therapeutic strategy for halting the progression of renal injury in IgAN patients.

In this study, plasma levels of ADMA were significantly higher in IgAN patients than in age-, sex- and mean eGFR-matched healthy volunteers (0.53 ± 0.14 vs. 0.43 ± 0.08 μM). The present finding has extended the previous observations showing that ADMA levels are increased even in stage 1 CKD patients [10, 11]. Proteins in the urine are reabsorbed by proximal tubular cells, which could stimulate protein turnover in these cell types. Furthermore, it has been known that ADMA generation is increased under the condition of high protein turnover [26]. Therefore, proteinuria may enhance ADMA production by tubular cells via increased protein turnover [2, 27]. This is one possible reason why plasma ADMA levels are increased even in mild to moderate CKD patients [11, 28], including our cases. Given that ADMA could elicit proteinuria as described above, proteinuria and ADMA may be correlated with each other and in the same pathogenetic pathway to renal damage.

In this study, 24 h-Ccr was not correlated with plasma ADMA levels. Further, it has been reported that only 5% of ADMA is recovered in the urine, when it is injected intravenously [29]. In addition, ADMA is extensively metabolized by DDAH rather than excreted into the urine [2, 30]. Therefore, it is unlikely that renal dysfunction could affect the ADMA clearance by the kidney. Proteinuria stimulates reactive oxygen species generation of tubular cells [31]. Oxidative stress could inactivate DDAH activity [32]. DDAH activity may be impaired in patients with CKD [2, 33], which could also explain the elevated plasma ADMA levels in these patients.

Study Limitations

First, this was a cross-sectional and retrospective study. Therefore, it did not elucidate the causal relationships among plasma ADMA levels, proteinuria, eGFR, renal tissue damage and disease progression. So, we did not know whether ADMA was mechanistically related to renal injury in IgAN patients. Second, we cannot exclude the possibility that use of RAS inhibitors could confound our present results, because plasma ADMA levels at baseline were higher in the patients who received RAS inhibitors than those without them ($p < 0.05$, 0.61 ± 0.15 vs. 0.51 ± 0.13 μM). The fact that the former suffered from more advanced renal disease compared with the latter (eGFR: 70.3 ± 36.4 vs. 89.7 ± 26.2 ml/min/1.73 m^2; proteinuria: 1.6 ± 1.2 vs. 0.78 ± 0.9 g/day) may explain the results. In this study, only 23/40 (57.5%) of the patients were on RAS inhibitors during the follow-up periods. Some patients were normotensive, had less proteinuria and/or wished for a baby. These are the reasons why not all the patients received RAS inhibitors. Third, some patients received conventional therapies such as steroid and tonsillectomy. This may also confound the relationship between ADMA and the decline in eGFR values in our subjects. Fourth, unfortunately, we cannot evaluate here the relationship between ADMA and annual reduction rates of 24 h-Ccr due to lack of urine samples. Fifth, eGFR slope is not a strong marker of renal risk, particularly in the eGFR range explored in the present study. Lastly, in the present study, plasma levels of symmetric dimethylarginine (SDMA), a structural isomer of ADMA, were significantly higher in IgAN patients than in healthy volunteers (0.41 ± 0.01 vs. 0.53 ± 0.03 μM, $p < 0.05$). Moreover, SDMA levels were associated with 24 h-Ccr, the severity of renal damage, and the 4-year changes in eGFR values (data not shown). Several clinical papers indicate that SDMA is associated with vascular damage and/or mortality [34, 35]. SDMA stimulates reactive oxygen species production of monocytes by acting on Ca^{2+} entry via store-operated calcium influx [36]. In addition, Kielstein et al. [37] showed that SDMA was a fast and early marker of decline in GFR in living-related kidney donors. These observations may support the possible role of SDMA in the progression of IgAN. Further study is needed to elucidate the mechanism by which SDMA was involved in renal injury in our subjects.

In conclusion, the present observations suggest that plasma ADMA levels could be a novel marker for renal tissue damage and a predictor of disease progression in young IgAN patients.

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Disclosure Statement

No conflict of interest to declare.

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Nifedipine inhibits advanced glycation end products (AGEs) and their receptor (RAGE) interaction-mediated proximal tubular cell injury via peroxisome proliferator-activated receptor-gamma activation

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ABSTRACT

There is a growing body of evidence that advanced glycation end products (AGEs) and their receptor (RAGE) interaction evokes oxidative stress generation and subsequently elicits inflammatory and fibrogenic reactions, thereby contributing to the development and progression of diabetic nephropathy. We have previously found that nifedipine, a calcium-channel blocker (CCB), inhibits the AGE-induced mesangial cell damage *in vitro*. However, effects of nifedipine on proximal tubular cell injury remain unknown. We examined here whether and how nifedipine blocked the AGE-induced tubular cell damage. Nifedipine, but not amlodipine, a control CCB, inhibited the AGE-induced up-regulation of RAGE mRNA levels in tubular cells, which was prevented by the simultaneous treatment of GW9662, an inhibitor of peroxisome proliferator-activated receptor-γ (PPARγ). GW9662 treatment alone was found to increase RAGE mRNA levels in tubular cells. Further, nifedipine inhibited the AGE-induced reactive oxygen species generation, NF-κB activation and increases in intercellular adhesion molecule-1 and transforming growth factor-beta gene expression in tubular cells, all of which were blocked by GW9662. Our present study provides a unique beneficial aspect of nifedipine on diabetic nephropathy; it could work as an anti-oxidative and anti-inflammatory agent against AGEs in tubular cells by suppressing RAGE expression via PPARγ activation.

1. Introduction

Diabetic nephropathy is a leading cause of end-stage renal disease, and accounts for disabilities and high mortality rates in patients with diabetes [1]. Diabetic nephropathy is characterized by functional and structural changes in the glomerulus, such as glomerular hyperfiltration, thickening of glomerular basement membranes and an expansion of extracellular matrix in mesangial areas [1]. However, it has recently been recognized that changes within tubulointerstitium are more important than glomerulopathy in terms of renal prognosis in diabetic nephropathy [2,3].

Glucose can react non-enzymatically with the amino groups of proteins to form reversible Schiff bases and then Amadori products [4,5]. These early glycation products undergo further complex reactions and rearrangements to become irreversibly cross-linked fluorescent protein derivatives termed advanced glycation end products (AGEs) [4,5]. The formation and accumulation of AGEs have been known to progress at an accelerated rate under diabetes [4,5]. Recent understandings of this process have revealed that the AGEs and their receptor (RAGE) interaction plays a role in the development and progression of diabetic nephropathy [6–11]. Indeed, engagement of RAGE by AGEs evokes oxidative stress generation and subsequently elicits inflammatory and fibrogenic reactions in renal cells, thereby being involved in diabetic nephropathy [6–11]. These observations suggest that blockade of the AGE–RAGE axis is a novel therapeutic target for preventing diabetic nephropathy.

Hypertension is an independent risk factor for diabetic nephropathy, and strict blood pressure control achieves a clinically important reduction in the risk of development and progression of diabetic nephropathy [12,13]. We have previously shown that nifedipine, a dihydropyridine-based calcium-channel blocker (CCB) widely used for the treatment of hypertension, reduces expression levels of monocyte chemoattractant protein-1 (MCP-1), a principal chemokine that mediates the recruitment of monocytes to inflammatory sites, in AGE-exposed human cultured mesangial cells [14,15]. These findings suggest that nifedipine

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could play a protective role against diabetic nephropathy partly through its anti-inflammatory properties via blockade of AGE-signaling. However, effects of nifedipine on AGE-induced proximal tubular cell injury remain unknown. In this study, we examined whether and how nifedipine blocked the AGE-elicited tubular cell damage *in vitro*.

2. Materials and methods

2.1. Materials

Nifedipine was generously gifted from Bayer-Pharma, Wuppertal, Germany. GW9662, an inhibitor of peroxisome proliferator-activated receptor-γ (PPARγ) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Bovine serum albumin (BSA) (essentially fatty acid free and essentially globulin free, lyophilized powder) was purchased from Sigma (St. Louis, MO, USA). D-Glyceraldehyde from Nakalai Tesque (Kyoto, Japan). Antibodies (Abs) directed against phospho-NF-κB p65 from Cell Signaling Technology (Boston, MA, USA).

2.2. Preparation of AGE-BSA

AGE-BSA was prepared as described previously [16]. In brief, BSA (25 mg/ml) was incubated under sterile conditions with 0.1 M glyceraldehyde in 0.2 M $NaPO_4$ buffer (pH 7.4) for 7 days. Then unincorporated sugars were removed by PD-10 column chromatography and dialysis against phosphate-buffered saline. Control non-glycated BSA was incubated in the same conditions except for the absence of reducing sugars. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan); no endotoxin was detectable. The extent of chemical modification was determined as described with 2,4,6-trinitrobenzenesulfonic acid as a difference in lysine residues of modified and unmodified protein preparations [17]. The extent of lysine modification (%) of modified BSA preparations was 65% for AGE-BSA.

