change in the conventional whole-cell mode but to a lesser extent than the perforated whole-cell mode, because ATP is supplied continuously through pipettes in whole-cell mode.

Time required until initiation of recordings after bringing the cells out of the $CO_2$ incubator was 21.0 ± 3.7 min ($n$ = 5) in perforated whole-cell mode and 11.7 ± 1.4 min ($n$ = 35) in conventional whole-cell clamp mode. The current density at time 0 for recordings was 156.7 ± 18.7 pA/pF ($n$ = 10) in the perforated mode and 166.6 ± 17.3 pA/pF ($n$ = 10, $P$ > 0.05) in the conventional mode. These results suggest that the Kv-channel activity is stable unless the membrane is voltage-clamped to depolarized potentials. In fact, we observed that Kv-channel current was down-regulated in a use-dependent manner and partially restored from the down-regulation after pauses without depolarized pulse at low glucose (Fig. 5C) and up-regulated (increase in the current) at high glucose (Fig. 5D).

Our results that glucose metabolism enhances the Kv-channel activity may suggest a negative-feedback regulation of action potentials in response to glucose stimulation. This may reduce an excess entry of $Ca^{2+}$ ions into the cell during action-potential bursting. Because there is no specific opener for Kv2.1 channels yet, we cannot evaluate effects of increase in Kv-channel current at high glucose on insulin secretion. Time required for 50% repolarization of action potential in β-cells was short during glucose stimulated electrical firings (48.8 ± 4.0 ms, $n$ = 6). The time constant for Kv-channel activation on depolarized pulses (6.6 ± 1.7 ms at −10 mV, $n$ = 5) was fast enough to influence action-potential durations. Thus, even in these short action-potential durations, both electrical activity and insulin secretion are reportedly influenced by Kv-channel blocker, guangxitoxin-1E [20]. We have previously observed that ghrelin, a gastric-derived hormone with potential stimulatory effects on feeding and growth hormone release, inhibits glucose-stimulated insulin secretion from pancreatic β-cells by an involvement of increase in Kv-channel current [14,15]. Whether insulin secretion is negatively regulated by an increase in Kv-channel availability at high glucose remains to be elucidated.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.05.050.

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Original Article

# SPARC is a Major Secretory Gene Expressed and Involved in the Development of Proliferative Diabetic Retinopathy

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***Aim***: Neovascularization is an important event in proliferative diabetic retinopathy (PDR), where various secretory proteins including multiple growth factors are considered to be involved in this process. We searched for secretory proteins expressed in a surgical specimen obtained from the eyes of patients with PDR.
***Methods***: We developed the oligo-cap signal sequence trap (SST) strategy which enables us to screen for secretory or membrane proteins from a minimal starting material. Using this method, we were able to screen a cDNA library constructed from a surgical specimen obtained from the eyes of the patients with PDR.
***Results***: Majority of the cloned cDNAs turned out to encode secreted protein acidic and rich in cystein (SPARC), strongly suggesting that SPARC is highly expressed in PDR. Analysis of vitreous fluid from various patients has shown that the concentration of SPARC protein is increased in patients with PDR. Furthermore, subretinal injection of recombinant SPARC adenovirus induced PDR-like changes in the rat eye.
***Conclusions***: Our results strongly suggested that SPARC is involved in the development of diabetic retinopathy (DR).

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**Key words;** Proliferative diabetic retinopathy, Neovascularization, Secreted protein acidic and rich in cystein

## Introduction

Diabetic retinopathy (DR) is one of the most frequent causes of blindness. Proliferative diabetic retinopathy (PDR) is a serious state in which the progression of DR is accelerated, and various secretory proteins, including multiple growth factors, are considered to be involved in this process. Among these, vascular endothelial growth factor (VEGF) has been suggested to be one of the most important factors to stimulate neovascularization[1], although additional factors are likely involved in the process of neovascularization in PDR. Also, we do not know the relative status of gene expressions of the secretory factors possibly involved in the development of PDR, which should be an important issue in understanding the process of the disease.

