C/EBP-binding sites. In the present study, we investigated C/EBP-δ, IL-6, COX-2, and MCP-1 expression in anti-Thy1.1 glomerulonephritis and evaluated the functional role of C/EBP-δ in proliferative activity and the induction of inflammatory gene expression in cultured MCs by using RNA interference against *C/EBP-δ*.

Methods

Induction of anti-Thy1.1 glomerulonephritis

The Animal Studies Committee of Ehime University approved the following experimental protocol. Anti-Thy1.1 glomerulonephritis was induced in Sprague-Dawley rats, weighing 200–250 g, by a single intravenous injection of anti-rat Thy1.1 monoclonal antibody (1 mg/kg; Cedarlane Laboratories, Hornby, Canada). Rats were kept at 25°C with free access to tap water and to a standard rat chow. One day and 7 days after the disease induction, rats were killed and renal cortical sections were taken, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and embedded in paraffin for immunohistochemical analysis.

Immunohistochemical analysis

Deparaffinized 4-μm-thick sections were stained with rabbit polyclonal antibodies raised against α-smooth muscle actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:150 dilution), C/EBP-δ (Santa Cruz Biotechnology, Inc.; 1:150 dilution) and IL-1β (Santa Cruz Biotechnology, Inc.; 1:150 dilution), goat polyclonal antibodies raised against IL-6 (Santa Cruz Biotechnology, Inc.; 1:100 dilution), COX-2 (Santa Cruz Biotechnology, Inc.; 1:100 dilution), and MCP-1 (Santa Cruz Biotechnology, Inc.; 1:100 dilution), and mouse monoclonal antibody raised against Thy1 (Santa Cruz Biotechnology, Inc.; 1:100 dilution). Immobilized antibodies were detected by the biotin-avidin-immunoperoxidase technique, using a Vectastain Universal Quick kit (Vector Laboratories, Burlingame, CA, USA), which includes biotinylated secondary antibody, which recognizes rabbit IgG, mouse IgG, and goat IgG, made in horse according to the manufacturer's instructions, with 3, 3′-diaminobenzidine as the chromogen. Sections then were counterstained with Mayer's hematoxylin and examined by light microscopy.

Cell culture

Primary MCs were isolated from Sprague-Dawley rats by a sieving method[11] and cultured in RPMI 1640 containing 20% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma Chemical, St. Louis, MO, USA), and insulin-transferrin-selenium G supplement (Invitrogen, Carlsbad, CA, USA). The integrity of the MC phenotype was confirmed regularly by immunostaining with anti-smooth muscle actin and anti-Thy1 antibodies. MCs were maintained at 37°C in an atmosphere of air and 5% CO_2 and used in the experiments at passages 4 to 10.

Short interfering RNA

The 23nt short interfering RNA (siRNA) duplex with two 3′ overhanging nucleotides of the rat *C/EBP-δ* mRNA coding region (AACGAGAAGCTGCATCAGCGT) was designed and produced by QIAGEN (Tokyo, Japan). Non-silencing (NS) siRNA (AATTCTCCGAACGTGTCAC GT) was used as a control (QIAGEN). In order to evaluate siRNA transfection efficiency into MCs, subconfluent MCs were transfected with 100 nM of fluorescein isothiocyanate-labelled NS siRNA (FITC-NS siRNA) or non-labelled NS siRNA, using RNAifect (QIAGEN) according to the manufacturer's specifications.

Western blot analysis

Subconfluent MCs were transfected with 100 nM of C/EBP-δ or NS siRNA. Subsequently, MCs were placed in RPMI 1640 supplemented with 0.1% FCS for 48 h and then treated with 10 ng/ml of IL-1β (Pepro Tech EC, London, UK) for 4 h. Nuclear proteins of MCs were extracted using a ReadyPrep protein extraction kit (cytoplasmic/nuclear) (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's specifications. Nuclear proteins (2 μg) were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinilidene difluoride (PVDF) membranes (Bio-Rad Laboratories). Membranes were probed for 1 h with rabbit polyclonal antibody raised against C/EBP-δ (Santa Cruz Biotechnology, Inc.; 1:150 dilution) at room temperature. Following incubation with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology, Inc.), C/EBP-δ expression was detected using enhanced chemiluminescence (ECL) Western blotting reagents (GE Healthcare Bio-Sciences, Fairfield, CT, USA).

Treatment with IL-1β and evaluation of mRNA expression by real-time PCR

MCs (2×10^5 cells per well) were seeded in six-well plates and cultured for 24 h. Cells were then transfected with 100 nM of C/EBP-δ or NS siRNA. Subsequently, MCs were placed in RPMI 1640 supplemented with 0.1% FCS for 48 h and then treated with 10 ng/ml of IL-1β for 24 h. Total RNA was isolated from cultured MCs by a single-step extraction method of ISOGEN (Nippon Gene, Tokyo, Japan) at various points and reverse-transcribed using a Cloned AMV FirstStrand cDNA Synthesis Kit (Invitrogen). A semiquantitative real-time PCR was performed using a LightCycler system (Roche Diagnostics, Mannheim, Germany) with primers designed specifically for each transcript (Table 1). Experimental samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR protocol. *Glyceraldehyde-3-phosphate dehydrogenase* cDNA was amplified and quantitated in each cDNA preparation to normalize the relative amounts of *C/EBP-δ*, *IL-6*, *COX-2*, and *MCP-1*.

Table 1. Design of the primers

Rat gene	Primer sequences	Annealing temperature
C/EBP-δ		
Forward	5′-GCCTTAGAGACTCCGAACGA-3′	62°C
Reverse	5′-CATGCGCAGTCTCTTCCTCT-3′	
IL-6		
Forward	5′-GTTGTGCAATGGCAATTCTG-3′	58°C
Reverse	5′-GAGCATTGGAAGTTGGGGTA-3′	
COX-2		
Forward	5′-ATCCTGAGTGGGATGACGAG-3′	58°C
Reverse	5′-TGAGCAAGTCCGTGTTCAAG-3′	
MCP-1		
Forward	5′-GCCAGATCTCTCTTCCTCCA-3′	59°C
Reverse	5′-AGGCATCACATTCCAAATCAC-3′	
GAPDH		
Forward	5′-TCCACCACCCTGTTGCTGTA-3′	58°C
Reverse	5′-ACCACAGTCCATGCCATCAC-3′	

C/EBP-δ, CCAAT/enhancer-binding protein-δ; IL-6, interleukin-6; COX-2, cyclooxygenase-2; MCP-1, monocyte chemoattractant protein-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

IL-6 in culture medium estimated by enzyme-linked immunosorbent assay (ELISA)

MCs (2×10^5 cells per well) were seeded in six-well plates and cultured for 24h. Cells were then transfected with 100nM of C/EBP-δ or NS siRNA. Subsequently, MCs were placed in RPMI 1640 supplemented with 0.1% FCS for 48h and then treated with 10ng/ml of IL-1β for 24h. The IL-6 concentration of the culture medium from C/EBP-δ- or NS siRNA-transfected MCs was determined by a solid-phase sandwich ELISA, using an Immunoassay Kit (BioSource International, Camarillo, CA, USA), according to a protocol supplied by the manufacturer.

Evaluation of the effect of C/EBP-δ knockdown upon cell proliferation by bromodeoxyuridine (BrdU) incorporation

MCs (1×10^4 cells per well) were seeded in 96-well plates, incubated for 24h and then transfected with 100nM of either C/EBP-δ or NS siRNA. Subsequently, MCs were serum-starved in RPMI 1640 supplemented with 0.1% FCS for 48h and then treated with 10ng/ml of IL-1β or 20ng/ml of platelet-derived growth factor (PDGF)-BB (Upstate Biotechnology, Lake Placid, NY, USA) for 24h. BrdU incorporation was determined by using a Cell Proliferation ELISA System (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's specifications.

Statistical analysis

Analysis of variance (ANOVA) with the Bonferroni-Dunn post-hoc test or Mann-Whitney's *U*-test was used to analyze differences between the two experimental groups and all data values were expressed as means ± SD. A *P* value less than 0.05 was considered to indicate statistical significance.

Results

C/EBP-δ and inflammatory protein expression in MCs in rat anti-Thy1.1 glomerulonephritis

In control rats, C/EBP-δ, IL-6, COX-2, and MCP-1 expressions were undetectable in glomeruli (Fig. 1a). On the other hand, C/EBP-δ was induced in glomeruli in rats with anti-Thy1.1 glomerulonephritis on day 1 (Fig. 1b). Immunohistochemical analysis also revealed α-smooth muscle actin expression, a marker for mesangial cell activation following injury,[12] in the glomeruli. IL-6, COX-2, and MCP-1 were also expressed at this time point (day 1). C/EBP-δ, IL-6, COX-2, and MCP-1 expression patterns were the same as that of Thy1, indicating that these inflammatory proteins were expressed in MCs. On day 7, when mesangial proliferation becomes prominent, the expression of each of C/EBP-δ, IL-6, COX-2, and MCP-1 was almost undetectable (Fig. 1c). These inflammatory proteins play an important role in the pathogenesis of this model at early stages.