2.3. Cells

Proximal tubular epithelial cells from human kidney were maintained in basal medium supplemented with 5% fetal bovine serum, 0.5 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 0.5 μg/ml epinephrine, 6.5 ng/ml triiodo-L-thyronine, 10 μg/ml transferrin, 5 μg/ml insulin, and GA-1000 according to the supplier's instructions (Clonetics Corp., San Diego, CA, USA) [18]. Cells at 3–5 passages were used for the experiments. AGE treatments were carried out in a serum-free basal medium containing 10 μg/ml transferrin and GA-1000.

2.4. Real-time reverse transcription-polymerase chain reactions (RT-PCR)

Tubular cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM nifedipine, 1 μM amlodipine or 500 nM GW9662 for 4 h. Then total RNA was extracted with RNAqueous-4PCR kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Applied Biosystems, Foster City, CA, USA) according to the supplier's recommendation. IDs of primers for human RAGE, intercellular adhesion molecule-1 (ICAM-1), transforming growth factor-β (TGF-β) and β-actin gene were Hs00153957_m1, Hs99999152_m1, Hs00171257_m1 and Hs99999903_m1, respectively.

2.5. Dihydroethidium (DHE) staining

Tubular cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM nifedipine or 500 nM GW9662 for 4 h, and then the cells were incubated with phenol red free Dulbecco's Modified Eagle Medium containing 3 μM DHE (Molecular Probes Inc., Eugene, OR, USA). After 15 min, the cells were imaged under a laser-scanning confocal microscope. Intensity of DHE staining in five different field of each sample was analyzed by microcomputer-assisted NIH image.

2.6. Immunostaining of phsopho-NF-κB p65

Tubular cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM nifedipine or 500 nM GW9662 for 4 h. Then the cells were fixed with 4% paraformaldehyde and washed with phosphate-buffered saline. The cells were stained with rabbit Abs raised against phospho-NF-κB p65. Phospho-NF-κB p65 was visualized with Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen) as described previously [19]. Nuclei were stained with diamidino-phenylindole (DAPI). The nuclear NF-κB-positive cells (purple) were imaged under a laser-scanning confocal microscope. Intensity of NF-κB staining in five different field of each sample was analyzed by microcomputer-assisted image J.

2.7. Statistics

All values were presented as means ± standard error (SEM). One-way ANOVA followed by the Scheffe *F*-test was performed for statistical comparisons; $p < 0.05$ was considered significant.

3. Results

RAGE is a signal transducing receptor for AGEs [6–11]. Indeed, engagement of RAGE with AGEs activates its down-stream signaling and subsequently evokes inflammatory and fibrogenic responses through reactive oxygen species (ROS) generation in various types of cells [6–11]. Therefore, we first examined the effect of nifedipine on RAGE gene expression in tubular cells. Nifedipine, but not amlodipine, a control CCB, decreased RAGE mRNA

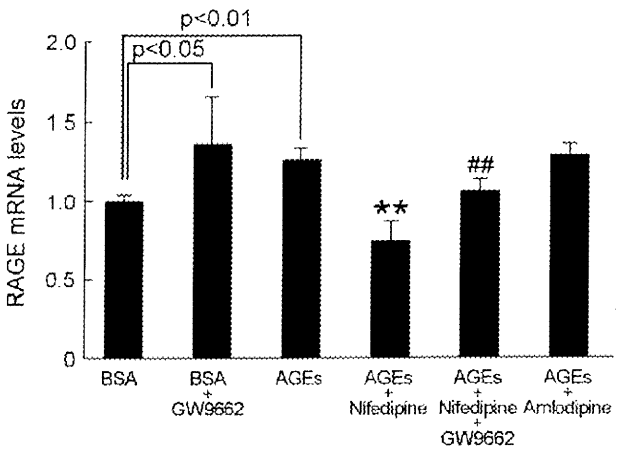

Fig. 1. Effects of nifedipine on RAGE gene expression in tubular cells. Cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM nifedipine, 1 μM amlodipine or 500 nM GW9662 for 4 h. Then total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of β-actin mRNA-derived signals and then related to the value obtained with non-glycated BSA. $^{**}p < 0.01$ compared to the value with AGEs alone. $^{\#\#}p < 0.01$ compared to the value with AGEs plus nifedipine. $N = 3$ per group.

levels in tubular cells, which was blocked by GW9662, an inhibitor of PPARγ (Fig. 1A). Further, GW9662 treatment alone was found to increase RAGE mRNA levels in tubular cells.

AGEs exert pleiotropic actions on various types of cells by inducing the generation of intracellular ROS through the interaction with cell surface receptor, RAGE [6–11]. So, we next investigated whether nifedipine could block the AGE-elicited ROS generation in tubular cells. As shown in Fig. 2, nifedipine decreased the AGE-elicited ROS generation in tubular cells, which was also prevented by the treatment with GW9662.

ROS in turn elicit transcriptional activation of NF-κB and induce a variety of inflammatory and fibrogenic gene expressions, thus being involved in diabetic nephropathy [8–11,19]. So, we next examined the effect of nifedipine on NF-κB activation in AGE-exposed tubular cells. As shown in Fig. 3, nifedipine inhibited the increase in NF-κB staining in AGE-exposed tubular cells, which was blocked by GW9662.

Further, nifedipine was found to inhibit the AGE-induced up-regulation of ICAM-1and TGF-β mRNA levels in tubular cells, which was also prevented by the treatment with GW9662 (Fig. 4A and B).

4. Discussion

In this study, we have demonstrated for the first time that nifedipine could block the AGE-induced up-regulation of ICAM-1 and TGF-β mRNA levels in human cultured proximal tubular cells by suppressing RAGE gene expression and subsequent ROS generation and NF-κB activation. In the present study, amlodipine, a control CCB, did not affect the AGE-induced RAGE gene expression in tubular cells. Further, GW9662, an inhibitor of PPARγ, blocked the effects of nifedipine on RAGE gene suppression in AGE-exposed tubular cells. These findings suggest that nifedipine could inhibit the deleterious effects of AGEs on tubular cells by reducing RAGE gene expression and may work as an anti-oxidative and anti-inflammatory agent against AGEs via PPARγ activation.

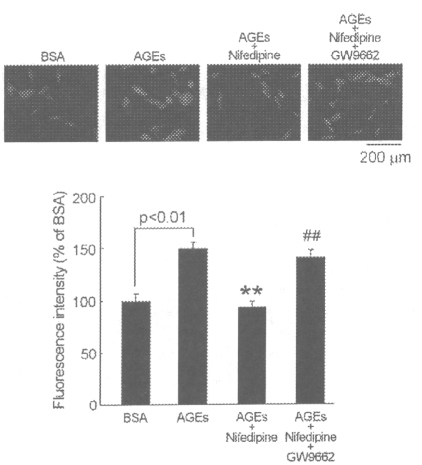

Fig. 2. Effects of nifedipine on ROS generation in tubular cells. Cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM nifedipine or 500 nM GW9662 for 4 h. Then the cells were incubated with DHE. Upper panel shows typical microphotographs of the cells. Lower panel shows quantitative data of ROS generation evaluated by fluorescent intensity. $^{**}p < 0.01$ compared to the value with AGEs alone. $^{\#\#}p < 0.01$ compared to the value with AGEs plus nifedipine. $N = 5$ per group.

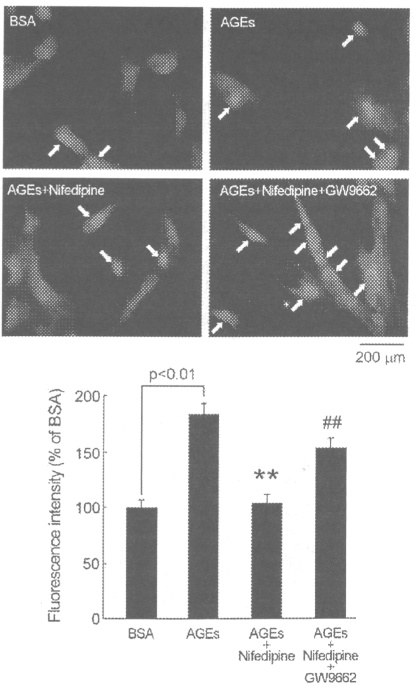

Fig. 3. Effects of nifedipine on NF-κB activation in tubular cells. Cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM nifedipine or 500 nM GW9662 for 4 h. Then intensity of NF-κB staining was measured. Upper panel shows typical microphotographs of the cells. Lower panel shows quantitative data. Arrows show NF-κB-positive nuclei. $^{**}p < 0.01$ compared to the value with AGEs alone. $^{\#\#}p < 0.01$ compared to the value with AGEs plus nifedipine. $N = 5$ per group.

Recently, PPARγ agonists have been reported to down-regulate basal as well as AGE-induced RAGE expression in endothelial cells and subsequently inhibit the AGE-induced MCP-1 and ICAM-1 expression, thus limiting the cells' susceptibility toward pro-inflammatory AGE effects [20,21]. In addition, we have recently found that nifedipine increases PPARγ transcriptional activity in mesangial cells [15]. These findings suggest that increased transcriptional activity of PPARγ by nifedipine may contribute to the reduction of RAGE gene expression in tubular cells as well, which could lead to the suppression of RAGE-down-stream pathway in these cell types. In this study, GW9662 treatment alone increased RAGE mRNA levels in tubular cells. As the case in endothelial cells, basal RAGE expression in tubular cells could be regulated by PPARγ activity.