Recently, we have developed the oligo-cap signal sequence trap (SST) method, which is an efficient strategy to clone cDNAs of secretory proteins. This strategy utilizes oligo-cap and PCR, which enables us to construct and screen a cDNA library starting from a small amount of RNA. Using this method, we were

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prompted to search for secretory proteins expressed in the minimal specimen obtained from surgery on a PDR patient. Our approach should provide direct and informative evidence of the genes expressed and possibly involved in the development of PDR.

## Materials and Methods

### Cloning of Secretory Factors Expressed in PDR

The oligo-cap SST method combines the Oligo-cap method and SST-REX (signal sequence trap by retrovirus-mediated expression screening system[2-4]. Starting tissue materials were obtained from vitreoretinal surgery of PDR patients under informed consent. Briefly, RNAs were extracted from the tissue obtained at surgery, and treated with the FirstChoice RLM-RACE procedure (Ambion). The RNA was then ligated to a RNA adaptor containing BstXI site. cDNA was synthesized from RNA ligated with the adaptor using a DNA primer containing the BstXI sites and random hexamers at the 3' end using the ReverScript II System (WAKO). The cDNA was then amplified by polymerase chain reaction, digested with BstXI, and inserted into the BstXI site of the pMX-SST vector plasmid. The cDNA library was amplified in DH10B cells (Invitrogen) and transfected into the packaging cell line PlatE to construct a high titer retroviral cDNA library. The library was infected to an interleukin-3 (IL-3)-dependent cell line, Ba/F3 cells. After infection, IL-3-independent Ba/F3 cells were selected in the medium without IL-3. The cDNAs were amplified from the genome extracted from IL-3-independent Ba/F3 cells and sequenced.

### Sample Collection

Vitreous fluid samples were obtained from patients who underwent vitreous surgery, after informed concent. Samples of vitreous fluid were collected into sterile tubes at the time of vitreoretinal surgery and immediately stored at −80℃ until analysis. Written informed consent was obtained from all patients, and the study was approved by the appropriate ethics committee. Vitreous fluids were assayed quantitatively for secreted protein acidic and rich in cystein (SPARC) by an osteonectin ELISA kit according to the manufacturer's instructions (Haematologic Technologies, Inc.).

### Animals

Sprague-Dawley rats were purchased from CLEA (Tokyo, Japan). Diabetes was induced by a single injection of STZ (60 mg/kg body wt; Sigma), dissolved in citrate buffer (PH4.5) into a tail vein. Long-Evans rats, 8 to 12 weeks of age, were obtained from CLEA (Tokyo, Japan). Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animal husbandry and animal experiments were consistent with the University of Tsukuba's Regulations of Animal Experiments and were approved by the Animal Experiment Committee, University of Tsukuba[5,6].

### *In Situ* Hybridization

DIG-labeled RNA probes for SPARC were created as follows. Part of the SPARC cDNA fragment was PCR-amplified using 5′-TTGCCACAAAGTG-CACCCT-3′ and 5′-ATTCGGTCAGCTCAGAG-TCCAG-3′ primers. The PCR fragment was cloned into pGEM-T Easy vector (Promega) and analyzed by sequencing. These plasmids were linearized with Nde I for an anti-sense probe to detect SPARC expression, and Nco I for a sense probe, as a negative control experiment. These linearized plasmids were transcribed *in vitro* with DIG-11-uridine-5′-triphosphate (Roche) along with 1 unit/μL T7 polymerase and SP6 polymerase (Promega). *In situ* hybridization was performed according to the manufacturer's protocol on the Non-radioactive *in Situ* Hybridization Application Manual (Roche).

### Delivery of Adenovirus

Long-Evans rats were used for subretinal injections of recombinant adenovirus. The animals were anesthetized by pentobarbital sodium delivered by intraperitoneal injection. The eyes were further treated with tropicamide and phenylephrine hydrochloride drops. A 30-gauge needle was used to make a shelving puncture of the sclera. A 30-gauge needle was injected in a tangential direction under an operating microscope. For histology and fluorescein angiogram studies, each eye was injected with 5 μL adenoviral stock containing $1 \times 10^7$ particles of recombinant adenoviruses expressing either GFP, SPARC, VEGF, or a combination of SPARC and VEGF, respectively, according to the procedure previously described[7-9]. Each adenoviral stock was delivered to the subretinal space. Immediately after subretinal injection, a circular bleb was usually observed under the operating microscope. The success of each subretinal injection was further confirmed by the observation of partial retina detachment, as seen by indirect ophthalmoscopy. Retinal detachment due to subretinal injections ranged over 1/4 to 1/2 of the retina.