Expression of *C/EBP-δ* and inflammatory genes during treatment with IL-1β

Because IL-1β was expressed in the glomeruli of anti-Thy1.1 glomerulonephritis on day 1 (Fig. 2), we used IL-β to stimulate cultured MCs. Comparison between a phase-contrast micrograph and a fluorescence micrograph showed that FITC-siRNA (green) was transfected in more than 90% of MCs (Fig. 3). Additionally, Western blot analysis showed that the induction of C/EBP-δ by IL-1β was ablated by *C/EBP-δ* siRNA (Fig. 4a). Then, the mRNA levels of *C/EBP-δ*, *IL-6*, *COX-2*, and *MCP-1* were monitored for 24h in order to identify the kinetic relationship between *C/EBP-δ* and inflammatory gene expression during treatment with IL-1β (10ng/ml). In the NS siRNA-transfected MCs, *C/EBP-δ* mRNA expression was markedly increased and peaked at 3h by 7.3-fold (Fig. 4b). In contrast, no induction of *C/EBP-δ* was observed in C/EBP-δ siRNA transfected

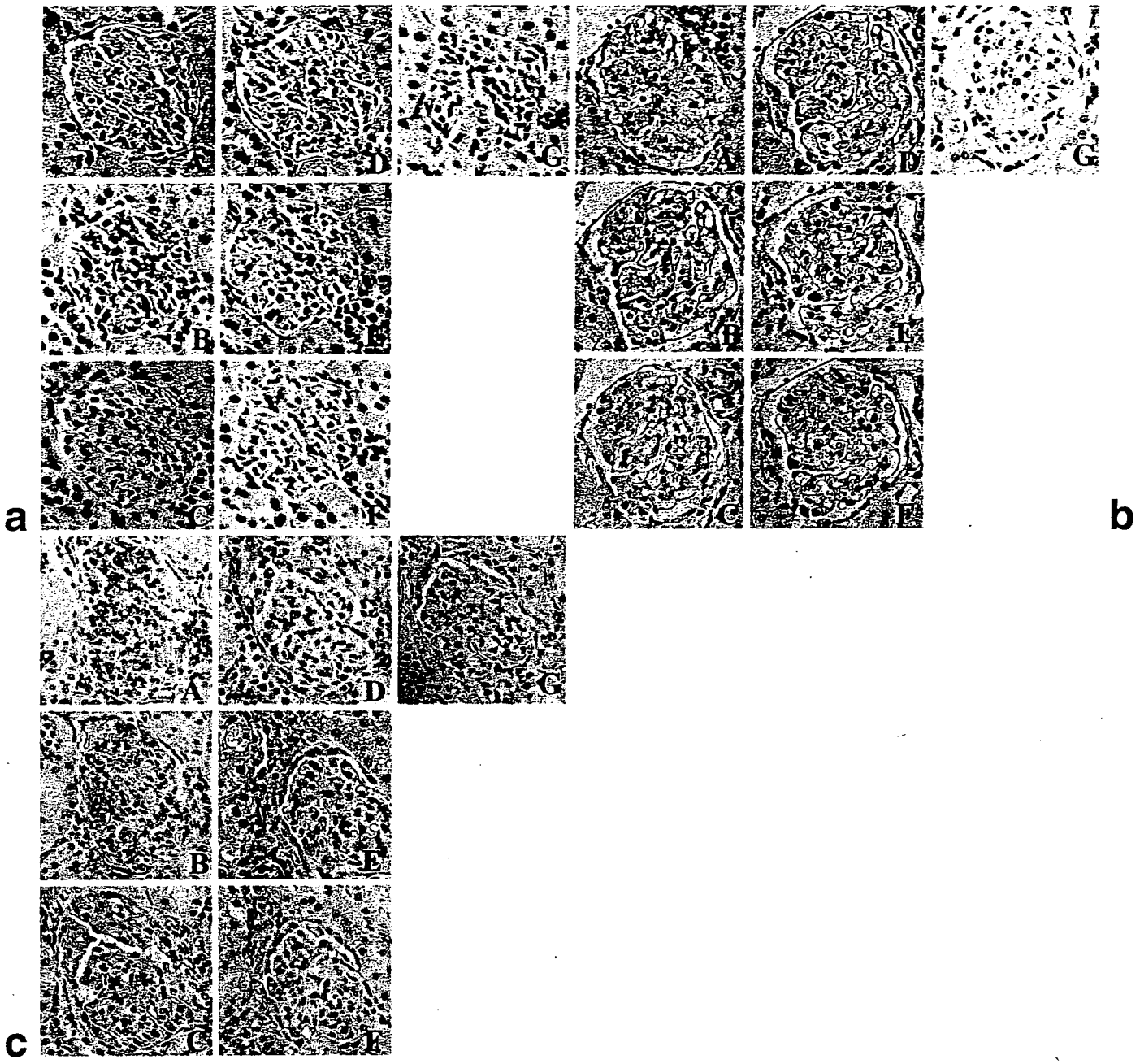

Fig. 1a–c. Immunohistochemical examination of renal tissue from anti-Thy1.1 glomerulonephritis rats. Serial sections of **a** control rats and **b** anti-Thy1.1 glomerulonephritis rats on day 1 and **c** on day 7. *A* Anti-smooth muscle α-actin antibody. *B* Anti-Thy1 antibody. *C* Anti-CCAAT/enhancer-binding protein-δ antibody. *D* Anti-interleukin-6 antibody. *E* Anti-cyclooxygenase-2 antibody. *F* Anti-monocyte chemoattractant protein-1 antibody. *G* Negative control: normal mouse IgG

MCs. Regarding the inflammatory cytokines, although *IL-6* mRNA was induced and peaked at 1 h in both NS siRNA-transfected MCs and C/EBP-δ siRNA-transfected MCs, the expression level in C/EBP-δ siRNA-transfected MCs was 57.7% lower compared to that in NS siRNA-transfected MCs (Fig. 4c). Similarly, the expression level of *COX-2* mRNA was increased by IL-1β in both NS siRNA-transfected MCs and C/EBP-siRNA-transfected MCs, but the mRNA levels in C/EBP-δ siRNA-transfected MCs were also much lower than those in NS siRNA-transfected MCs; they were reduced by 85.7% at 6 h (Fig. 4d). We also evaluated *MCP-1* mRNA expression. *MCP-1* expression was barely evident in quiescent MCs, but was markedly induced and reached peak levels at 1 h following treatment with IL-1β. The induction of *MCP-1* in C/EBP-δ siRNA-transfected MCs was much lower than that seen in NS siRNA-transfected MCs, with a 69.3% reduction evident at 1 h (Fig. 4e).

IL-6 in culture medium from MCs after treatment with IL-1β

In quiescent MCs, the IL-6 concentrations in the culture media from both C/EBP-δ siRNA- and NS siRNA-transfected MCs were lower than the measurable range. The IL-6 concentration after treatment with IL-1β in the culture medium from C/EBP-δ siRNA-transfected MCs (7.37 ± 4.3 pg/ml) was much lower than that in the culture medium from NS siRNA-transfected MCs (25.2 ± 3.4 pg/ml) (Fig. 5). Suppression of C/EBP-δ resulted in the reduction of IL-6 production.

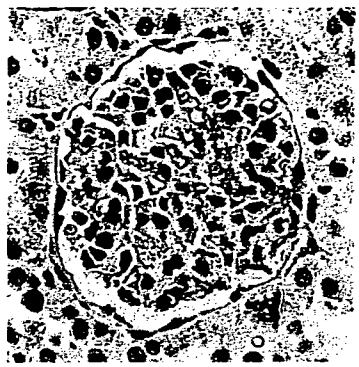

Fig. 2. Glomerular interleukin (IL)-1β expression in anti-Thy1.1 glomerulonephritis rats on day 1

Cell proliferation and treatment with IL-1β and PDGF-BB

Treatment with IL-1β induced a 10.7% increase in cell proliferation in untreated MCs and a 9.2% increase in NS siRNA transfected MCs. In contrast, IL-1β did not increase cell proliferation in C/EBP-δ siRNA-transfected MCs (Fig. 6a). In addition, treatment with PDGF-BB induced a 155.7% increase in cell proliferation compared with untreated MCs. PDGF-BB also induced a 96.0% increase in C/EBP-δ siRNA-transfected MCs and a 150.5% increase in NS siRNA-transfected MCs (Fig. 6b). Thus C/EBP-δ knockdown attenuated the MC proliferation activity stimulated by IL-1β and PDGF-BB.