Chronic tubulointerstitial damage in the kidney, including interstitial inflammation and fibrosis, is more important than glomerulopathy in terms of renal prognosis in diabetic nephropathy [2,3]. Further, renal ICAM-1 and TGF-β overexpressions are involved in tubulointerstitial injury [18,22–24]. These observations

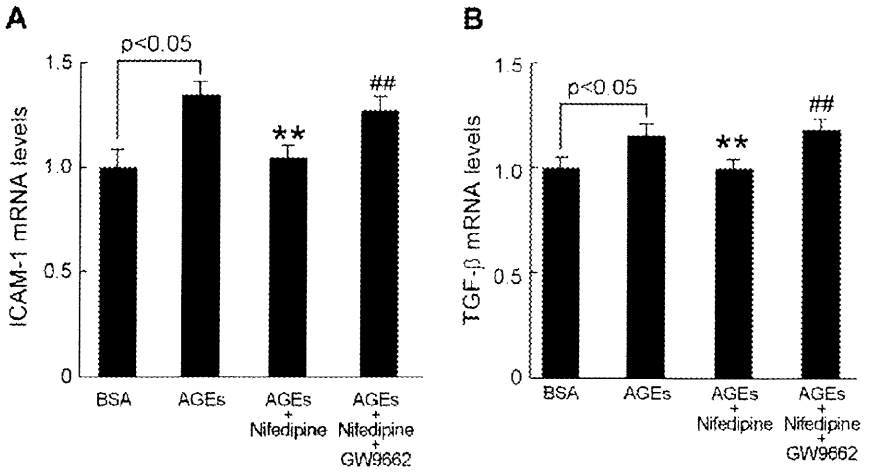

Fig. 4. Effects of nifedipine on ICAM-1 (A) and TGF-β (B) gene expressions in tubular cells. Cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM nifedipine or 500 nM GW9662 for 4 h. Then total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of β-actin mRNA-derived signals and then related to the value obtained with non-glycated BSA. $^{**}p < 0.01$ compared to the value with AGEs alone. $^{\#\#}p < 0.01$ compared to the value with AGEs plus nifedipine. $N = 3$ per group.

suggest that nifedipine may play a protective role against diabetic nephropathy through its unique PPARγ-modulating properties. With this regard, in J-MIND study, an open-label, randomized prospective trial to compare the effect of long-acting nifedipine or enalapril on onset and progression of nephropathy in hypertensive patients with type 2 diabetes [25], long-acting nifedipine and enalapril had a similar protective effect on the development and progression of diabetic nephropathy.

In vitro-modified AGEs were prepared by incubating BSA with glyceraldehyde for 1 week; this process produces relatively highly-modified proteins in comparison to those *in vivo*. However, it is unlikely that extensively-modified, unphysiologic AGEs that were formed under the *in vitro*-conditions may exert non-specific and toxic effects on tubular cell damage for the following reasons: (1) we have previously found that immunological epitope of glyceraldehyde-modified AGEs is actually present in serum of diabetic patients and that the concentration (100 μg/ml) of *in vitro*-prepared AGEs used here were comparable with those of the *in vivo* diabetic situation and (2) pre-incubation of AGE-containing media with 1 mg/ml polymyxin B, an inhibitor of endotoxin for 30 min did not affect the AGE-induced ROS generation [26–28]. Further, although the clinical plasma concentration of nifedipine is reported to 0.5 μM [15,29], localized concentrations of nifedipine are likely to be above 0.5 μM because of its high affinity to plasma membranes [15,29]. So, the concentration of nifedipine having beneficial effects on tubular cells (1 μM) may be comparable to the therapeutic levels, which are achieved in the treatments of patients with hypertension.

Conflict of interest statement

None declared.

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Interstitial Foxp3-positive T cells may predict renal survival in patients with myeroperoxidase anti-neutrophil cytoplasmic antibody-associated glomerulonephritis

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SUMMARY

1. Regulatory T cells (T_{reg}) and cytotoxic T cells (CTL) are involved in various immune diseases. However, the prognostic impact of T_{reg} and CTL in patients with myeroperoxidase anti-neutrophil cytoplasmic antibody-associated glomerulonephritis (MPO-ANCA-GN) is not well known. Therefore, in the present study, we examined the relationship between expression of forkhead box P3 (Foxp3) and T cell intracytoplasmic antigen (TIA)-1, T_{reg} and CTL markers and renal survival in patients with MPO-ANCA-GN.

2. Forty patients with MPO-ANCA-GN and 10 patients with minimal change nephrotic syndrome (MCNS) underwent physical examination, determination of blood chemistry and renal biopsy. Immunohistochemical staining for Foxp3 and TIA-1 was performed on paraffin-embedded renal sections.

3. Although almost all patients received standard immunosuppressive treatment for 6 months, seven MPO-ANCA-GN patients needed maintenance haemodialysis (HD), whereas 33 patients did not (non-HD). Both Foxp3- and TIA-1-positive cells were detected in the interstitium and glomeruli of MPO-ANCA-GN patients, whereas they were rarely detected in patients with MCNS. The total crescent rate was significantly higher in the HD group than in the non-HD group (35.9 ± 3.5 vs 65.8 ± 7.4, respectively). In the interstitium, the age-adjusted Foxp3/TIA-1 ratio was significantly higher in the non-HD group than in the HD group (0.016 ± 0.016 vs 0.004 ± 0.008, respectively; $P < 0.05$). The Foxp3/TIA-1 ratio, but not the Foxp3/CD3 ratio, remained significantly higher in the non-HD group than in the HD group even after adjustment for crescent rate. Age- and total crescent rate-adjusted renal survival rates were higher in patients with a Foxp3/TIA-1 ratio ≥ 0.06 than in patients with a Foxp3/TIA-1 ratio < 0.06 ($P = 0.02$).

4. The results of the present study suggest that T_{reg} could play a protective role against MPO-ANCA-GN and that a decreased Foxp3/TIA-1 ratio in interstitial areas may predict future renal failure in patients with MPO-ANCA-GN.

Key words: anti-neutrophil cytoplasmic antibodies, forkhead box P3, prognosis, regulatory T cells, T cell intracytoplasmic antigen-1.

See editorial commentary on page 877

INTRODUCTION

Anti-neutrophil cytoplasmic antibodies (ANCA) are circulating antibodies directed mainly against neutrophil granules and monocyte lysosomes.[1] Anti-neutrophil cytoplasmic antibodies with specificity for myeloperoxidase (MPO), proteinase 3 (PR3) or other antigens defines the condition known as ANCA-associated vasculitis (ANCA-V).[1,2] In particular, in Japan, MPO-ANCA-V is more common than other types of ANCA-V[3] and MPO-ANCA-associated glomerulonephritis (MPO-ANCA-GN) often has a progressive clinical course that leads to renal death.[2] Therefore, clarifying predictive markers for MPO-ANCA-GN is important for the treatment of MPO-ANCA-GN. With regard to the prognosis for ANCA-GN patients, Okano *et al.* [4] have shown that global sclerosis as a histological feature is an indicator of a poor prognosis. However, Hogan *et al.* [5] have reported that lesions in renal biopsies are not predictive of renal survival, except for interstitial sclerosis. Thus, debate remains regarding the predictive factors for MPO-ANCA-GN.

Infiltration of inflammatory cells is involved in the pathogenesis of ANCA-GN.[6] Of the inflammatory cells, regulatory T cells (T_{reg}) have been reported to be prognostic markers for several immune diseases, including rheumatoid arthritis (RA)[7] and renal allograft rejection.[8] However, whether infiltrating T_{reg} have a role in the renal prognosis of ANCA-GN is not known. Forkhead box P3 (Foxp3), a member of the forkhead family of transcription factors, is involved in the development and differentiation of T_{reg}. [9,10] Therefore, in the present study, we examined the relationship between expression of Foxp3

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and cytotoxic T cell intracytoplasmic antigen (TIA)-1, T_{reg} and cytotoxic T cells (CTL) markers, and renal survival in patients with MPO-ANCA-GN.

METHODS

Patients

From 1994 to 2005, 61 patients with MPO-ANCA-GN underwent renal biopsy in Kurume University Hospital. Any patients who died as a result of infection, gastrointestinal bleeding or alveolar haemorrhage within 2 months of the biopsy were excluded from the present study. Thus, a total of 40 patients was enrolled in the study. Ten patients with minimal change nephrotic syndrome (MCNS) without any complications were also enrolled as controls. All patients were diagnosed with microscopic polyangitis according to the Chapel Hill Nomenclature (http://www.arupconsult.com/images/NomenclatureandDefintiionsofVasculitisChapelHill.pdf, accessed 9 July 2009) and all patients were found to be MPO-ANCA positive. Medical records were reviewed at the time of hospital admission. The following parameters were determined: serum creatinine, estimated glomerular filtration rate (eGFR), ANCA titre, proteinuria, Birmingham vasculitis activity score (BVAS), blood pressure (BP) and haemoglobin. The eGFR for men was calculated as follows:

$$\text{eGFR (mL/min per 1.73 m}^2) = 0.741 \times 175 \times \text{Age}^{-0.203} \times \text{Cr}^{-1.154}$$

For women, we used the calculation as follows:

$$\text{eGFR (mL/min per 1.73 m}^2) = 0.741 \times 175 \times \text{Age}^{-0.203} \times \text{Cr}^{-1.154} \times 0.742.$$

All participants provided written informed consent to undergo renal biopsy and to participate in the study. However, the Ethics Committee of Kurume University Hospital was not convened until 2002, so the study did not have ethics approval prior to 2002 (although patients did provide consent to participate in the study).

