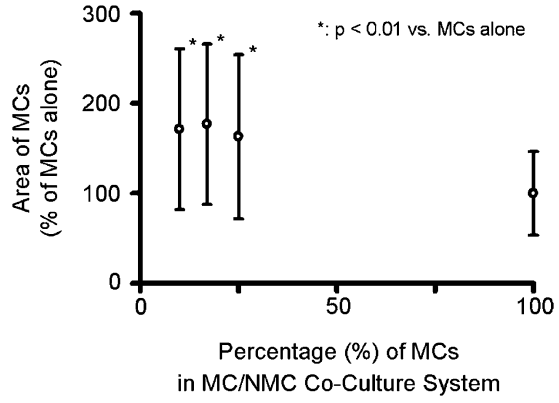

Fig. 9. *Comparison of MC size in isolated MC cultures and in MC/NMC co-cultures. Data are presented as means ± SD.*

ensured that the purity of tested cells was preserved well. Samples whose cells have been stimulated in a particular way, as in NMC stimulation of MCs, can thus be quantified for protein and/or DNA content without contamination by the stimulating cell type, or cells can be tested for the incorporation of specific tracers. Samples from our co-culture system, therefore, facilitated examination of stimulated cell-type–specific gene expression (*i.e.*, RT-PCR, Northern blot, and DNA array analyses) and protein expression patterns (*i.e.*, Western blot analysis). Thus, the co-culture system is useful for investigating cardiac pathophysiologies and, more specifically, as an experimental model for pharmacotherapy. In addition, the percentages of MCs, NMCs, and other components of the co-culture system can be easily changed to achieve the desired experimental conditions; for example, adult MCs and NMCs instead of neonatal MCs and NMCs. Some cellular features between adult and neonatal cardiac cells, including the features concerning cardiac hypertrophy, are different (*8*, *20*) and therefore remain to be investigated. However, the insert well MC/NMC co-culture system does not permit direct physical MC-NMC communication and therefore cannot be used to evaluate these aspects of cell physiology that may play certain roles in cardiac physiology and pathologies (*9*). Furthermore, the system cannot be used to evaluate the specific contributions of MCs and NMCs to the production of humoral factors, such as TGF-β and Ucn, which both cell types have been reported to secrete (*1*, *5*, *7*).

The present study showed that under co-culture conditions, [^{3}H]-leucine incorporation by MCs increased while [^{3}H]-thymidine incorporation by NMCs decreased. It was also demonstrated that MC cells were significantly larger when co-cultured with NMCs. Consequently, protein synthesis markedly increased by MCs in co-cultures containing 17% and 10% NMCs. However, a higher ratio of NMCs to MCs in the co-culture did not affect MC size. A possible explanation for the discrepancy between the increase in [^{3}H]-leucine incorporation by MCs and their cell size or protein content may be that the increased number of NMCs facilitated the synthesis of rapid-turnover proteins, because [^{3}H]-thymidine incorporation by MCs (*i.e.*, reflecting the number of live MCs) was not affected by the increased percentage of NMCs significantly. Increased [^{3}H]-thymidine incorporation by NMCs in these MC/NMC co-cultures (*i.e.*, 17% and 10% NMC) compared with 25% MCs (model of normal heart ratio) suggests that the number of NMCs may increase significantly and therefore change from the "normal" ratio during the culture period. Correspondingly, the percentage of MCs may decrease in these co-cultures, indicating that these co-cultures at the experimental time point examined may represent more advanced fibrotic stages of cardiac disease. The results confirm that cardiac fibrosis induced by the loss of MCs may facilitate cardiac hypertrophy and further fibrosis. Moreover, given the large increase in ANP and BNP production in co-cultures containing 25% MCs, the synthesized proteins might not contribute significantly to an increase in cardiac cell protein content because these natriuretic peptides have anti-hypertrophic properties (*21*). Although it is unclear why BNP synthesis by MCs was increased compared with ANP secretion in co-cultures containing 10% MCs, one possible reason is the increased activity of neutral endopeptidase and differences in the resistance of ANP and BNP to neutral endopeptidase in the MC/NMC co-cultures containing 17% and 10% MCs (*22*, *23*). ANP mRNA is expressed by neonatal MCs and NMCs, but ANP production by MCs is much higher than that of NMCs (*8*). Although the production of BNP by NMCs is disputed (*16*), the amount of BNP synthesized by NMCs,