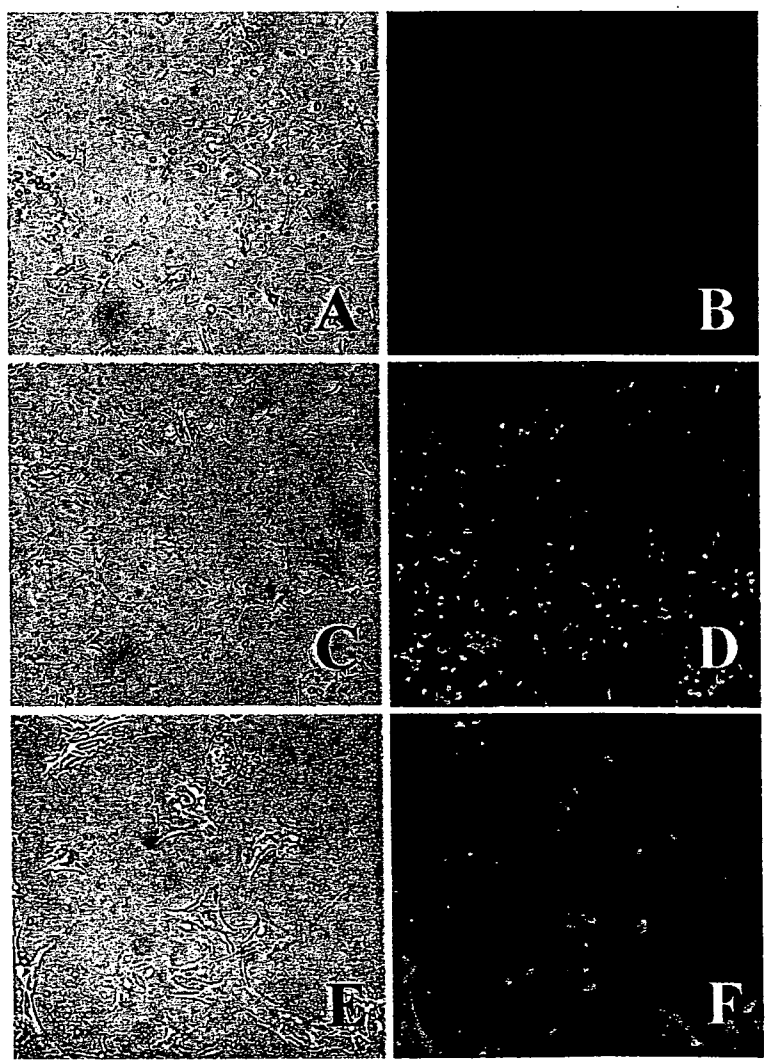

Fig. 3A–F. Efficiency of short interfering (si) RNA transfection into cultured mesangial cells. **A** Phase-contrast micrograph of mesangial cells with non-labelled NS siRNA transfection; **B** fluorescence micrograph of same field; **C** Phase-contrast micrograph of mesangial cells with fluorescein isothiocyanate-labelled NS siRNA transfection; **D** fluorescence micrograph of same field; **E** High-power view of phase-contrast micrograph of mesangial cells with fluorescein isothiocyanate-labelled NS siRNA transfection; **F** fluorescence micrograph of same field

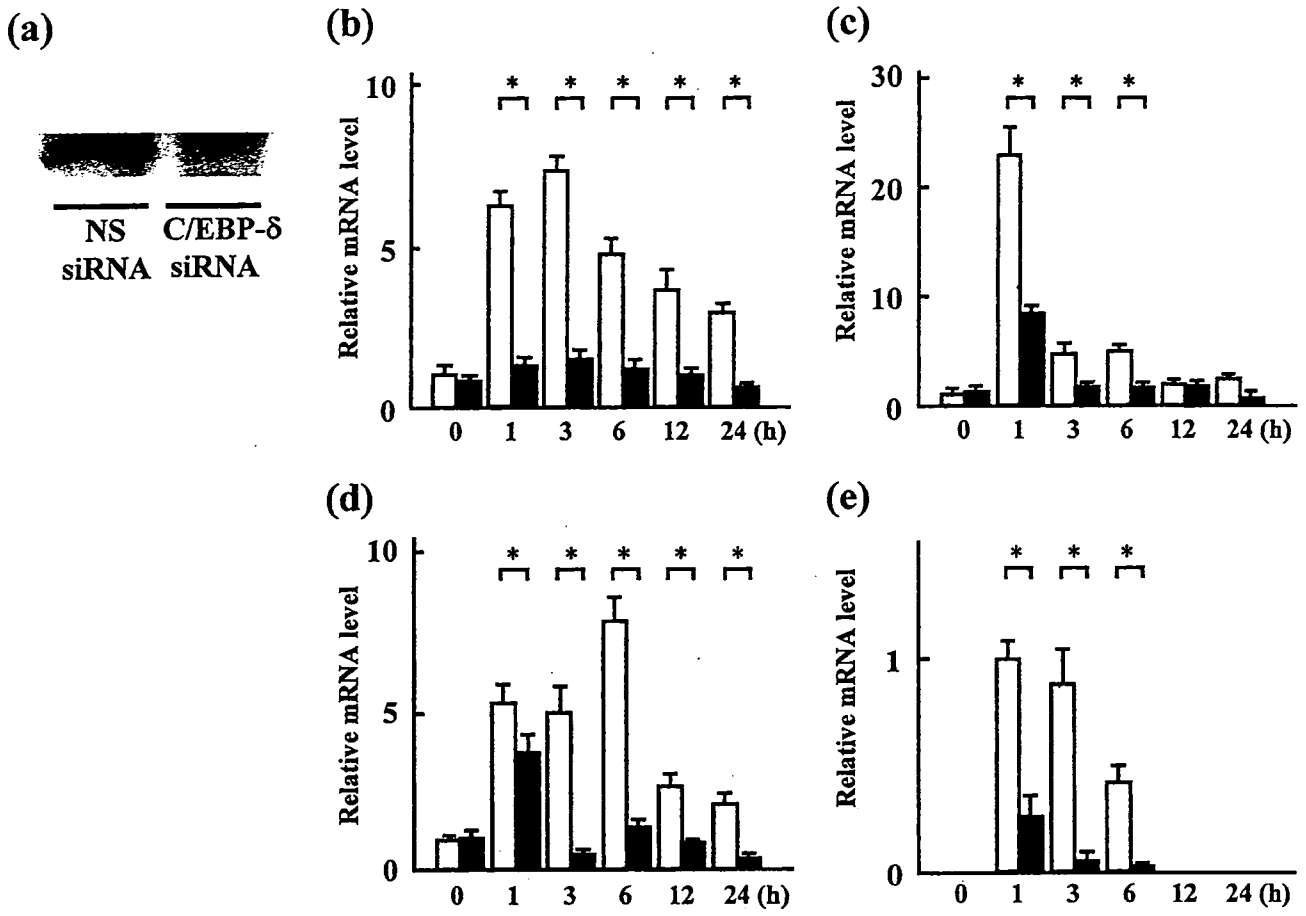

Fig. 4a–e. Effect of CCAAT/enhancer-binding protein-δ (C/EBP-δ) knockdown on inflammatory gene induction by interleukin (IL)-1β in cultured mesangial cells. **a** C/EBP-δ protein expression. After treatment with 10ng/ml of IL-1β for 4h, nuclear proteins (2μg) were extracted from mesangial cells transfected with non-silencing short interfering RNA (NS siRNA) or C/EBP-δ siRNA and subjected to Western blot analysis to detect C/EBP-δ. Time-courses of mRNA induction of **b** *C/EBP-δ*, **c** *IL-6*, **d** *cyclooxygenase-2*, and **e** *monocyte chemoattractant protein-1* are shown. RNAs were isolated from mesangial cells transfected with NS siRNA or C/EBP-δ siRNA at various time points after the addition of IL-1β (10ng/ml) and subjected to real-time polymerase chain reaction (PCR) for assessment of each mRNA expression. *Glyceraldehyde-3-phosphate dehydrogenase* cDNA was amplified and quantified in each cDNA preparation to correct for variability in RNA recovery and for efficiency of reverse transcription. *Open bars*, mesangial cells transfected with NS siRNA; *solid bars*, mesangial cells transfected with C/EBP-δ siRNA. Results are expressed as means ± SD; $n = 5$. $^*P < 0.01$ versus mesangial cells transfected with NS siRNA at the same point. Data values are expressed as a fold increase in reference to NS siRNA-transfected mesangial cells at 0h (**b–d**) or 1h (**e**)

Discussion

C/EBP-δ was originally identified as a member of the C/EBP family, which belongs to leucine zipper transcription factors.[13] C/EBP-δ expression is undetectable in normal cells or tissues, but its expression is known to be rapidly induced by inflammatory cytokines such as IL-β and lipopolysaccharide.[14,15] In the present study, we demonstrated that C/EBP-δ was expressed in glomerular MCs in anti-Thy1.1 glomerulonephritis rats on day 1. Moreover, IL-6, COX-2, and MCP-1 were also expressed in MCs. These proteins were not evident in control rats and were transiently expressed in rats with anti-Thy1.1 glomerulonephritis, with expression disappearing by day 7. C/EBP-δ was thought to play an important role during the early stages of anti-Thy1.1 glomerulonephritis. IL-β was expressed in the glomeruli of anti-Thy1.1 glomerulonephritis rats on day 1 (Fig. 2) and is known as an important cytokine in the pathogenesis of a number of glomerular diseases such as IgA nephropathy[16] and crescentic glomerulonephritis,[17] and it is reported that treatment with an IL-1 receptor antagonist reduced MC proliferation and glomerular hypercellularity,[18] so we used IL-1β as a pro-inflammatory stimulant for cultured MCs. Because PDGF-BB is a potent inducer of MC proliferation and is reported to be increased in patients with various forms of glomerulonephritis,[19] we used PDGF-BB, in addition to IL-1β, to assess cell proliferative activity. The effect of C/EBP-δ silencing should also be studied in more potently induced MC proliferation.