Histopathological findings

For light microscopy, renal specimens were bouin fixed, paraffin embedded, sectioned and stained with haematoxylin–eosin, periodic acid-Schiff (PAS), periodic acid-methenamine (PAM) silver and azocarmin-aniline blue. Glomerular and interstitial inflammatory cell infiltration was analysed as described previousl.[11] Global sclerosis and the total crescent formation rate were evaluated and crescent rate was subdivided into two types: cellular and total crescent rate.[11] Tubulointerstitial damage was scored as follows, as described previously:[12] 0, none; 1, mild (0–25%); 2, moderate (25–50%); 3, severe (> 50%).

Immunohistochemical staining for CD3, TIA-1 and Foxp3 was performed on 3–4 μm sections.[13] Deparaffinized sections were then incubated with the primary antibodies murine anti-human CD3 monoclonal antibody (mAb; 1 : 100 dilution; Novocastra, Newcastle, UK), TIA-1 mAb (1 : 200 dilution; Immunotech, Marseille, France) and Foxp3 mAb (1 : 40 dilution; Bioscience, San Diego, CA, USA).[13] After sections had been washed with phosphate-buffered saline (PBS), they were incubated with horseradish peroxidase (HRP) polymer (Dako, Tokyo, Japan). Staining was visualized by incubation with diaminobenzidine (DAB; Dako). The number of all positively stained cells in the cortical glomeruli and interstitium was counted at a magnification of ×400 and by an observer blinded to the treatment group. Results are expressed as mean ± SEM.

Statistical analysis

Because of the skewed distributions, a natural logarithmic (ln) transformation was performed for tubulointerstitial CD3/mm^2, TIA-1/mm^2, Foxp3/mm^2, TIA-1/CD3, Foxp3/CD3, Foxp3/glomerular cell number, Foxp3/CD3, Foxp3/TIA-1, cellular crescent rate, proteinuria, ANCA titre and BVAS. Mean values of parameters for patients requiring maintenance haemodialysis (HD group) and those not (non-HD group) were compared using analysis of covariance adjusted for age. Statistical significance was defined as $P < 0.05$. An analysis of the variables related to renal survival in ANCA-GN patients was performed using the Cox proportional risks model. Survival analyses were adjusted for age. All statistical analyses were performed with using SPSS (SPSS, Chicago, IL, USA).

RESULTS

Clinical characteristics of the patients

Although almost all patients received standard immunosuppressive treatment (starting at 1 mg/kg per day prednisolone and then tapered) for 6 months, seven ANCA-GN patients required HD due to renal death, whereas 33 patients did not (non-HD). Of the 33 patients in the non-HD group, 19 required other immune suppressants (i.e. mizoribine (n = 5) and cycrophosphamide (n = 14)). Of the seven patients in the HD group, three required other immune suppressants (i.e. cycrophosphamide (n = 2) and cyclosporin (n = 1)). There was no significant difference in therapy between the non-HD and HD groups. The clinical characteristics of non-HD and HD patients are given in Table 1. Patients who required HD were older and anaemic compared with non-HD subjects ($P < 0.05$). There were no signficant differences between the two groups in terms of ANCA titre, BVAS, BP, eGFR and CRP levels (Table 1).

Kidney immunostaining for Foxp3, TIA-1 and CD3 in patients with ANCA-GN and MCNS

In most biopsy specimens from patients with ANCA-GN, CD3-positive cells (Fig. 1a) and TIA-1-positive cells (Fig. 1b) were observed extensively in the interstitial areas of the kidney. In contrast, only a few Foxp3-positive cells (Fig. 1c) were observed in this area. Intense infiltration of CD3-positive cells (Fig. 2a) and TIA-1-positive cells (Fig. 2b) was also seen in both the crescents and glomerular capillaries in biopsy specimens from patients with ANCA-GN. Much smaller numbers of Foxp3-positive cells (Fig. 2c) were observed in the glomeruli of patients with ANCA-GN. These inflammatory cells were rarely seen in the interstitial and glomerular areas of kidneys from patients with MCNS (Table 2).

Immunohistochemistry in the non-HD and HD groups

Compared with patients in the non-HD group, age-adjusted Foxp3/CD3 and Foxp3/TIA-1 ratios were significantly lower in the

Table 1 Clinical characteristics of non-HD and HD patients

	non-HD	HD
Number (male/female)	33 (10/23)	7 (2/5)
Age (years old)	66.5 ± 8.1*	74.6 ± 4.50
Proteinuria (g/day)†	1.12 ± 1.19	0.90 ± 0.40
eGFR (mL/min)	28.9 ± 26.4	19.9 ± 27.0
ANCA titer†	460 ± 427	455 ± 266
BVAS†	19.3 ± 5.90	18.1 ± 3.8
Systolic BP (mmHg)	140 ± 21.4	147 ± 21.4
Diastolic BP (mmHg)	77.5 ± 11.7	79.7 ± 12.9
Hemoglobin (g/dL)	9.09 ± 1.56*	8.60 ± 1.79
CRP (mg/dL)	7.73 ± 5.24	7.07 ± 3.50

Data were shown as mean ± SD. *$P < 0.05$ versus HD patients.
†Log-transformed values were used.

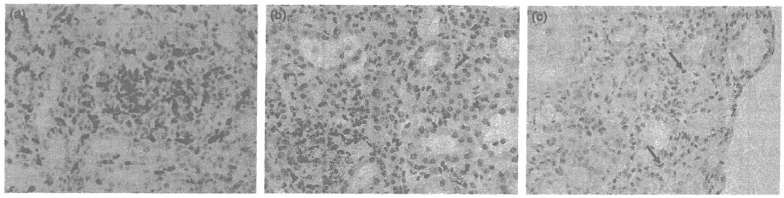

Fig. 1 Tubulointerstitial T cells in anti-neutrophil cytoplasmic antibody-associated glomerulonephritis (ANCA-GN). (a) Marked infiltration of CD3-positive T cells in the tubulointerstitium; (b) cytotoxic T cells (T cell intracytoplasmic antigen-1); (c) regulatory T cells (Foxp3; arrows). (Original magnification ×400.)

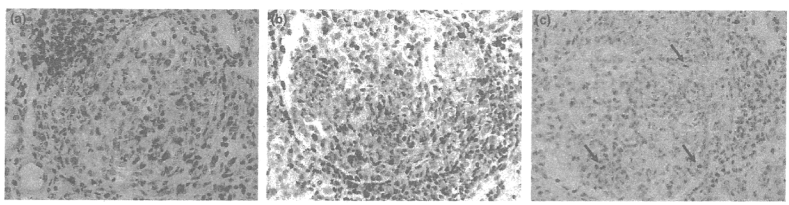

Fig. 2 Glomerular T cells in anti-neutrophil cytoplasmic antibody-associated glomerulonephritis (ANCA-GN). Marked infiltration of (a) CD3-positive T cells and (b) cytotoxic T cells (T cell intracytoplasmic antigen-1) is seen in both the crescent area and glomerular capillaries. (c) A few regulatory T cells are seen in the glomeruli (Foxp3; arrows). (Original magnification ×400.)

Table 2 Histopathological parameters in MCNS, non-HD and HD patients

	MCNS (n = 10)	non-HD (n = 33)	HD (n = 7)	P-value
Interstitium				
CD3 (/mm^{2†})	8.96 ± 3.71	322 ± 50.0	385 ± 54.5	0.87
TIA-1 (/mm^{2†})	3.27 ± 2.81	122 ± 25.8	130 ± 39.8	0.65
Foxp3(/mm^{2†})	0	8.14 ± 2.60	3.76 ± 0.97	0.06
TIA-1/CD3†	0.34 ± 0.11	0.38 ± 0.06	0.34 ± 0.10	0.47
Foxp3/CD3†	0	0.025 ± 0.003*	0.001 ± 0.003	< 0.05
Foxp3/TIA-1	0	0.016 ± 0.016*	0.004 ± 0.008	< 0.05
Tubulointerstitial damage score	0	2.06 ± 0.14	2.00 ± 0.38	0.53
Glomeruli				
Foxp3 (/glomerulus†)	0	0.75 ± 1.16	0.61 ± 1.39	0.71
Foxp3/CD3†	0	0.33 ± 1.26	0.32 ± 1.78	0.95
Foxp3/TIA-1†	0	0.36 ± 1.28	0.36 ± 1.66	0.57
Total crescent rate† (%)	0	35.9 ± 3.54*	65.8 ± 7.40	< 0.05
Cellular crescent rate† (%)	0	44.4 ± 9.29	17.8 ± 12.3	0.13

†Log-transformed values were used. *P < 0.05 versus HD patients. Tubulointerstitial damage score; 0 = no, 1 = mild, 2 = moderate, 3 = severe.

interstitium in samples from HD patients (P < 0.05; Table 2; Fig. 3a,b). However, there were no significant differences in Foxp3, Foxp3/CD3 and Foxp3/TIA-1 ratios in the glomeruli between the two groups. The interstitial Foxp3/TIA-1 ratio, but not the Foxp3/CD3 ratio, remained significantly different between the two groups after adjustment by total crescent rate (P < 0.05). The number of Foxp3-positive cells in the interstitium also tended to be higher in patients in the non-HD compared with HD group (P = 0.06). There was no significant difference in the TIA-1/CD3 ratio between the two groups (P = 0.47). There was no significant difference between the HD and non-HD groups in terms of tubulointerstitial damage score and glomerular sclerosis; however, the total crescent rate was significantly lower in patients in the non-HD compared with HD group (Table 2). Using the Cox proportional risks model revealed that the age- and total crescent rate-adjusted renal survival rate was significantly higher in patients with a Foxp3/TIA-1 ratio ≥ 0.06 compared with patients with a Foxp3/TIA-1 ratio < 0.06 (P = 0.02; Fig. 4). There was no significant association between the Foxp3/TIA-1 ratio and the reduction in eGFR in patients in the non-HD group.