which was estimated to be about 0.5 ng/90×10^5 NMCs according a previous study (*16*), seems to be very low compared with the total amount of BNP (measured by EIA in the culture medium of the insert well) found in MC/NMC co-cultures (about 84 ng/culture for 25% MCs, 33 ng/culture for 17% MCs, and 70 ng/culture for 10% MCs). The present study showed that neither ANP- nor BNP-LI measured by specific EIA kits was detectable in the maximum number of NMC cultures, indicating that NMC production of ANP and BNP was negligible compared with the levels produced by MCs. The present study also showed that three humoral factors—Ang II, TGF-β1, and ET-1—are synthesized by NMCs, but that the percentage of MCs present had no significant effect on Ang II, TGF-β1, or ET-1 synthesis in the MC/NMC co-culture system. Based on the results of RT-PCR analyses for components of the renin-angiotensin system, angiotensinogen mRNA was detected in NMCs but not in MCs. In addition, as angiotensinogen mRNA was barely detectable in MCs isolated from the MC/NMC co-culture system, angiotensinogen from MCs was unlikely to be a significant source of Ang II production in each of the MC/NMC co-culture systems. Therefore, angiotensinogen most likely originated predominantly from NMCs. Although production of Ang II by NMCs may be contentious because of the low expression of renin mRNA and cross reactivity of the Ang II RIA kit (0.9%) to renin substrate, attenuation of [^{3}H]-thymidine incorporation by NMCs and [^{3}H]-leucine incorporation by MCs in the MC/NMC co-culture system by RNH6270 and temocaprilat indicate that *de novo* production of Ang II (in our culture system) may be independent of the usual renin or prorenin-uptake systems (*24*). The effects of RNH6270 on [^{3}H]-leucine incorporation by MCs suggest that MCs could not produce Ang II without the influence of NMCs, as angiotensinogen mRNA was barely expressed by MCs alone. However, the present results indicate that Ang II may be synthesized even in the closed *in vitro* system of the MC/NMC co-culture system. In other words, the complete components of the local Ang II–synthesizing system may exist in the heart, despite the controversial concepts on the presence of cardiac renin (*24*, *25*), and the local Ang II–synthesizing system may be involved in the hypertrophy of MCs indirectly. Together with the reported pharmacokinetics of olmesartan medoxomil in human subjects (*26*, *27*) and the present results, that RNH6270 (10^{-7} mol/L and 10^{-6} mol/L) attenuated [^{3}H]-leucine incorporation by MCs in the MC/NMC co-culture system containing 17% MCs to the insignificant level of that seen in the vehicle of the MC/NMC co-culture system containing 25% MCs, indicate a beneficial therapeutic potential of the blockade of Ang II type 1 receptor on hypertensive patients in addition to the improvement of their blood pressure. This indicates that regionally produced Ang II may be also involved in cardiac hypertrophy, and that olmesartan may have an anti-hypertrophic effect against pressure-independent cardiac hypertrophy in addition to previously reported effects on pressure overload (*28*).

The exact cellular source of TGF-β1 in the MC/NMC co-culture system is unclear due to its possible auto-induction in MCs by TGF-β1 secreted from NMCs (*1*). The results indicate that the influence of such humoral factors may be increased correspondingly as the number of NMCs is increased, and may thus affect incorporation of [^{3}H]-leucine by MCs. On the other hand, the altered [^{3}H]-thymidine incorporation by NMCs in MC/NMC co-cultures may be attributed to increases in natriuretic peptide secretion in each of the MC/NMC co-culture systems. Therefore, the results show that the evaluation under co-culture conditions is far more useful than the examination of isolated single cell culture systems, especially in the *in vitro* simulation for the effects of pharmacological agents on pathological conditions, because a lot of known (or currently unknown) factors emanating from both MCs and NMCs can be studied concomitantly.

The co-existence of MCs in the NMC co-culture system reduced the [^{3}H]-thymidine incorporation by NMCs significantly. These findings may be attributable simply to the antiproliferative properties of ANP and BNP, because the concentration of these natriuretic peptides increased slightly as the content of MCs in the co-culture increased. These results suggest that cardiac fibrosis could accelerate cardiac hypertrophy and fibrosis independently of mechanical stress on MCs. Humoral factors secreted by NMCs could thus induce a hypertrophic response in cardiocytes (*3*), even for an MC percentage identical to that seen in the heart of an animal model for hypertension (17.6% of muscle nuclei and 82.0% of connective tissue nuclei were reported in the hearts of hypertensive animals) (*10*). The increase in the percentage of NMCs in experimental animal models could result in overproduction of humoral factors implicated in cardiomyocyte hypertrophy, such as ET-1 and TGF-β, in spite of the increased secretion by MCs of ANP and BNP (*2*). Consequently, the inhibitory actions of ANP and BNP on hypertrophy and fibrosis may be reduced.