In C/EBP-δ siRNA-transfected MCs, *IL-6* induction by IL-1β was significantly lower than that in NS siRNA-transfected MCs (Fig. 4c). This result indicates a key role for the transcription factor C/EBP-δ in *IL-6* mRNA induction in MCs. It is of interest, however, that the peak level of *IL-6* mRNA induction preceded that of *C/EBP-δ*, and this suggests that additional transcription factors such as nuclear factor (NF)-κB may be functional in the induction of *IL-6* mRNA. Recently, heterodimer formation between C/EBP-δ and the NF-κB p65 in mesangial cells has been reported to be important in *IL-6* gene transcription,[20] and it could be predicted that silencing C/EBP-δ would also inhibit such a heterodimer formation, thereby resulting in a reduction of IL-6 induction.

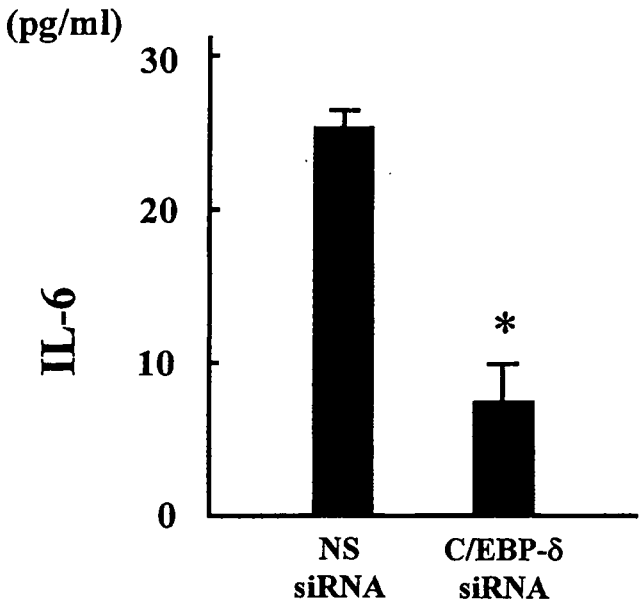

Fig. 5. Effect of CCAAT/enhancer-binding protein-δ (C/EBP-δ, knockdown on levels of interleukin (IL)-6 in the culture medium from mesangial cells. The levels of IL-6 in the culture medium from mesangial cells transfected with non-silencing short interfering RNA (NS siRNA) or C/EBP-δ siRNA after treatment with 10 ng/ml of IL-1β for 24 h were determined by enzyme-linked immunosorbent assay. Results are expressed as means ± SD; $n = 5$. $^*P < 0.01$ versus mesangial cells transfected with NS siRNA

COX-2 mRNA induction was also reduced by C/EBP-δ silencing through 24 h (Fig. 4d). The difference in *COX-2* induction between NS siRNA-transfected MCs and C/EBP-δ siRNA-transfected MCs increased after 3 h and *C/EBP-δ* mRNA reached peak levels at this time. There are consensus binding sites for C/EBP as well as for NF-κB within the *COX-2* promoter of human, rat, and mouse.[21] *COX-2* induction before 3 h seemed to mainly depend on additional transcription factors to C/EBP-δ as well as *IL-6*, while C/EBP-δ was thought to be the main transcription factor for COX-2 after 3 h. *MCP-1* mRNA expression was hardly detectable in unstimulated MCs but was markedly induced after IL-1β stimulation. Similar to the results seen for *IL-6* and *COX-2*, silencing C/EBP-δ reduced *MCP-1* mRNA levels to some extent at each time point (Fig. 4e). MCP-1 is transcribed not only by C/EBP-δ but also by NF-κB, which has been reported to rapidly activate MCP-1 within 30 min.[22,23] The suppression of *MCP-1* mRNA was strongest at 3 h, when *C/EBP-δ* reached peak levels in control MCs.

IL-6 acts as an autocrine growth factor for MCs,[24] so we evaluated the IL-6 concentration in the culture medium after treatment with IL-1β with or without silencing C/EBP-δ. Silencing C/EBP-δ reduced IL-6 release from MCs (Fig. 5). Moreover silencing C/EBP-δ reduced MC proliferation (Fig. 6). The reduction of IL-6 release from MCs may have affected the reduced MC proliferation by silencing C/EBP-δ.

In conclusion, C/EBP-δ, IL-6, COX-2, and MCP-1 were expressed in the mesangial area of glomeruli in the early stages of anti-Thy1.1 glomerulonephritis. In vitro, C/EBP-δ regulated the expression of these inflammatory genes as well as the proliferation of MCs.

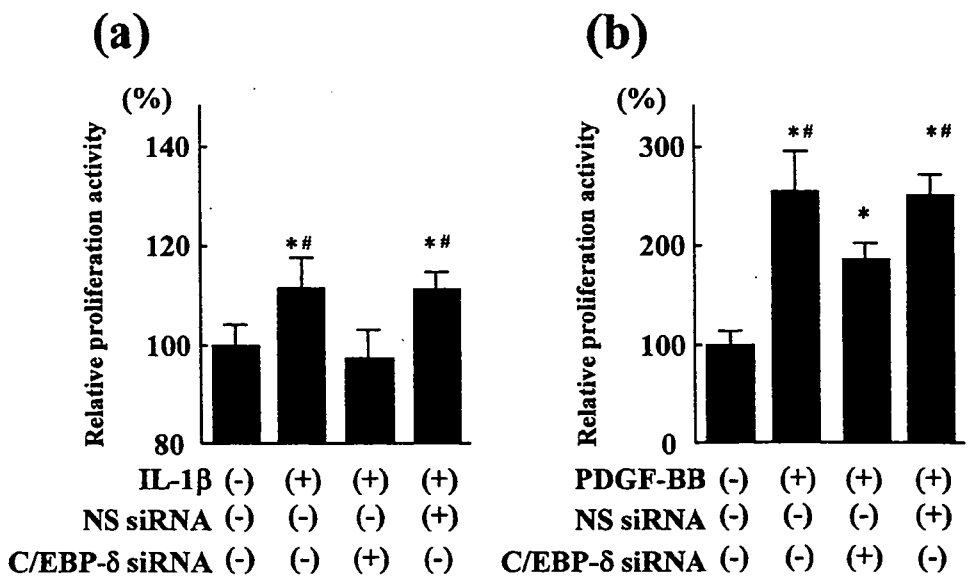

Fig. 6a,b. Effect of CCAAT/enhancer-binding protein-δ (C/EBP-δ) knockdown on mesangial cell proliferation. Cell proliferation in response to **a** interleukin (IL)-1β or **b** platelet-derived growth factor (PDGF)-BB in mesangial cells transfected with non-silencing short interfering RNA (NS siRNA) or C/EBP-δ siRNA. Cells were treated with **a** IL-1β (10 ng/ml) or **b** PDGF-BB (20 ng/ml) for 24 h and cell proliferation was evaluated by bromodeoxyuridine incorporation. Results are expressed as means ± SD; $n = 10$. $^*P < 0.01$ versus control mesangial cells. $^\#P < 0.01$ versus mesangial cells transfected with C/EBP-δ siRNA

Acknowledgments This study was supported by the Department of Biological Resources, INCS, Ehime University.

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Antioxidative effects of azelnidipine on mesangial cell proliferation induced by highly concentrated insulin

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Abstract

Insulin resistance combined with hyperinsulinemia is involved in the generation of oxidative stress. There is known to be a relationship between increased production of reactive oxygen species and the diverse pathogenic mechanisms involved in diabetic vascular complications including nephropathy. The present study found that high doses of insulin affect mesangial cell proliferation through the generation of intracellular reactive oxygen species and the activation of cell signaling pathways. We also examined whether azelnidipine, a dihydropyridine-based calcium antagonist with established antioxidant activity, has the potential to inhibit mesangial cell proliferation. Cell proliferation was increased in a dose-dependent manner by high doses of insulin (0.1–10 μM), but was inhibited by 0.1 μM azelnidipine. Phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 was found to be increased by insulin in a dose-dependent manner (0.1–10 μM). This increased phosphorylation of ERK-1/2 was inhibited by treatment with 0.1 μM azelnidipine. Intracellular oxidative stress was also increased by insulin stimulation in a dose-dependent manner (0.01–10 μM), and 0.1 μM azelnidipine was found to block intracellular reactive oxygen species production more effectively than 0.1 μM nifedipine. The NAD(P)H oxidase inhibitor, apocynin (0.01–0.1 μM), prevented insulin-induced mesangial cell proliferation. Taken together, these results suggest that azelnidipine inhibits insulin-induced mesangial cell proliferation by inhibiting the production of reactive oxygen species. Given these pharmacological characteristics, azelnidipine may have the potential to protect against the onset of diabetic nephropathy and slow its progression.

Keywords: Insulin; Mesangial cell; Extracellular signal-regulated kinase; Azelnidipine; Oxidative stress

1. Introduction

Diabetic nephropathy is the leading cause of end-stage renal failure, accounting for 35–40% of all new cases requiring dialysis therapy worldwide (Locatelli et al., 2004; Mogensen and Cooper, 2004). Patients with early-stage type 2 diabetes mellitus have insulin resistance, which in combination with compensatory hyperinsulinemia accelerates the clustering of coronary risk factors and the development of coronary artery disease (Despres et al., 1996; Pyorala et al., 1998; Reaven, 2003).