DISCUSSION

The salient findings of the present study are as follows: (i) Foxp3/CD3 and Foxp3/TIA-1 ratios in the interstitium are significantly higher in patients in the non-HD than HD group, with the difference in the Foxp3/TIA-1 ratio remaining significant even adjustment for total crescent rate; (ii) there is no correlation between the TIA-1/CD3 and renal death; and (iii) renal survival rate was significantly higher in MPO-ANCA-GN patients with a Foxp3/TIA-1 ratio ≥ 0.06 compared with patients with a Foxp3/TIA-1 ratio < 0.06. These observations suggest that immunohistochemical analysis of Foxp3 and TIA-1 could provide information that is useful for predicting renal death in patients with ANCA-GN.

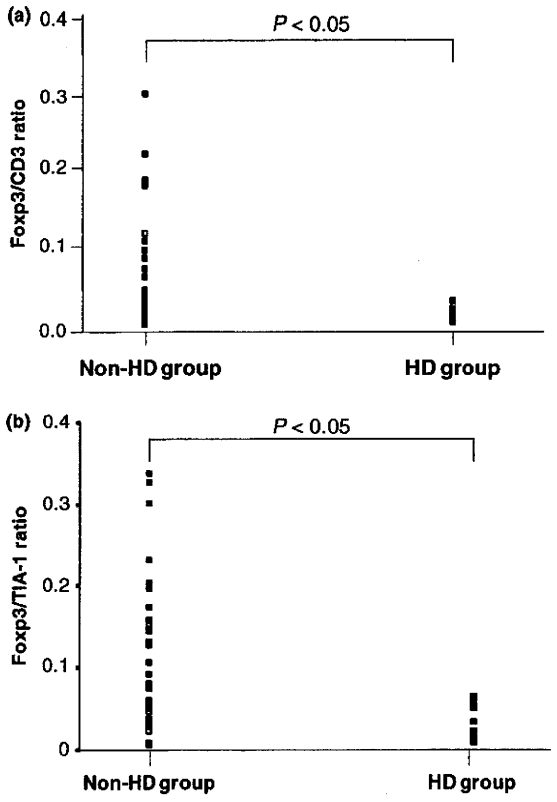

Fig. 3 (a) Foxp3/CD3 and (b) Foxp3/T cell intracytoplasmic antigen (TIA)-1 ratios in patients requiring maintenance haemodyalisis (HD group) and those not (non-HD group). Analysis of covariance adjusted for age was performed using log-transformed values for Foxp3/CD3 and Foxp3/TIA-1 ratios.

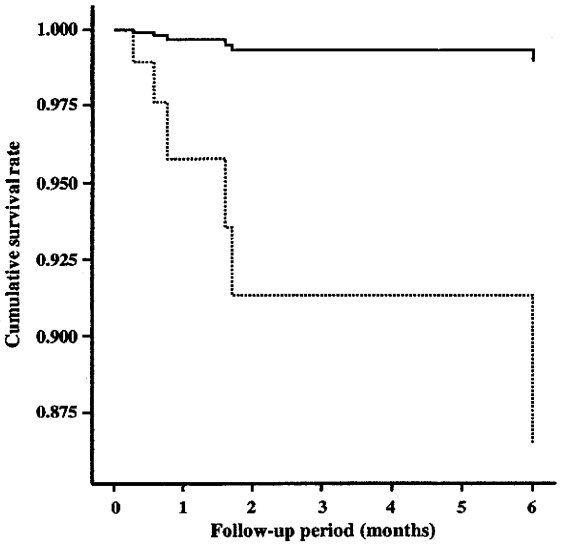

Fig. 4 Cox proportional model for renal survival rate adjusted for age and total crescent rate in patients with a Foxp3/T cell intracytoplasmic antigen (TIA)-1 ratio ≥ 0.06 (—; $n = 21$) compared with patients with a Foxp3/TIA-1 ratio < 0.06 (·····; $n = 19$).

To our knowledge, there has been no study examining the relationship between Foxp3 expression levels and renal prognosis in patients with ANCA-GN. Marinaki *et al.*[14] reported that there were no differences in circulating Foxp3 levels between ANCA-V patients and age- and sex-matched healthy controls. Furthermore, although T_{reg} infiltration was associated with RA activity in the synovial fluid, the number of $CD4^+$ $CD25^+$ T cells, one of the markers for T_{reg}, in peripheral blood did not differ significantly between RA patients and normal controls.[7] These observations suggest that local infiltration of T_{reg} could play a more important role in tissue damage and that tissue, but not circulating, T_{reg} numbers may be a prognostic marker for various inflammatory diseases, including ANCA-GN.

In the present study, although there was no correlation between the number of Foxp3- or TIA-1-positive cells and renal death, there was a signficant correlation between the Foxp3/CD3 and Foxp3/TIA-1 ratios renal death. These findings are consistent with those of Bestard *et al.*[8], who showed that there was a positive correlation between the Foxp3/CD3 ratio and graft function 2 years after renal transplantation. Because CD3 is known as a total T cell marker, it may be considered that the Foxp3/CD3 ratio more exactly reflects the extent of the shift of inflammatory cells into T_{reg} in ANCA-GN. Furthermore, in the present study, the interstitial Foxp3/TIA-1 ratio, but not the Foxp3/CD3 ratio, remained a significant factor indicating renal survival after adjustment with crescent rate. These observations suggest that the Foxp3/TIA-1 ratio in the interstitium could be a novel prognostic marker for renal death in ANCA-GN.

Hogan *et al.*[5] previously reported that glomerular crescent formation and sclerosis are not predictive of renal survival in ANCA- GN. It has also been shown that neither cellular crescent formation nor the MPO-ANCA titre are correlated with mortality.[15] These observations are consistent with the findings of the present study, in which neither MPO-ANCA titre nor cellular crescent rate were correlated with renal prognosis. However, total crescent rate was found to be associated with renal death in ANCA-GN. We do not know the reasons underlying these differences between the present study and previous studies. The could be due to differences in the study population, ethnicity and immunosuppressive therapies.

In the present study, the TIA-1/CD3 ratio was not correlated with renal death in ANCA-GN. These findings are consistent with the report of van Es *et al.*[16], who showed that, although higher TIA-1 levels in renal tubules were associated with renal progression in early stages of IgA nephropathy, there were no significant differences in TIA-1 cell numbers between progressors and non-progressors. These findings suggest that the expression of interstitial T_{reg} may be more important than CTL to determine the prognosis of renal diseases, including MPO-ANCA-GN.

In conclusion, we have demonstrated, for the first time, that interstitial Foxp3-positive T cells are associated with renal survival in ANCA-GN patients. These observations suggest that T_{reg} may play a protective role against ANCA-GN.

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Development of Enzyme-Linked Immunosorbent Assay System for PEDF and its Clinical Utility

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Abstract: Pigment epithelium-derived factor (PEDF) is reported to play a protective role against diabetic vascular complications through its anti-oxidative properties. However, since a commercially available kit is not suitable for measurement of serum PEDF in humans, kinetics and regulation of serum PEDF are not known in these devastating disorders. Therefore, we developed a simple, specific and reliable method for measurement of serum PEDF in humans using a competitive enzyme-linked immunosorbent assay (ELISA) system. Assay linearity was shown intact with 50~300-fold dilution of urea-pretreated serum by phosphate-buffered saline. The recovery ratio of added recombinant human PEDF in serum was 94.2 ± 1.7 %. Inter- and intra-assay coefficient of variations of the ELISA were 4.7 and 7.3 %, respectively. When we measured serum PEDF levels in a general population, PEDF levels were elevated in proportion to the accumulation of the number of the components of the metabolic syndrome. Further, the percent changes in serum levels of PEDF during 1-year observational periods were positively correlated with those of body mass index (BMI) in patients with type 2 diabetes. In addition, PEDF mRNA levels in cultured adipocytes were increased in parallel to the BMI values of subjects from which adipocytes were derived, especially in omental adipocytes. These observations suggest that PEDF is generated from adipose tissues and could be increased as a counter-system against vascular cell damage in humans. PEDF may be one of the useful biomarkers for vascular injury in high-risk patients.