In conclusion, the results of the present study show that decreases in the ratio of MCs to NMCs could facilitate cardiac hypertrophy and fibrosis, *i.e.*, cardiac remodeling without mechanical stress, and change the pathophysiological properties of MCs, such as their natriuretic peptide secretory patterns. However, secretion from NMCs or production in NMCs of humoral factors, such as ET-1, Ang II, and TGF-β1, was not significantly affected by the co-culture with MCs. The finding here also indicates that locally *de novo*–synthesized Ang II may be involved in cardiomyocyte hypertrophy, even though its production may be independent of the renin or pro-renin uptake system typical for the *in vivo* synthesis of Ang II.

The insert well MC/NMC co-culture system described here has several notable features that could prove useful in experimental studies:

1) The system can be used in the evaluation of MC- or NMC-specific incorporation of radioactive tracers under conditions simulating desired physiological or pathological states.

2) The system can be used in the evaluation of MC- or NMC-specific expression of mRNA or protein by RT-PCR, Northern blot, or Western blot methods. Samples from each cell type can be prepared without contamination by other cells that form part of the co-culture.

3) MCs and NMCs can be easily separated, and any humoral factor–related cross talk between cells can be preserved well. If necessary, the percentages of MCs, NMCs, and components of the co-culture system can be easily changed to achieve desired experimental conditions (for example, adult MCs and NMCs instead of neonatal MCs and NMCs).

However, limitations of the insert well MC/NMC co-culture system should also be noted:

1) The effects of direct cell-to-cell contact between MCs and NMCs cannot be evaluated.

2) It is difficult to identify which cell type—MCs, NMCs, or both—is responsible for the secretion of a particular humoral factor (as in the cases of TGF-β and Ucn, which are secreted by both cell types).

On the basis of the findings presented here, the insert well MC/NMC co-culture system will make it possible to investigate the cellular cross talk between MCs and NMCs for its involvement in a variety of cardiac pathologies, and will be particularly useful as an experimental model for cardiac pharmacotherapy.

Acknowledgements

The authors thank Y. Takada and Y. Inada for their excellent technical assistance and Professor O. Ohara (English Department, the Jikei University School of Medicine, Tokyo, Japan) for his help with the manuscript preparation. The authors also thank Daiichi-Sankyo Co. Ltd. for providing RNH6270 and temocaprilat, although they were not involved in the study design, experiments, data analysis, or data interpretation of this study.