One of the causes of diabetic organopathy associated with hyperinsulinemia is oxidative stress. In diabetes mellitus, the generation of oxidative stress is increased by hyperglycemia (Baynes and Thorpe, 1999) and hyperinsulinemia (Sarafidis and Ruilope, 2006). Oxidative stress has also been reported to be a potent mediator of diabetic kidney disease because it activates protein kinase C-mitogen activated protein kinase (MAPK) (Brownlee, 2001; Ha and Kim, 1999). Extracellular signal-regulated kinase (ERK)-1/2, a MAPK, is activated in a variety of cell types by a diverse range of extracellular stimuli, and functions by connecting the nucleus to signals derived from cell membrane receptors. The ERK-1/2 pathway is activated primarily by growth factors and participates significantly in cell proliferation (Robinson and Cobb, 1997; Widmann et al., 1999).

Glomerulosclerosis associated with mesangial cell proliferation and excessive accumulation of extracellular matrix proteins is a central pathophysiological feature of diabetic nephropathy. Glomerulosclerosis ultimately leads to renal

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failure (Wesson, 1988), and therefore, the clinical suppression of mesangial overgrowth is critical to prevent renal failure.

Dihydropyridine-based calcium channel blockers are a class of drugs used widely in the treatment of hypertension and whose dihydropyridine ring can donate electrons to a propagating radical and reduce it to a non-reactive form. Azelnidipine is a dihydropyridine-based calcium channel blocker which was developed recently in Japan and which is a highly lipid soluble drug with enhanced vascular affinity (Yoram and Ahuva, 1995). We hypothesized that it may inhibit insulin-induced mesangial cell proliferation by directly decreasing oxidative stress. In the present study, we examined the antioxidative effect of azelnidipine on the insulin-induced proliferation of mesangial cells.

2. Materials and methods

2.1. Materials

Sprague–Dawley rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan). Azelnidipine was generously provided by Sankyo Co., Ltd. (Tokyo, Japan), and tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1.3-benzene disulfonate) was purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). A cell proliferation enzyme-linked immunosorbent assay (ELISA) 5-bromo-2′-deoxyuridine (BrdU) colorimetric kit (Biotrak Kit) was purchased from GE Healthcare Bio-Sciences Corporation (Little Chalfont, Buckinghamshire, UK). Phospho p44/42 MAPK (Thr202/Thr204) antibody and p44/42 MAPK antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) Western blotting reagent was purchased from GE Healthcare Bio-Sciences KK (Fairfield, CT, USA). 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) was purchased from Invitrogen (Eugene, Oregon, USA).

2.2. Cell culture and treatment

Primary mesangial cells were isolated from Sprague–Dawley rats using a sieving method (Misra, 1972) and then cultured in RPMI 1640 containing 20% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and insulin–transferrin–selenium G supplement (Invitrogen Co., Carlsbad, CA, USA). The mesangial cells were maintained at 37 °C in an atmosphere of air and 5% CO_2. Subconfluent cells were used in the experiments at passages 4 to 10. Before each experiment, the cells were serum-starved in RPMI 1640 containing 0.1% fetal calf serum. The quiescent mesangial cells were treated with azelnidipine (0.01–0.1 μM), apocynin (0.01–0.1 μM) or nifedipine (0.1 μM) (Sigma Chemical Co., St. Louis, MO, USA) for 24 h, after which they were treated with 0.001–10 μM insulin solution (Sigma) containing one of these drugs. All animal experiments were approved by our University Animal Care Committee and were carried out following the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23; revised 1985).

2.3. Cell proliferation

A colorimetric assay was performed based on the cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases in viable cells as described in our previous report (Okura et al., 1998). The cells were seeded in 96-well plates and treated with azelnidipine or apocynin for 24 h before stimulation with insulin for 24 h. A 10-μl aliquot of WST-1 solution was added to each well, followed by incubation for 2 h at 37 °C. The plate was read on an ELISA reader (Multiskan Bichromatic; Labsystems, Helsinki, Finland) at 450 nm, with a reference wavelength of 650 nm. Data were expressed as the percentage absorbance relative to untreated controls.

2.4. DNA synthesis

DNA synthesis was measured using a cell proliferation ELISA BrdU colorimetric kit following the protocol described by Yang et al. (2001). The cells were seeded in 96-well plates, treated with azelnidipine for 24 h, and stimulated with insulin for 24 h. They were then labeled with BrdU for 3 h at 37 °C and, after washing, the cells were fixed and stained with anti-BrdU antibody for 90 min at 37 °C. After three washes, the substrate, tetramethylbenzidine, was added, followed by incubation for 30 min. A blocking solution (1 M H_2SO_4) was then added, and the absorbance of the samples was measured in an ELISA reader at 450 nm with a reference wavelength of 690 nm.

2.5. Western blotting

Western blotting analysis was performed following the protocol described by Fukuoka et al. (1999). Briefly, the cells were seeded and treated with azelnidipine for 24 h, followed by treatment with insulin for 10 min. The cells were then snap-frozen in liquid nitrogen, lysed in ice-cold lysis buffer and dissolved in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Protein content was assayed by the Bradford method. Ten μg of protein was added in each lane and resolved by SDS-PAGE, then blotted on polyvinylidene difluoride (PVDF) membranes incubated with phospho p44/42 MAPK (Thr202/Thr204) antibody or total p44/42 MAPK antibody. The membranes were subsequently washed three times and incubated in horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody. Blots were detected using ECL Western blotting reagents.

2.6. Oxidative stress

Oxidative stress was evaluated by visualizing the intracellular generation of H_2O_2. The cells were seeded in 96-well plates. After treatment with azelnidipine or nifedipine for 24 h, the cells were stimulated with insulin for 10 min, washed with PBS and then incubated in the dark for 30 min with 20 μM of DCF-DA. DCF-DA diffuses into cells and is hydrolyzed into

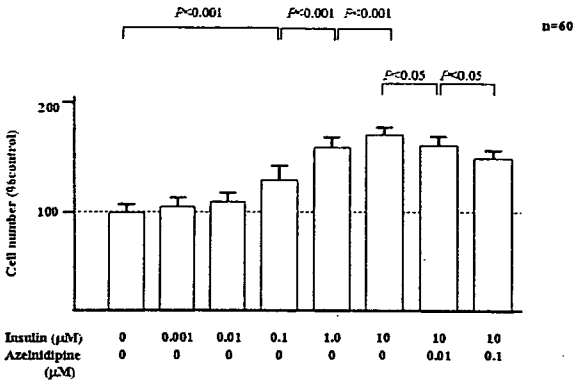

Fig. 1. Effects of insulin on mesangial cell proliferation and the inhibitory effect of azelnidipine. Cell proliferation was estimated by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1.3-benzene disulfonate). Cell proliferation was increased by treatment with insulin in a dose-dependent manner (0.1–10 μM), and was inhibited by treatment with azelnidipine. Values are expressed as mean±S.D. (n=60 for each group).

non-fluorescent 2′,7′-dichlorofluorescein (DCFH). The reactive oxygen species, including H_2O_2, superoxide and .OH, oxidized non-fluorescent intracellular DCFH into highly fluorescent dichlorofluorescein (DCF). DCF fluorescence was quantified in a multiplate fluorometer (Wallac ARVO SX; PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Furthermore, to evaluate the *in situ* production of reactive oxygen species, the cells were seeded in chamber slides and treated with azelnidipine or nifedipine for 24 h and then stimulated with insulin for 10 min. The slides were incubated with 20 μM of DCF-DA in a light-protected humidified chamber at 37 °C for 30 min. The DCF image was obtained by fluorescence microscopy (PROVIS AX; Olympus, Tokyo, Japan).

2.7. Statistical analysis

Analysis of variance with the Bonferroni–Dunn post hoc test was used to analyze differences between the 2 experimental groups. All values are expressed as mean±S.D., and statistical significance was defined as $P<0.05$.

3. Results

3.1. Effects of azelnidipine on cell proliferation and DNA synthesis induced by insulin

Insulin was found to increase mesangial cell proliferation, as estimated by WST-1, in a dose-dependent manner over the concentration range of 0.1–10 μM (Fig. 1). Doses of 0.1 and 1 μM azelnidipine inhibited by 8% and 18%, respectively, the mesangial cell proliferation induced by 10 μM insulin (Fig. 1). Insulin also caused a concurrent increase in DNA synthesis, as estimated by BrdU incorporation, with this effect being dose-dependent at concentrations above 0.1 μM. Furthermore, this effect with 10 μM insulin was attenuated by 32% under 0.1 μM of azelnidipine (Fig. 2).