### Fluorescein Angiography

Rats were anesthetized and perfused intravenously with 0.4 mL PBS containing 50 mg/mL FITC-

labeled dextran (Sigma) as described[10].

## Results

### Oligo-Cap SST Screening of the PDR cDNA Library

From the initial screen, we obtained 291 cDNAs of putative secretory or membrane proteins. Of these, 144 clones were found to be SPARC (**Table 1A**). Independent screening was repeated to confirm the result, using surgical tissue from another patient, which also demonstrated 137 of 169 cloned cDNAs of secreted proteins to be SPARC (**Table 1B**). These results strongly suggested that SPARC is highly expressed and may be a major player in the proliferative retinal tissue of PDR.

### SPARC is Increased in Vitreous Fluid from Patients with Proliferative Diabetic Retinopathy

To confirm the observation obtained from the oligo-cap SST screen, we next measured the concentration of SPARC in vitreous fluid obtained from patients who underwent vitreous surgery. The concentration of SPARC in vitreous samples from 64 patients undergoing vitreous surgery (40 with PDR, 13 with diabetic vitreous hemorrhage (DM VH), and 11 with diabetic macular edema (DME)), and 8 nondiabetic patients who received vitrectomy for macular hole (MH) were analyzed. As expected, SPARC was significantly increased in patients with PDR (285.3 ng/mL average), compared to patients with MH (124.8 ng/mL average) ($p<0.005$), and DME (127.6 ng/mL) ($p<0.005$) (**Fig. 1**). SPARC concentration was also shown to be increased in the vitreous of DM VH (326.1 ng/mL) compared to MH ($p<0.01$), and with DME ($p<0.001$). There were no significant differences between the vitreous of DME and MH.

### Localization of SPARC in Streptozotocin-Induced Diabetes in Rat Eyes

*In situ* hybridization was performed to analyze the expression of SPARC and VEGF in sections of eyes from STZ-induced diabetic rats. Previous studies demonstrated that VEGF expression in the retina of STZ-induced diabetic rats was upregulated predominantly in the ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL)[11].

The expression level of SPARC was shown to be markedly increased in the retina of diabetic rats at 6 months after STZ injection. Increased expression of SPARC was observed in INL and ONL of diabetic rats compared to control rats (**Fig. 2A, B**). We also confirmed that the expression of VEGF in the retina of STZ-induced diabetic rat at 6 months was upregulated in INL and ONL, where no significant VEGF expression was observed in the retina of control rats (**Fig. 2C, D**). Our results suggested that SPARC expression is increased in retina exposed to high glucose.

### Fluorescein Angiography of AdCMV.SPARC-Injected Eyes

In order to test whether SPARC may play a significant role in the progression of proliferative diabetic retinopathy, we injected recombinant adenoviruses that express SPARC, VEGF and GFP into the RPE of rats. Fluorescein angiogram was performed to detect possible vascular leakage to see if these adenoviruses have a permeability effect on blood vessels. Twenty-two eyes of pigmented Long-Evans rats were subjected to subretinal injection with AdCMV.SPARC, AdCMV.VEGF, or both AdCMV.SPARC and AdCMV.VEGF. Each eye was injected with $1 \times 10^7$ particles. All animals were analyzed 4 weeks after injection. Fluorescein angiograms representative of a control phosphate-buffered saline-injected eye (**Fig. 3A**) and a control virus (AdCMV.GFP)-injected eye, are shown (**Fig. 3B**). Previous studies demonstrated that leakage by fluorescein angiography is observed in AdCMV.VEGF-injected eyes[8, 9, 12]. Partial leakage was also seen in our results of AdCMV.VEGF-injected eyes (**Fig. 3E**). A similar leaky area was demonstrated in AdCMV.SPARC-injected eyes (**Fig. 3C, D**). In eyes injected with both AdCMV.SPARC and VEGF, relatively massive leakage was observed (**Fig. 3F**). These data demonstrated that forced expression of SPARC in the RPE had a permeability effect on blood vessels which is comparable to, and additive to, the effect of VEGF.