Keywords: ELISA, PEDF, metabolic syndrome, insulin resistance, CKD.

INTRODUCTION

Pigment epithelium-derived factor (PEDF), a multifunctional serine proteinase inhibitor, was first identified in cultured human retinal pigment epithelial cells as a factor which possessed potent neuronal differentiating activity [1]. It has been reported that PEDF, which has anti-angiogenic, anti-oxidant, and anti-inflammatory effects, is widely expressed in a variety of organs [1-5]. PEDF has also been found in vitreous, and its levels were decreased in angiogenic eye diseases, suggesting that loss of PEDF in the eye is functionally important in the pathogenesis of proliferative diabetic retinopathy [6]. We have recently found that PEDF prevents neuronal derangements and vascular hyperpermeability in early diabetic retinopathy *via* inhibition of NADPH oxidase-driven oxidative stress generation in rats [4]. PEDF inhibited retinal endothelial cell (EC) growth and migration and suppressed ischemia-induced retinal neovascularization as well [7,8]. Further, PEDF is also shown to block advanced glycation end product (AGE)- or angiotensin II-induced EC injury *in vitro* [9,10]. These observations suggest that PEDF could play a protective role against diabetic vascular complications through its anti-oxidative properties. However, since a commercially available kit was not suitable for the measurement of serum PEDF in humans, a little was known about the kinetics and regulation of serum PEDF in these devastating disorders. Here we developed a simple, specific and reliable method for measurement of serum PEDF in humans using a competitive enzyme-linked immunosorbent assay (ELISA) system. In this review, we provided information about our assay system for PEDF and discussed the clinical utility of the measurement of PEDF in various disorders.

DEVELOPMENT OF ELISA SYSTEM FOR HUMAN PEDF

Assay for Serum PEDF

Serum PEDF measurements were performed with the competitive ELISA on the following procedures. A 96-well microtiter plate (Nalge Nunc International, Japan) was coated by overnight incubation at 4 °C with an anti-PEDF monoclonal antibody (Transgenic, Kumamoto, Japan). Fifty μl of serum was pretreated with 200 μl of 8 M urea at 4 °C for 1 h and then each sample was 50-fold diluted with 10 mM phosphate-buffered saline pH 7.4, 0.25 % bovine serum albumin and 0.05 % Tween-20. After washing the microtiter plate, 100-μl aliquots of standard recombinant human PEDF proteins (0.5-300 ng/ml; Chemicon International, Temecula, CA, USA) or diluted serum were added to the wells and then the plate was incubated at room temperature for 2 h. Then the well was washed four times, and 50 μl of biotinylated anti-human PEDF polyclonal antibody (R&D Systems, Minneapolis, MN, USA) was added to each well. After 2-h incubation at room temperature, the plate was incubated with 100 μl of HRP-conjugated streptavidin solution (Zymed, South

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1566-5240/10 $55.00+.00

San Francisco, CA, USA) at room temperature for 30 min. Then the well was washed again, and 50 µl of chromogenic substrate solution (Dako, Tokyo, Japan) was added to each well and the plate was incubated with shaking at room temperature for 15 min. After the color was developed, 50 µl of reaction stopper was added. The plate was read at 450 nm using a microplate reader (Emax, Molecular Devices, Bucher Biotec AG-Basel, Switzerland). Inter- (n=17) and intra-assay (n=14) coefficient of variations of the ELISA were 4.7 and 7.3 %, respectively.

Standard Curve of PEDF ELISA

Appropriate calibration curves were obtained with recombinant human PEDF proteins. Fig. (**1**) shows a typical standard curve; linearity was shown at the concentrations ranging from 20-300 ng/ml. PEDF levels of 250-fold diluted samples were within this range, typically ranging from 40 to 80 ng/ml, which corresponded to 10-20 µg/ml of serum PEDF.

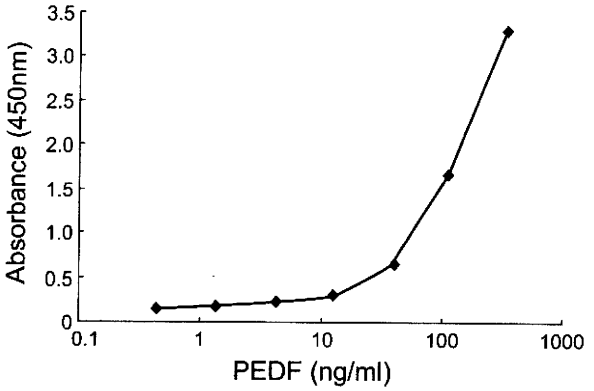

Fig. (1). Standard curve of PEDF ELISA. Seven concentrations of recombinant PEDF were applied to the ELISA system.

Specificity of PEDF ELISA System

Sera were obtained from 5 persons with different concentrations of PEDF. Urea-treated 50-fold diluted sera (range between 40 and 120 ng/ml) were serially diluted with phosphate-buffered saline. The assay linearity was shown intact with serial dilution (Fig. **2**).

Recovery of PEDF in Serum

To evaluate the recovery ratio of PEDF to serum, three concentrations of recombinant PEDF (75-166.6 ng/ml) were added to serum and PEDF in each serum was determined. Newly added PEDF was fully recovered by this ELISA system. Recovery of the recombinant PEDF in sera was 94.2 ± 1.7 % (mean ±SD).

SUPERIORITY OF OUR ELISA SYSTEM

Although a commercially available PEDF ELISA kit can be obtained from Chemicon International, Inc. (Temecula, CA), it is good for measuring PEDF of vitreous fluid, but not for serum. Indeed, we have confirmed that serum PEDF can not be accurately detected with this kit as described below, even if we performed ELISA according supplier's instruction. Therefore, here we developed a modified ELISA method for measuring human serum PEDF. We demonstrated that our modified method was a simple, specific, reliable and suitable because (1) it easily permitted determination of serum PEDF within the quantitative ranges (Fig. **1**), (2) the monoclonal antibody used in this system was specific to PEDF and showed negligible cross-reactivity with other proteins [11], (3) the assay linearity was intact with 50~300-fold dilution of urea-pretreated serum by phosphate-buffered saline (r^2=0.999) (Fig. **2**), (4) the recovery ratio of added recombinant human PEDF in serum was more than 90 %, and (5) the inter- and intra-assay coefficient of variations were low. Further, since the concentrations of PEDF of serum and platelet-poor plasma did not differ with our ELISA system (n=5; 12.1 ± 3.3 vs 11.2 ± 2.8 µg/ml), both serum and plasma samples were available for ours.

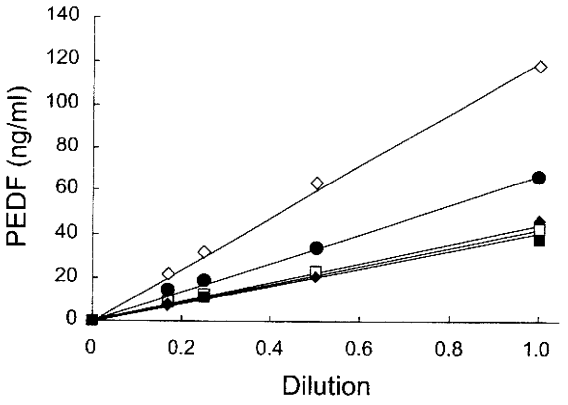

Fig. (2). Dilution curve of serum PEDF. Five sera with different concentration of PEDF were prepared. Urea-treated 50-fold diluted sera were serially diluted with phosphate-buffered saline. The assay linearity was shown intact with serial dilution.

In our assay, we found that pretreatment of serum with 8 M urea, whose process is lacking in Chemicon kit, was indispensable for measuring human serum PEDF because serum PEDF levels detected by the commercial kit are three orders of magnitude lower (10-30 ng/ml) than those by our ELISA system. These observations suggest that most of circulating PEDF in serum may exist as a protein-bound form. To clarify what proteins could bind to PEDF in the circulation and regulate its biological activity would be helpful for further understanding the pathophysiological role of PEDF in health and disease.

SERUM PEDF LEVELS AND THE METABOLIC SYNDROME

When subjects with no histories of coronary or peripheral artery disease (n=196; age 65.7±9.3 years

old; men:women, 71:125) underwent a complete history and physical examination, determination of blood chemistries, and serum levels of PEDF, PEDF was found to show a normal distribution, ranging from 8 to 24 μg/ml with the mean of 14.6±3.2 μg/ml (Fig. **3**) [12]. The average serum levels of PEDF in men were higher than those in women (15.4±3.2 μg/ml in men and 14.1±3.1 μg/ml in women), and serum PEDF levels were much higher than those in human vitreous (1.2-1.5 μg/ml) [6,13]. Univariate analysis revealed significant correlations of PEDF with body mass index (BMI), waist circumference, HDL-cholesterol (inversely), triglycerides, glucose, insulin, and uric acid. In a stepwise multiple regression analysis, uric acid ($p<0.001$), waist circumference ($p=0.009$), insulin ($p=0.019$) and triglycerides ($p=0.028$) remained significant and were independently related to serum PEDF levels ($R^2=0.239$) [12]. Further, PEDF levels were significantly higher in proportion to the accumulation of the number of the components of the metabolic syndrome [12]. These observations suggest that PEDF is strongly and specifically associated with the metabolic syndrome.