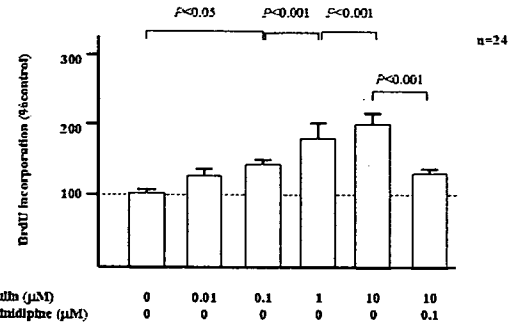

Fig. 2. Effects of insulin on DNA synthesis and the inhibitory effect of azelnidipine. DNA synthesis estimated by BrdU incorporation was increased by insulin treatment in a dose-dependent manner, and 0.1 μM of azelnidipine treatment inhibited this increment. Values are expressed as mean±S.D. (n=24 for each group).

3.2. Effects of azelnidipine on p44/42 MAPK phosphorylation induced by insulin

Since p44/42 MAPK activation is a key step in the proliferation of a variety of cells, we assessed the activation status of p44/42 MAPK on insulin-treated mesangial cells using a phosphor-specific antibody of p44/42 MAPK that reacted only with the phosphorylated (Thr202 and Tyr204) and activated forms of p44/42 MAPK. We then examined p44/42 MAPK activity after 10 min stimulation with insulin over the concentration range of 0.001–10 μM. P44/42 MAPK phosphorylation was found to increase gradually in proportion to the concentration of insulin, and increased significantly at concentrations above 0.1 μM compared to control levels (Fig. 3). We then examined the effects of azelnidipine on p44/42 MAPK phosphorylation induced by 10 μM of insulin. Azelnidipine (0.1 μM) was found to block p44/42 MAPK phosphorylation, maintaining it at almost non-stimulated control level, but did not affect total p44/42 MAPK expression (Fig. 3).

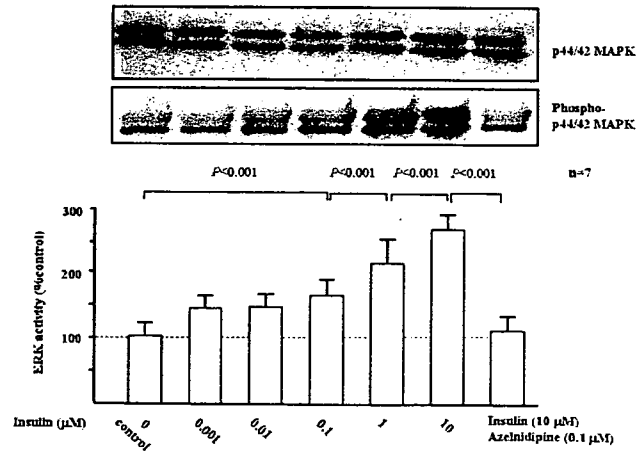

Fig. 3. Effect of insulin on ERK-1/2 phosphorylation and the inhibitory effect of the calcium channel blocker azelnidipine in mesangial cells. ERK-1/2 phosphorylation was increased by insulin in a dose-dependent manner (0.01–10 μM). Azelnidipine (0.1 μM) attenuated this effect, almost returning the samples to control values. Values are expressed as mean±S.D. (n=7 for each group).

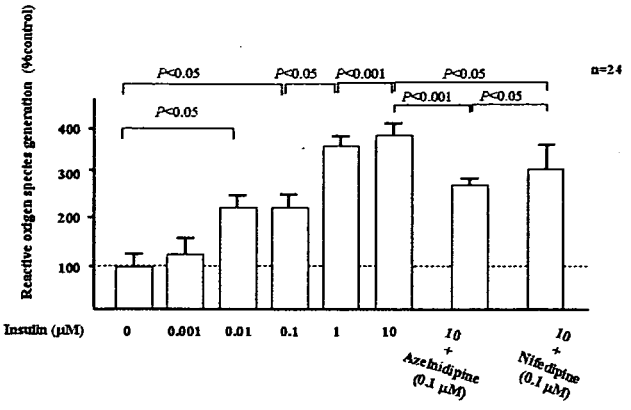

Fig. 4. Effect of insulin on the induction of reactive oxygen species and the inhibitory effect of the calcium channel blocker azelnidipine in mesangial cells. Intracellular oxidative stress was detected with DCF-DA. The production of reactive oxygen species was increased by insulin stimulation in a dose-dependent manner. Treatment with 0.1 μM of azelnidipine or 0.1 μM of nifedipine for 24 h inhibited the induction of DCF fluorescence produced by 10 μM of insulin in mesangial cells, compared with untreated controls. Values are expressed as mean±S.D. (n=24 for each group).

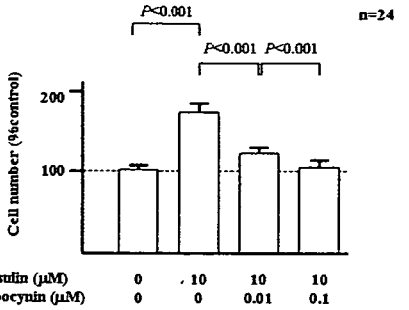

Fig. 6. The inhibitory effects of apocynin on cell proliferation induced by insulin. Cell proliferation was examined by WST-1 and was found to increase under insulin treatment; this increase was inhibited treatment in a dose-dependent manner by apocynin (0.01–0.1 μM). Values are expressed as mean±S.D. (n=24 for each group).

3.3. Effect of azelnidipine on reactive oxygen species induced by insulin

In order to examine whether insulin affects the production of reactive oxygen species, mesangial cells were incubated with 0.001–10 μM insulin for 10 min. Increasing concentrations (0.01–10 μM) of insulin were shown to produce more reactive oxygen species in a dose-dependent manner, as estimated by the fluorescence levels of DCF (Fig. 4). We next examined whether the calcium channel blockers azelnidipine and nifedipine inhibited the production of reactive oxygen species following insulin stimulation. The cells were incubated with 0.1 μM azelnidipine or 0.1 μM nifedipine for 24 h, and then with 10 μM of insulin for 10 min, before incubation with DCF-DA. Treatment with either azelnidipine or nifedipine reduced reactive oxygen species levels by 22% and 11%, respectively. The antioxidant effect of azelnidipine was significantly greater than that of nifedipine (P<0.05) (Figs. 4 and 5).

3.4. Effects of apocynin on mesangial proliferation

In order to assess the effect of the reactive oxygen species blocker on insulin-induced mesangial cell proliferation, mesangial cells were pre-treated with the antioxidant apocynin, and then stimulated with 10 μM insulin for 10 min. As shown in Fig. 6, apocynin (0.01–0.1 μM) prevented insulin-induced mesangial cell proliferation in a dose-dependent manner.

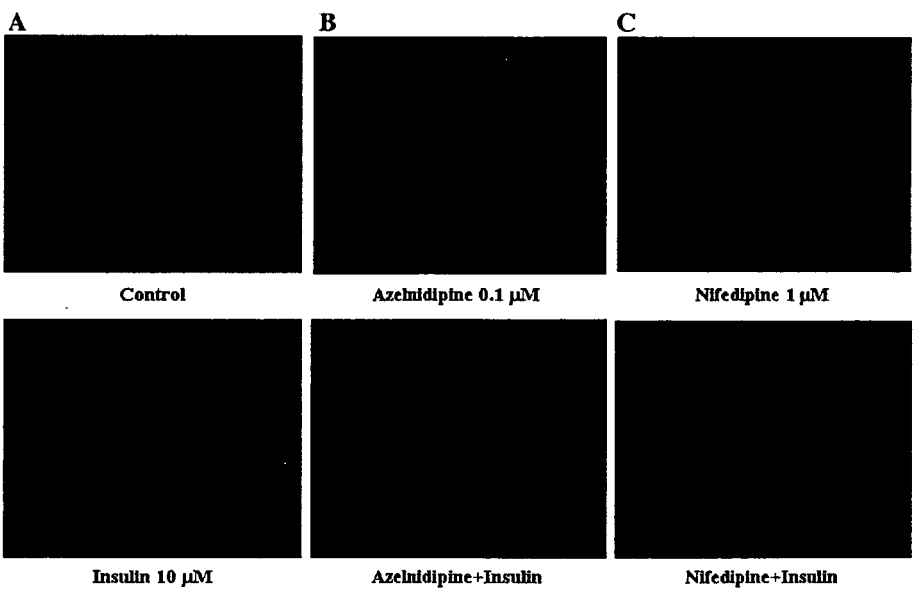

Fig. 5. *In situ* labeling of the production of reactive oxygen species induced by insulin and the inhibitory effect of the calcium channel blockers, azelnidipine and nifedipine. Representative fluorescent images of mesangial cells in the presence of DCF-DA for 30 min after stimulation with 10 μM insulin for 10 min. Insulin stimulation increased the level of intracellular reactive oxygen species, which was then decreased by azelnidipine or nifedipine treatment; azelnidipine was found to be more effective than nifedipine.

4. Discussion

The present study found that highly concentrated insulin stimulates the production of reactive oxygen species, and activates p44/42 MAPK (ERK-1/2) and cell proliferation in mesangial cells. We also demonstrated that azelnidipine, a calcium channel blocker, inhibits the production of insulin-stimulated reactive oxygen species more effectively than nifedipine.