### Histological Analysis of AdCMV.SPARC-Injected Eyes

To determine whether the leakage detected by fluorescein angiography is from previous new blood vessel formation and not due only to leakage from existing blood vessels, eyes were harvested 4 weeks postinjection and analyzed by histology. Normal rat retina is shown in **Fig. 3G**. Eyes injected with AdCMV.GFP, as a control, did not show any change, except for a small area around the injection site (**Fig. 3H**). Detachment of the neural retina from the RPE cell layer on both sides of the scar occurred during fixation and, provided all cell layers were present, was considered normal.

Development of choroidal neovascularization (CNV) (white arrow) was observed in histological analysis of an AdCMV.VEGF-injected eye (**Fig. 3K**), which is consistent with a previous report[9].

In AdCMV.SPARC-injected eyes, preretinal neo-

**Table 1.** Oligo-cap SST-selected clones

A: Initial screen of Oligo-cap SST-selected clones

| No. | ACCESSION | DEFINITION | Number of clones |
|---|---|---|---|
| 1 | NM_003118 | SPARC/osteonectin | 144 |
| 2 | NM_000088 | Collagen, type I, alpha 1 (COL1A1) | 19 |
| 3 | NM_002294 | Lysosomal-associated membrane protein 2 (LAMP2) | 7 |
| 4 | NM_003299 | Tumor rejection antigen (gp96) 1 (TRA1) | 7 |
| 5 | NM_013697 | Mus musculus transthyretin (Ttr) | 7 |
| 6 | NM_000491 | Complement component 1, q subcomponent, beta polypeptide polypeptide | 4 |
| 7 | NM_001908 | Cathepsin B (CTSB) | 4 |
| 8 | BC053656 | EGF-like repeats and discoidin I-like domains 3 | 4 |
| 9 | NM_002290 | Laminin, alpha 4 (LAMA4) | 4 |
| 10 | NM_000758 | Colony stimulating factor 2 (granulocyte-macrophage) (CSF2) | 4 |
| 11 | NM_001711 | Biglycan (BGN) | 3 |
| 12 | NM_139279 | Multiple coagulation factor deficiency 2 (MCFD2) | 3 |
| 13 | NM_020199 | Chromosome 5 open reading frame 15 (C5orf15) | 2 |
| 14 | NM_001425 | Epithelial membrane protein 3 (EMP3) | 2 |
| 15 | BC014410 | EGF-containing fibulin-like extracellular matrix protein 1 | 2 |
| 16 | NM_020650 | Reticulocalbin 3, EF-hand calcium binding domain | 2 |
| 17 | NM_002593 | Procollagen C-endopeptidase enhancer (PCOLCE) | 2 |
| 18 | NM_024756 | Multimerin 2 (MMRN2) | 2 |
| 19 | NM_006184 | Nucleobindin 1 (NUCB1) | 2 |
| 20 | NM_000041 | Apolipoprotein E (APOE) | 1 |
| 21 | NM_020405 | Plexin domain containing 1 (PLXDC1) | 1 |
| 22 | NM_002414 | CD99 antigen (CD99) | 1 |
| 23 | NM_177924 | N-acylsphingosine amidohydrolase (acid ceramidase) 1 (ASAH1) | 1 |
| 24 | NM_007085 | Follistatin-like 1 (FSTL1) | 1 |
| 25 | NM_023105 | Fibroblast growth factor receptor 1 (FGFR1) | 1 |
| 26 | NM_001849 | Collagen, type VI, alpha 2 (COL6A2) | 1 |
| 27 | BC010507 | CD14 antigen | 1 |
| 28 | NM_020010 | Mus musculus cytochrome P450, 51 (Cyp51) | 1 |
| 29 | NM_078474 | BBP-like protein 2 (BLP2) | 1 |
| 30 | NM_005211 | Colony stimulating factor 1 receptor (CSF1R) | 1 |
| 31 | NM_018259 | Tetratricopeptide repeat domain 17 (TTC17) | 1 |
| 32 | NM_001961 | Eukaryotic translation elongation factor 2 (EEF2) | 1 |
| 33 | NM_172355 | Membrane cofactor protein (MCP) | 1 |
| 34 | NM_003123 | Sialophorin (gpL115, leukosialin, CD43) (SPN) | 1 |
| 35 | NM_003278 | Tetranectin (plasminogen binding protein) (TNA) | 1 |
| 36 | NM_021983 | Major histocompatibility complex, class II, DR beta 4 (HLA-DRB4) | 1 |
| 37 | NM_001219 | Calumenin (CALU) | 1 |
| 38 | AF159295 | Serine/threonine protein kinase Kp78 | 1 |
| 39 | NM_212482 | Fibronectin 1 (FN1) | 1 |
| 40 | D14043 | MGC-24 | 1 |
| 41 | HSA276485 | Putative integral membrane transporter protein (LC27 gene) | 1 |
| | Other | | 45 |
| | Total | | 291 |