References

1. Flanders KC, Holder MG, Winokur TS: Autoinduction of mRNA and protein expression for transforming growth factor-βs in cultured cardiac cells. *J Mol Cell Cardiol* 1995; **27**: 805–812.
2. Harada M, Itoh H, Nakagawa O, *et al*: Significance of ventricular myocytes and nonmyocytes interaction during cardiocyte hypertrophy: evidence for endothelin-1 as a paracrine hypertrophic factor from cardiac nonmyocytes. *Circulation* 1997; **96**: 3737–3744.
3. Harada M, Saito Y, Kuwahara K, *et al*: Interaction of myocytes and nonmyocytes is necessary for mechanical stretch to induce ANP/BNP production in cardiocyte culture. *J Cardiovasc Pharmacol* 1998; **31** (Suppl 1): S357–S359.
4. Ikeda K, Tojo K, Tokudome G, *et al*: Possible involvement of endothelin-1 in cardioprotective effects of benidipine. *Hypertens Res* 2000; **23**: 491–496.
5. Ikeda K, Tojo K, Oki Y, Nakao K: Urocortin has cell-proliferative effects on cardiac non-myocytes. *Life Sci* 2002; **71**: 1929–1938.
6. Ikeda K, Tojo K, Otsubo C, *et al*: 5-Hydroxytryptamine synthesis in HL-1 cells and neonatal rat cardiocytes. *Biochem Biophys Res Commun* 2005; **328**: 522–525.
7. Ikeda K, Tojo K, Otsubo C, *et al*: Effects of urocortin II on neonatal rat cardiac myocytes and non-myocytes. *Peptides* 2005; **26**: 2473–2481.
8. Horio T, Tokudome T, Maki T, *et al*: Gene expression, secretion, and autocrine action of C-type natriuretic peptide in cultured adult rat cardiac fibroblasts. *Endocrinology* 2003; **144**: 2279–2284.
9. Camelliti P, McCulloch, Kohl P: Microstructured cocultures of cardiac myocytes and fibroblasts: a two dimensional *in-vitro* model of cardiac tissue. *Microsc Microanal* 2005; **11**: 249–259.
10. Brandt SP: Microgenomics: gene expression analysis at the tissue-specific and single-cell levels. *J Exp Bot* 2005; **56**: 495–505.
11. Diks SH, Peppelenbosch MP: Single cell proteomics for personalised medicine. *Trends Mol Med* 2004; **10**: 574–577.
12. Zak R: Cell proliferation during cardiac growth. *Am J Cardiol* 1971; **31**: 211–219.
13. Mizuno M, Sada T, Ikeda M, *et al*: Pharmacology of CS-866, a novel nonpeptide angiotensin II receptor antagonist. *Eur J Pharmacol* 1995; **285**: 181–188.
14. Smith PK, Krohn RI, Hermanson GT, *et al*: Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985; **150**: 76–85.
15. Cameron VA, Rademaker MT, Ellmers LJ, Espiner EA, Nicholls MG, Richards AM: Atrial (ANP) and brain natriuretic peptide (BNP) expression after myocardial infarction in sheep: ANP is synthesized by fibroblasts infiltrating the infarct. *Endocrinology* 2000; **141**: 4690–4697.
16. Tsuruda T, Boerrigter G, Huntley B, *et al*: Brain natriuretic peptide is produced in cardiac fibroblasts and induces matrix metalloproteinases. *Circ Res* 2002; **91**: 1127–1134.
17. Miyata N, Park F, Li XF, Cowley AW Jr: Distribution of angiotensin AT1 and AT2 receptor subtypes in the rat kidney. *Am J Physiol* 1999; **277**: F437–F446.
18. Tsubou Y, Kobayashi N, Mita S, Yoshida K, Matsuoka H: Celiprolol inhibits mitogen-activated protein kinase and endothelin-1 and transforming growth factor-β1 gene in rats. *Eur J Pharmacol* 2002; **457**: 85–93.
19. Fang X, Song Y, Zemans R, Hirsch J, Matthay MA: Fluid transport across cultured rat alveolar epithelial cells: a novel *in vitro* system. *Am J Physiol* 2004; **287**: L104–L110.
20. Jans SW, de Jong YF, Reutelingsperger CP, van der Vusse GJ, van Bilsen M: Differential expression and localization of annexin V in cardiac myocytes during growth and hypertrophy. *Mol Cell Biochem* 1998: **178**: 229–236.
21. Pandey KN: Biology of natriuretic peptides and their receptors. *Peptides* 2005; **26**: 901–932.
22. Smith MW, Espiner EA, Yandle TG, Charles CJ, Richards AM: Delayed metabolism of human brain natriuretic peptide reflects resistance to neutral endopeptidase. *J Endocrinol* 2000; **167**: 239–246.
23. Fielitz J, Dendorter A, Pregla R, *et al*: Neutral endopeptidase is activated in cardiomyocytes in human aortic valve stenosis and heart failure. *Circulation* 2002; **105**: 286–289.
24. Danser AHJ, Saris JJ: Prorenin uptake in the heart: a prereq-

uisite for local angiotensin generation? *J Moll Cell Cardiol* 2002; **34**: 1463–1472.
25. Wollert KC, Drexler H: The renin-angiotensin system and experimental heart failure. *Cardiovasc Res* 1999; **43**: 838–849.
26. Schwocho LR, Masonson HN: Pharmacokinetics of CS-866, a new angiotensin II receptor blocker, in healthy subjects. *J Clin Pharmacol* 2001; **41**: 515–527.
27. Laeis P, Püchler K, Kirch W: The pharmacokinetic and metabolic profile of olmesartan medoxomil limits the risk of clinically relevant drug interaction. *J Hypertens* 2001; **19** (Suppl): S21–S32.
28. Jia N, Okamoto H, Shimizu T, *et al*: A newly developed angiotensin II type 1 receptor antagonist, CS866, promotes regression of cardiac hypertrophy by reducing integrin β1 expression. *Hypertens Res* 2003; **26**: 737–742.