In the present study, doses of more than 0.1 μM insulin induced mesangial cell proliferation. Under normal fasting conditions, the physiological concentration of insulin in the blood is 0.001 μM or less. On the other hand, postprandial insulin concentrations found in patients with insulin resistance is between 0.01 and 0.1 μM, the levels which were adopted for the present study. It is well known that insulin administered at the appropriate concentrations stimulates nitric oxide (NO) production in endothelial cells (Baron, 1994). NO functions not only as a potent vasodilator but also as an antiatherogenic factor by inhibiting platelet adhesion and aggregation (Cockcroft, 2005). However, in the present study, doses greater than 0.01 μM insulin increased the production of intracellular reactive oxygen species in mesangial cells, indicating that high concentrations of insulin are harmful to the cells.

Mahadev et al. (2004) report the potential role of Nox 4, a homologue of gp91phox and a subunit of NAD(P)H oxidase complex, in the generation of reactive oxygen species due to stimulation with 0.1 μM insulin in 3T3-L1 preadipocytes. We also found that apocynin, a NAD(P)H oxidase inhibitor, prevents the mesangial cell proliferation induced by highly concentrated insulin. Apocynin is a methoxy-substituted catechol obtained from the medicinal herb *Picroria kurroa*. It inhibits NAD(P)H oxidase by impeding the assembly of p47phox and p67phox subunits within the membrane NAD(P)H oxidase complex (Meyer and Schmitt, 2000). The present results suggest that highly concentrated insulin induces NAD(P)H oxidase and increases the production of intracellular superoxide. However, the present study did not examine the mechanism of NAD(P)H oxidase activation by highly concentrated insulin in mesangial cells. Further study is needed to clarify this issue.

NAD(P)H oxidase activation leads in turn to the activation of ERK-1/2 (Goldstein et al., 2005). Lin et al. (2006) report that NAD(P)H oxidase activation induced Ras phosphorylation, which acts as a crucial regulator in the transmission of ERK-1/2 signaling by high glucose and advances glycation products in mesangial cells. We also confirmed that doses greater than 0.01 μM of insulin activate ERK-1/2 in mesangial cells through NAD(P)H activation. However, we cannot exclude the possibility that insulin activation of ERK-1/2 is directly or indirectly regulated by other membrane-bound signaling molecules.

Recently, several studies have reported that azelnidipine reduces the intracellular production of reactive oxygen species. Matsui et al. (2005) demonstrated that azelnidipine inhibited the generation of angiotensin II-induced reactive oxygen species in human adult skin microvascular endothelial cells. Moreover, azelnidipine reduced the intensity of 1,1′-diphenyl-2-picrylhydrazyl (DPPH) free radicals as determined by an electron spin resonance (ESR) spectrometer, and inhibited superoxide and hydroxyl radical-scavenging activity measured by an ESR assay using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap (Naito et al., 2006). Although the mechanism by which azelnidipine acts and scavenges radicals is not fully understood, it is generally accepted that oxidation of the dihydropyridine ring can donate electrons to the radicals, reducing them to a non-reactive form (Cominacini et al., 2003).

In the present study, we showed that azelnidipine blocked the production of intracellular reactive oxygen species more effectively than nifedipine. We attribute this difference in the antioxidative effect to variations in the chemical structure of the two drugs. The reason for the stronger antioxidative action of azelnidipine may be due to its higher lipid solubility and vascular membrane affinity (Yoram and Ahuva, 1995).

In conclusion, high concentrations (over 0.01 μM) of insulin induced the production of reactive oxygen species, leading to the activation of ERK-1/2 and mesangial cell proliferation. Azelnidipine, a highly lipid soluble dihydropyridine-based calcium channel blocker, preferentially blocked the insulin-induced production of reactive oxygen species, resulting in an attenuation of mesangial cell proliferation. These results suggest that azelnidipine may have the potential to protect against the onset of diabetic nephropathy and delay its progression.

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Design, statistical analysis and sample size calculation of dose response study of telmisartan and hydrochlorothiazide

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Abstract

Many patients with hypertension take some antihypertensive drugs with complementary mechanisms of action to lower their blood pressure and achieve the therapeutic goals reducing the risk of cardiovascular events. Telmisartan, angiotensin II receptor blocker, and hydrochlorothiazide, diuretic are two antihypertensive drugs that have a well-recognized clinical efficacy. Their combination is expected to be one of the most appropriate therapies for hypertensive patients. However there is no information to show the effective dose combination of two drugs for the Japanese patients with mild to moderate hypertension. Therefore, the prospective, randomized, double-blinded study was planed for showing the dose response surface of two components. The 3 by 3 factorial design was applied for this purpose and the approach for calculating sample size was proposed. This study was registered with ClinicalTrial.gov (NCT00153049).

Keywords: Factorial design; Combination therapy; Telmisartan; Hydrochlorothiazide; Dose response surface; Sample size calculation

1. Introduction

The amount of hypertensive patients is increasing globally. The concomitant diseases of these patients are also more complicated. In the Hypertension Optimal Treatment (HOT) study [1], which recruited patients with higher grades of hypertension (grades 2 and 3) and defined therapeutic goals of a diastolic blood pressure (DBP) below 80 mm Hg, a single antihypertensive therapy was successful in only 25% of patients. Therefore, some combination therapies for hypertension are being used recently to control blood pressure of patients or to reduce the adverse events of high dose single therapy. Combination therapies are able to be administrated into hypertensive patients even if both mono components have been approved. In addition, some guidelines of treatment for hypertensive patients recommend combination therapies [2–4]. However the appropriate doses for combination therapy are not shown at present. We

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planned to investigate the appropriate doses of combination therapy for telmisartan and hydrochlorothiazide (HCTZ) for patients with hypertension. Telmisartan is an angiotensin II receptor blocker (ARB). ARBs provide the multiple actions against many of the risk factors for renal disease or cardiovascular disease [5,6].

The factorial design is recommended to show the dose response relationship of combination therapy [7,8]. However the 2 by 2 factorial design is not sufficient to show the dose response surface of the combination therapy since only two groups (0 and a certain dose) can be set. As the dose groups of both components have to be added, the total sample size automatically increases. Therefore, some studies which include combination therapy did not show the dose response surface [9–14]. The dose response relationship of a drug was evaluated in patients who took a fixed dose of the other drug in those studies.

On the other hand, the 4 by 4 factorial study was conducted to show the dose response surface for the combination of Irbesartan, angiotensin II receptor blocker and HCTZ. And the excellent dose response surface was shown in this study [15]. That surface was very informative to determine the appropriate dose for each patient. As for telmisartan, the 4 by 5 factorial study was conducted in the United States (US) [16]. The results of this study showed the dose response surface of telmisartan and HCTZ for mild to moderate hypertensive patients. Therefore, our study could be planed using these information. The 3 by 3 factorial design was applied to the study which showed the dose response surface for the Japanese patients with mild to moderate hypertension, as well as the safety (including the effects on serum uric acid, potassium, glucose, and lipid levels) of combination. And sample size was calculated using the US data to compare the dose response surface. Section 2 shows the design of our study. Next the statistical analysis method is shown in Section 3. Finally, we propose the method for sample size calculation in the factorial study.

2. Design

In a randomized, double-blinded, placebo-controlled factorial design study conducted in around 50 centers, the dose response surface of telmisartan and HCTZ was investigated in patients with mild to moderate hypertension. Both components have been approved as antihypertensive drug in Japan. The basic design in this study was similar to that in the US study in order to compare the dose response surface. The study had a 4-week placebo run-in period and an 8-week treatment period. The dose groups of telmisartan were determined as 0, 40 mg, and 80 mg based on the approval dose for hypertension. Meanwhile, the dose groups of HCTZ were determined as 0, 6.25 mg, and 12.5 mg (Table 1). Those are below half dose of approval since the high dose treatment of HCTZ induced severe metabolic adverse events [17].

A dose response study needs to be conducted in Japan as there is a difference of approved dose regime between Japan and the US or European countries. One of the adverse events for combination therapy is hypotension. If the patient takes an overdose of antihypertensive drugs, hypotension could occur. We have no data to assess whether telmisartan 80 mg/HCTZ 12.5 mg is an overdose for the Japanese patients.

Table 2 shows the dose groups set in the US factorial study. This study included the combination of 5 telmisartan dose groups and 4 HCTZ dose groups. Groups combined with telmisartan 160 mg or HCTZ 25 mg were not needed in the Japanese study based on the change of blood pressure, frequency or severity of adverse event, and change of laboratory parameters. Since we used the information of the US study to calculate the sample size and compare a dose response surface, the primary endpoint, measuring method of blood pressure, treatment period, and inclusion and exclusion criteria basically corresponded to those of the US study.

2.1. Primary endpoint

The change of trough supine diastolic blood pressure (SuDBP) after the 8-week treatment from the baseline value was determined as a primary endpoint as used in the US study. Blood pressure of supine position was measured in the

Table 1
Dose group of Japanese factorial study

Telmisartan (mg)/HCTZ (mg)			
	0/0	40/0	80/0
	0/6.25	40/6.25	80/6.25
	0/12.5	40/12.5	80/12.5

Table 2
Dose group of US factorial study

Telmisartan (mg)/HCTZ (mg)					
	0/0	20/0	40/0	80/0	160/0
	0/6.25	20/6.25	40/6.25	80/6.25	160/6.25
	0/12.5	20/12.5	40/12.5	80/12.5	160/12.5
	0/25.0	20/25.0	40/25.0	80/25.0	160/25.0

same arm of individual patients using the standard mercury sphygmomanometer at each time. This method is less variable than other measuring by some equipment. All blood pressure measurements were made using WHO criteria [18] to the nearest 2 mm Hg. For diastolic pressure, phase V (disappearance of Korotkoff sounds) was used. Three measurements were taken at 1 to 2 min intervals after the patient was asked to stand for 2 min and one standing measurement was taken. The mean value of these three measurements was applied to calculate the change.