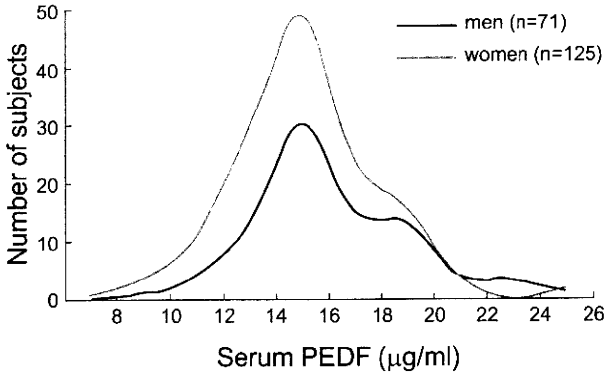

Fig. (3). Distributions of serum PEDF levels in men and women.

In patients with type 2 diabetes, we found that the waist circumference, triglycerides, creatinine, and tumor necrosis factor-α (TNF-α) were significant independent determinants of serum PEDF levels and that 1-year changes in PEDF were positively correlated with those in body mass index (BMI) [14]. In addition, PEDF mRNA levels in cultured adipocytes were increased in parallel to the BMI values of subjects from which adipocytes were derived, especially in omental adipocytes [14]. Recently, it is reported that PEDF is synthesized by adipose tissue and its level is down-regulated during the differentiation process to mature adipocytes [15]. Since this expression pattern is opposite to that of adiponectin and similar to that of TNF-α, adipocyte PEDF production may be increased in visceral obesity in contrast to the case of adiponectin [16]. Taken together, the present findings suggest that immature adipocytes in visceral adipose tissues may be one of the main origins of serum PEDF in humans.

We have very recently found that PEDF level is an independent determinant of homeostasis model assessment of IR (HOMA-IR), a marker of insulin resistance in elderly patients with essential hypertension [17]. When mean PEDF levels stratified by HOMA index tertiles were compared using analysis of covariance adjusted for age and sex, a linear and significant tend ($p=0.027$) was observed. Given the fact that PEDF possesses anti-oxidative, anti-inflammatory and insulin-sensitizing properties [5-10], these observations further support the concept that serum PEDF levels may be elevated as a counter-system against insulin resistance and vascular injury in patients with the metabolic syndrome, elderly hypertension and/or type 2 diabetes.

SERUM PEDF LEVELS AND RENAL FAILURE

The clearance of PEDF was not yet characterized, and the relation of serum PEDF levels to renal function was still unknown. Therefore, we examined whether serum levels of PEDF were elevated in patients with end-stage renal disease (ESRD). When we measured serum PEDF levels in 50 ESRD patients (age 60.1±10.7 years old; men:women, 27:23) and 50 age- and sex-matched healthy subjects, serum PEDF levels were found to be much higher in ESRD patients than those in healthy controls (36.3±5.7μg/ml vs 14.5±22.3μg/ml, $p<0.001$) [18]. Moreover, blood urea nitrogen (BUN) was the independent determinant of PEDF in ESRD patients. These observations suggest that serum PEDF concentrations were substantially higher in ESRD patients than in controls and that PEDF might be excreted mainly through the kidney. As described above, the fact that serum creatinine level is one of the independent determinants of serum PEDF level in type 2 diabetic patients [14] further supports the concept that kidney may be a main organ for the clearance of PEDF in the circulation.

SERUM PEDF LEVELS AND SYMPATHETIC NERVE ACTIVITY

Although high heart rate is known to be associated with metabolic risk factors for atherosclerosis [19], the correlation between serum level of PEDF and resting heart rate remains to be elucidated. So, we further examined their relationship in Japanese outpatients. Four hundred two consecutive outpatients (141 male and 261 female; mean age 74.9±9.9 years old) underwent a complete history and physical examination, determination of blood chemistries and PEDF levels. In multiple regression analysis, smoking ($p=0.003$), use of β-blockers ($p=0.010$, inversely), presence of atrial fibrillation ($p=0.014$), PEDF ($p=0.018$) and glucose levels ($p=0.033$) were independently correlated to resting heart rate [19]. Further, when mean serum PEDF levels were stratified by resting heart rates, a linear and significant trend ($p=0.025$) was observed. The underling molecular mechanism by which PEDF was an independent determinant of resting heart rate remains unclear. However, since increase in heart rate is known to enhance oxidative

stress generation *via* both symptomatic nerve activity-dependent and independent pathways [20,21], serum PEDF level may be elevated in response to increased heart rate in order to counter-act against oxidative stress injury in humans. In addition, PEDF expression levels were reported to significantly decrease in the sympathectomized retina, and norepinephrine increased PEDF secretion by retinal pigment epithelial cells [22]. These findings suggest the sympathetic nerve regulation of PEDF expression *in vivo* and that PEDF may play some role in sympathetic nerve activity.

CONCLUSIONS

We described here the clinical utility of our ELISA system for human PEDF. PEDF may be one of the useful biomarkers for oxidative stress and vascular injury in high-risk patients.

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Irbesartan Inhibits Albumin-Elicited Proximal Tubular Cell Apoptosis and Injury *In Vitro*

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Abstract: There is accumulating evidence that proteinuria is not merely a biomarker for the progression of chronic kidney disease (CKD), but also a mediator of this devastating disorder. Indeed, albumin, one of the major components found in proteinuria, causes proinflammatory and profibrotic changes in cultured proximal tubular cells. Further, numerous studies have demonstrated the active participation of the renin-angiotensin system (RAS) in the pathogenesis of CKD as well. However, the role of the RAS in albumin-elicited tubular cell damage remains to be elucidated. Therefore, in this study, we studied whether and how irbesartan, an angiotensin II type 1 receptor blocker, could inhibit albumin-elicited proximal tubular cell apoptosis and injury *in vitro*. Bovine serum albumin (BSA) increased oxidative stress generation in human cultured proximal tubular cells, which was blocked by the treatment with irbesartan. Irbesartan was also found to block the BSA-induced apoptotic cell death as well as up-regulation of plasminogen activator inhibitor-1 and transforming growth factor-β mRNA levels in tubular cells. The present study suggests that there could exist a pathophysiological crosstalk between the RAS and albumin overload in proximal tubular cell apoptosis and damage. Blockade of the RAS by irbesartan may play a protective role against tubular cell injury by attenuating the deleterious effects of albumin.

Keywords: CKD, renin-angiotensin system, irbesartan, tubular injury.

INTRODUCTION

Proteinuria is one of the most common findings in chronic kidney disease (CKD) [1,2]. Although persistent proteinuria shows the existence of glomerulopathy, it has recently been recognized that changes within tubulointerstitium are more important than glomerulopathy in terms of renal prognosis in patients with CKD [1,2]. Further, there is accumulating evidence that proteinuria is not merely a biomarker for the progression of CKD, but also a mediator of this devastating disorder [3,4]. Indeed, albumin, one of the major components found in proteinuria, causes proinflammatory and profibrotic changes in cultured proximal tubular cells [3,4]. Further, clinical data show a positive correlation of the extent of proteinuria with the severity of tubulointerstitial damage in CKD patients [3,4].

Numerous studies have demonstrated the active participation of the renin-angiotensin system (RAS) in the pathogenesis of CKD as well [1]. The renoprotective effects of the inhibitors of the RAS are largely ascribed to its blood pressure (BP)-lowering properties [1]. However, a recent clinical study suggests the pleiotropic effects of the RAS inhibitors, *that is,* beyond BP-lowering effects, on diabetic nephropathy [5]. Indeed, it has been shown that irbesartan, an angiotensin II type 1 receptor blocker (ARB), significantly prevents the progression of nephropathy in patients with type 2 diabetes, compared with calcium channel blocker, amlodipine with an equipotent BP-lowering property [5]. These observations suggest that the inhibition of the RAS itself is a therapeutic target for CKD. However, the role of the RAS in albumin-elicited tubular cell damage remains to be elucidated. Therefore, in this study, we studied whether and how irbesartan at the therapeutic concentration could inhibit albumin-elicited proximal tubular cell apoptosis and injury *in vitro*.

MATERIALS AND METHODS

Materials

Irbesartan was generously gifted from Dainippon Sumitomo Pharma (Tokyo, Japan). BSA (essentially fatty acid free and essentially globulin free, lyophilized powder) was purchased from Sigma (St. Louis, MO, USA).

Preparation of BSA

BSA was prepared as described previously [6]. Briefly, BSA (25 mg/ml) was incubated under sterile conditions in 0.2 M $NaPO_4$ buffer (pH 7.4) for 7 days. Then contaminants were removed by PD-10 column chromatography and dialysis against phosphate-buffered saline. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan); no endotoxin was detectable.

Cells

Proximal tubular epithelial cells from human kidney were maintained in basal medium supplemented with 5% fetal bovine serum, 0.5 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 0.5 μg/ml epinephrine, 6.5 ng/ml triiodo-L-thyronine, 10 μg/ml transferrin, 5 μg/ml insulin, and GA-1000 according to the supplier's instructions (Clonetics Corp., San Diego, CA, USA). Cells at 3-5 pas-

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sages were used for the experiments. BSA treatments were carried out in a serum-free basal medium containing 10 μg/ml transferrin and GA-1000.