2.2. Patient

Male and female patients aged 20 to 80 years with mild to moderate essential hypertension were randomized into 9 groups in our study. Patients who showed a mean value of SuDBP during the run-in period of more than 94 mm Hg and less than 115 mm Hg were included into the study. Mean SuDBP could not vary by >10 mm Hg over during the last 2 weeks of the 4-week placebo run-in period. Additionally, mean supine systolic blood pressure (SuSBP) had to be between 140 and 200 mm Hg at the randomization.

Patients were excluded from the study if they violated the inclusion criteria or met the exclusion criteria. These criteria were set to show the fair treatment effect for patients with hypertension and minimize the risk of adverse events and the worse for patient's conditions. These criteria in our study were also set similarly to the US study. The consistency of them was crucial to make the comparability of treatment effect between two studies.

All patients gave written consent to participate in the study, which was approved by an Institutional Review Board at each center.

2.3. Randomization

This study had the 9 groups to investigate the dose response surface after the 4-week placebo run-in period. The number of patients in each group was not large. Therefore, the patient background and baseline characteristics which affect blood pressure were balanced by the assignment of the allocation center. The allocation center was located out of the sponsor.

The allocation center randomized a newly registered patient to each treatment regimen so that the treatment regimens were equally distributed in both each study site and all study sites among 9 groups throughout the study. Patients were registered to the allocation center twice, once for registration of enrolment and the other for allocation. All patients who signed the informed consent form were registered immediately after the first visit of this study. The investigator filled in a registration form after the informed consent was given and sent it out to the allocation center. The registration form included the inclusion and exclusion criteria in this study. After receiving the registration form, the allocation center sent the registration report to the investigator. The investigator confirmed that a patient satisfied the criteria and sent the s registration form to the allocation center again after the 4-week placebo run-in period. The allocation center allocated the patient so that the randomized patients were distributed almost equally among 9 groups and determined the medication number. The registration report including the medication number was sent to the investigator. The randomization of patient was conducted based on the Zelen rule [19].

3. Statistical analysis

The statistical analyses were done from the intention to treat principle. Full analysis dataset (FAS) which was generally explained in the ICH E9 guideline [20] was defined as the analysis dataset for efficacy and the following procedures was analyzed.

3.1. Generation of the model for the primary endpoint

The following model was basically fitted into data provided from the study.

$$y_i = \alpha_0 + \alpha_1 \mathrm{Dose_T} + \alpha_2 \mathrm{Dose_{HCTZ}} + \alpha_3 \mathrm{Dose_T^2} + \alpha_4 \mathrm{Dose_{HCTZ}^2} + \alpha_5 \mathrm{Dose_T}{*}\mathrm{Dose_{HCTZ}} + \varepsilon_i$$

where

$\mathrm{Dose_T}$: Magnitude of the telmisartan dosage (0, 40, and 80 mg)
$\mathrm{Dose_{HCTZ}}$: Magnitude of HCTZ dosage (0, 6.25, and 12.5 mg)
y : Change of SuDBP
ε_i : Error term due to model specifications.

Each coefficient was tested at a 5% level. The null hypotheses and the alternative hypotheses were:

$\mathbf{H_0}$. Coefficient=0 v.s.

$\mathbf{H_1}$. Coefficient $\neq 0$

However the main effect (first order) was be included in the model if the coefficients of interaction or second order remain.

3.2. Comparison of terms in models

Terms in the model constructed by the Japanese study were compared with those in the US model (see below). By assessing whether the same terms were included in both models, we were able to conclude that dose response surface in the Japanese population was similar to that in the US population if the same terms were included in the two models.

US model:

$$y_i = \alpha_0' + \alpha_1' \mathrm{Dose_T} + \alpha_2' \mathrm{Dose_{HCTZ}} + \alpha_3' \mathrm{Dose_T^2} + \varepsilon_i$$

where

$\mathrm{Dose_T}$: Magnitude of the telmisartan dosage (0, 20, 40, 80 and 160 mg)
$\mathrm{Dose_{HCTZ}}$: Magnitude of HCTZ dosage (0, 6.25, 12.5 and 25 mg)
y : Change of SuDBP
ε_i : Error term due to model specifications.

4. Sample size

Two types of simulation were done to calculate sample size for this study using data of the US study. The mean value and variance of each group in US study are shown in Table 3. One is the parametric method (Section 4.1) and the

Table 3
Mean and standard deviation of trough supine diastolic blood pressure

		Telmisartan		
		0 mg	40 mg	80 mg
HCTZ	0 mg	n=73	n=75	n=77
		−3.8 (7.6)	−10.7 (8.4)	−11.5 (8.9)
	12.5 mg	n=73	n=70	n=73
		−7.3 (7.4)	−12.6 (9.4)	−14.9 (7.2)

mean (standard deviation): mm Hg.

other the non-parametric method (Section 4.2). The sample size was determined to be 51 patients per group, 459 patients in total from the results of two types of simulation.

4.1. Assumption of normal distribution

We assumed SuDBP in each group was distributed normally with a mean and a variance of each group in the US study. The data of size N were independently sampled from the normal distribution with the mean and variance in each group of the US study. A fitted multiple regression model was constructed from these sampled data. These samplings were replicated 1000 times using the bootstrap method [21]. A model was constructed at each replication. The number of models which simultaneously included dose effect of telmisartan and HCTZ was counted. The number of sampled data was determined as an appropriate sample size with 80% power when the sum of these models was over 800. When N equals to 49, 802 out of 1000 models included two dose effects.

4.2. Application of empirical distribution

Around 70 patients were included in each group in the US study. These data were assumed as true population. We therefore used individual data in the US study to calculate the sample size of the Japanese study. The data of size N were independently extracted from each group in the US study. A fitted multiple regression model was constructed from these sampled data. These samplings were replicated 1000 times using the bootstrap method. A model was constructed at each replication. The number of models which simultaneously included dose effect of telmisartan and HCTZ was counted. The number of sampled data was determined as an appropriate sample size with 80% power when the sum of these models was over 800. When N equals to 51, 816 out of 1000 models included two dose effects.

In case of the parametric method, the sample size was 49. Meanwhile, the sample size was 51 when assuming the empirical distribution. Therefore, the sample size was conservatively determined to be 51 per group.

5. Discussion

Hypertensive patients are commonly treated combination therapy of antihypertensive drugs. However there is no information for the appropriate combined dose of each component. The dose administrated into patients is adjusted based on the management for practical blood pressure of each patient in the case of the combination therapy. The results of mono-therapy are also being used. But the combination therapy may show the multiplicative effect. Therefore, the factorial design was proposed as an appropriate method to evaluate the dose response relationship for combination therapy [22]. The n by m factorial design must be applied to show the dose response surface since both components have more than two dose groups. Increasing the groups dictates the increase of sample size as well. For the case where detail information for dose response relationship is investigated, then the sample size must be large. We could determine the investigational dose groups and decrease our sample size by appropriately selecting dose groups using the information of the US study. From a regulatory point of view, the information of dose response relationship is addressed to be evaluated in the target population [23,24]. And a dose response study often seems to be an ideal bridging study to show the similarity between two regions in the ICH E5 guideline [25]. In our case, to investigate the dose response relationship of the Japanese population using the same primary endpoint and study design was considered to be appropriate for comparing with those of the US population.

In addition, we can theoretically investigate the dose response relationship of three components by the factorial design. However determining the appropriate dose combination for hypertensive patients would be much more complicated. Dose adjustment of all three components must be carefully monitored when more than two antihypertensive drugs are administrated into patients. We do not recommend planning these kinds of studies.

The dose response surface of the combination therapy cannot be easily estimated prior to starting the study since we do not know if the middle dose of Drug A plus the high dose of Drug B is more effective than the high dose of Drug A plus the middle dose of Drug B and vice versa. And multiplicity is needed to be adjusted as several pairwise comparisons are conducted. Therefore, the calculation of sample size was difficult at the planning of the study although the pre-definition for sample size is absolutely important in the clinical study. We proposed the approach to calculate the sample size based on simulations. Fortunately, we had the factorial data in the US study and we could apply two

proposed approaches. Even if the case where factorial studies are not done, we could determine the mean value and variance by searching previous data or published articles for information. The sample size could be calculated by applying the proposed method in Section 4.1 by using the mean value and variance in each dose group estimated from the survey.

The fact that the primary endpoint, measuring methods, inclusion and exclusion criteria of patients, and study period in our study corresponded to those in the US study used for sample size calculation leads us to the expected results in our own study.

In conclusion, this study with the high power to show the dose response surface of the combination therapy was informative for determining the appropriate doses of two drugs.

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