Reactive Oxygen Species (ROS) Generation

Tubular cells were treated with 100 μg/ml BSA in the presence or absence of 1 μM irbesartan for 4 h or 24 h, and then the cells were incubated with phenol red free Dulbecco's Modified Eagle Medium containing 3 μM dihydroethidium (DHE) (Molecular Probes Inc., Eugene, OR, USA). After 15 minutes, the cells were imaged under a laser-scanning confocal microscope. Intensity of DHE staining in 5 different field of each sample was analyzed by microcomputer-assisted NIH image. Intracellular ROS generation is evaluated by the intensity of DHE staining (arbitrary units).

Measurement of Apoptotic Cell Death of Tubular Cells

Tubular cells were treated with 100 μg/ml BSA in the presence or absence of 1 μM irbesartan for 4 h or 24 h. Then the cells were lysed and the supernatant was analyzed in an enzyme-linked immunosorbent assay (ELISA) for DNA fragments (Cell Death Detection ELISAplus, Roche Molecular Biochemicals, Mannheim, Germany) as described previously [7].

Real-Time Reverse Transcription-Polymerase Chain Reactions (RT-PCR)

Tubular cells were treated with 100 μg/ml BSA in the presence or absence of 1 μM irbesartan for 4 h or 24 h. Then total RNA was extracted with RNAqueous-4PCR kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Applied Biosystems, Foster city, CA, USA) according to the suplier's recommendation. IDs of primers for human plasminogen activator inhibitor-1 (PAI-1), transforming growth factor-β (TGF-β) and β-actin gene were Hs00171257_m1, Hs00171257_m1 and Hs99999903_m1, respectively.

Statistics

All values were presented as means ± standard error (SEM). One-way ANOVA followed by the Scheffe F test was performed for statistical comparisons; $p<0.05$ was considered significant.

RESULTS

We first investigated whether irbesartan could inhibit ROS generation in tubular cells. As shown in Fig. (**1A**), BSA increased ROS generation in tubular cells, which was blocked by the treatment with irbesartan. We next investigated the effects of irbesartan on apoptotic cell death of BSA-exposed tubular cells. As shown in Fig. (**1B**), 4-h treatment with BSA significantly induced apoptotic cell death of tubular cells, which was inhibited by irbesartan. Further, irbesartan was found to inhibit the BSA-induced up-regulation of PAI-1 and TGF-β mRNA levels in tubular cells (Fig. **1C**).

DISCUSSION

In this study, we demonstrated for the first time that irbesartan, an ARB inhibited the BSA-elicited apoptosis and fibrogenic gene expressions (PAI-1 and TGF-β gene expressions) in cultured human proximal tubular cells probably through its anti-oxidative properties. Recently, Takao *et al.* reported that BSA increased angiotensin II generation and oxidative stress production by human proximal tubular cells, both of which were markedly suppressed by the treatment with an ARB, olmesartan or valsartan [8]. Further, angiotensin II has been known to stimulate the expressions of PAI-1 and TGF-β in several cell lines [9]. Therefore, our present study suggests that there could exist a pathophysiological crosstalk between the RAS and albumin overload in proximal tubular cell apoptosis and damage. Blockade of the RAS by irbesartan may play a protective role against tubular injury by attenuating the deleterious effects of albumin.

Chronic tubulointerstitial damage in the kidney, including tubular atrophy and interstitial inflammation fibrosis, is more important than glomerulopathy in terms of renal prognosis in CKD [1,2]. Further, PAI-1 is a major physiological inhibitor of the plasminogen activator/plasmin system, a key regulator of fibrinolysis and extracellular matrix turnover [9]. In addition, a growing body of evidence has implicated the TGF-β system as a major etiologic agent in the pathogenesis of tubulointerstitial fibrosis in CKD [1,2]. These observations suggest that BP lowering-independent renoprotective effects of irbesartan observed in type 2 diabetic patients [5] could be ascribed, at least in part, to its albumin overload blockade properties.

In the present study, although BSA increased ROS generation in tubular cells in a time-dependent manner (Fig. **1A**), apoptosis was significantly increased only in the 4-h BSA-treated cells, but not 24-h ones (Fig. **1B**). These observations suggest that early phase of BSA-elicited ROS generation may be involved in apoptosis of proximal tubular epithelial cells. It would be helpful to measure apoptotic cell death of tubule cells using a different method like annexin V.

In this study, we chose the concentration of BSA at 100 μg/ml because the level is within the range of microalbuminuria. Therefore, it is unlikely that BSA used here may exert non-specific and toxic effects on tubular cell damage. Further, the peak plasma concentration of irbesartan is reported to be about 1-2 μM [10]. So, the concentration of irbesartan having beneficial effects on tubular cells used in the present experiments (1 μM) may also be comparable to the therapeutic levels which are achieved in the treatments for patients with hypertension. Taken together, our present study provides a novel beneficial aspect of irbesartan on CKD; it could work as an agent against the albumin overload and may play a protective role against the progression of CKD.

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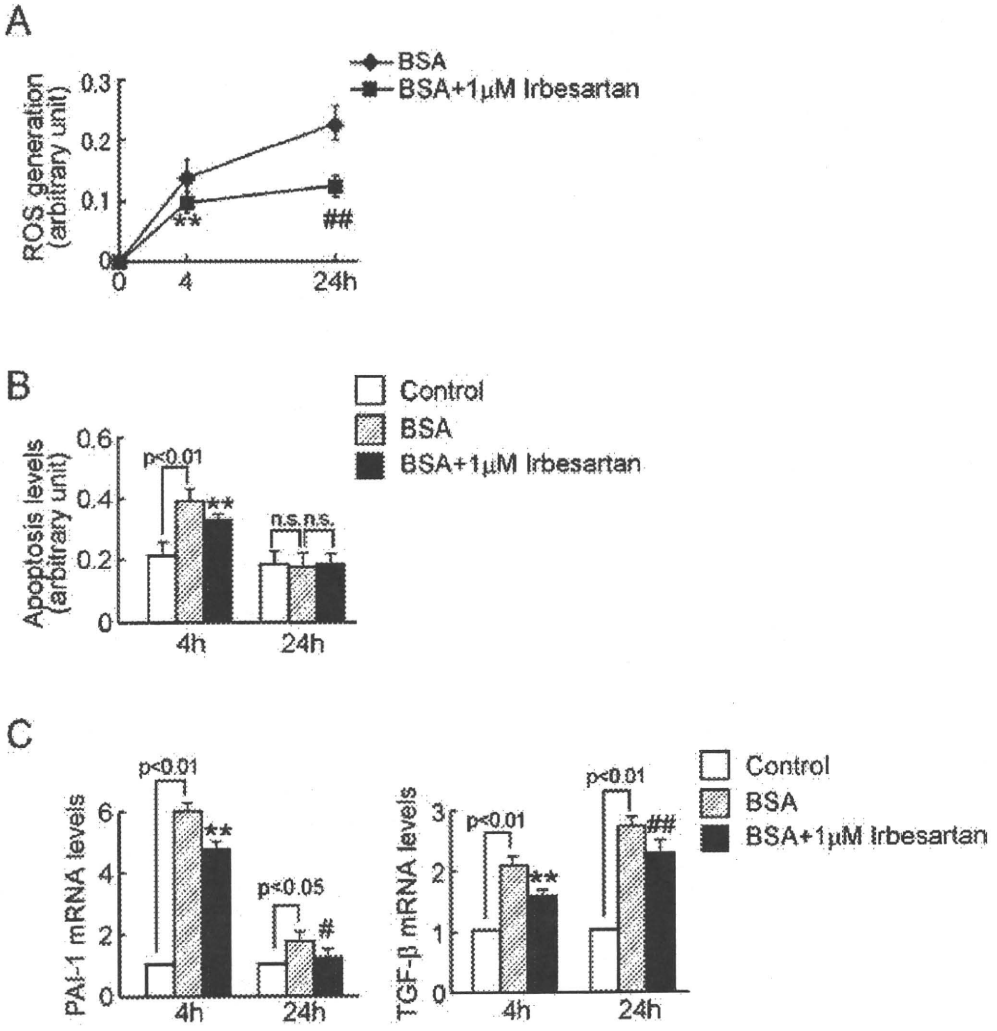

Figure 1. Effects of irbesartan on ROS generation **(A)**, apoptotic cell death **(B)**, and PAI-1 and TGF-β gene expressions **(C)** in tubular cells. Cells were treated with 100 μg/ml BSA in the presence or absence of 1 μM irbesartan for 4 h or 24 h. Then the cells were incubated with DHE (A). Cell were lysed and the the supernatant was analyzed in an ELISA for DNA fragments **(B)**. **(C)** Total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of β-actin mRNA-derived signals and then related to the value obtained with the control. **, $p<0.01$, compared to the value with 4 h-BSA treatment alone. # and ##, $p<0.05$ and $p<0.01$, compared to the value with 24 h-BSA treatment alone, respectively. **(A)** $N=5$ per group. **(B)** $N=4$ per group. **(C)** $N=3$ per group. n.s.; not significant.

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