vascularization (dark arrowheads), proliferation of RPE (dark arrows) and the growth of blood vessels from the retinal vasculature into inner plexiform layers (asterisk), were demonstrated (**Fig. 3I, J**). In eyes injected with both AdCMV.SPARC and VEGF viruses, both of the changes seen in either AdCMV.SPARC- or

B: Second screen of Oligo-cap SST-selected clones

| No. | ACCESSION | DEFINITION | Number of clones |
|---|---|---|---|
| 1 | NM_003118 | SPARC/osteonectin | 137 |
| 2 | NM_001235 | Serine (or cysteine) proteinase inhibitor, clade H, member 1 (SERPINH1) | 6 |
| 3 | NM_000758 | Colony stimulating factor 2 (granulocyte-macrophage) (CSF2) | 5 |
| 4 | BC013858 | Clone IMAGE:3869909 | 4 |
| 5 | NM_022555 | Major histocompatibility complex, class II, DP beta 1 (HLA-DRB3) | 2 |
| 6 | NM_001032 | Ribosomal protein S29 (RPS29) | 2 |
| 7 | X02422 | Ig lambda L-chain V (VI)-J-C. | 2 |
| 8 | NM_003144 | Signal sequence receptor, alpha (SSR1) | 1 |
| 9 | HSA276485 | Putative integral membrane transporter protein (LC27 gene) | 1 |
| 10 | NM_013995 | Lysosomal-associated membrane protein 2 (LAMP2) | 1 |
| 11 | BC026246 | Alpha-2-macroglobulin | 1 |
| 12 | NM_024689 | Hypothetical protein FLJ14103 (FLJ14103) | 1 |
| 13 | NM_004438 | EphA4 (EPHA4) | 1 |
| 14 | BC056418 | Melanoma cell adhesion molecule | 1 |
| 15 | AK129843 | Highly similar to 6-phosphofructokinase, type C (EC2.7.1.11) | 1 |
| 16 | MMU564877 | Cryptdin related sequence peptide (CRS4C3dgene), strain C3H/HeN | 1 |
| | Other | | 2 |
| | Total | | 169 |

Identification of a secreted protein, SPARC, by Oligo-cap SST of proliferative tissue cDNAs.
A: 144 of 291 cDNA clones of the initial Oligo-cap SST-selected clones were the SPARC gene. B: 137 of 169 cDNA clones of the second Oligo-cap SST-selected clones were the SPARC gene.

AdCMV.VEGF-injected eyes were seen (**Fig. 3L**). The results of AdCMV.SPARC-injected eyes suggest that the overexpression of SPARC can develop preretinal neovascularization.

## Discussion

Neovascularization is an important event in PDR, where various secretory proteins, including multiple growth factors, are considered to be involved in this process. Recently, we developed the Oligo-cap SST strategy which enables us to screen for secretory or membrane proteins from minimal starting material. Using this method, we were able to screen a cDNA library constructed from a surgical specimen obtained from the eyes of patients with PDR. The majority of cloned cDNAs were found to encode SPARC, strongly suggesting that this gene is highly expressed in PDR. Surprisingly, VEGF was not cloned in our experiment, probably because the signal sequence is located at 1032 to 1109 base of the mRNA and was difficult to amplify by PCR; therefore, our result does not exclude the high expression of VEGF in PDR. To confirm our findings, concentrations of SPARC protein in vitreous samples from 64 diabetic patients undergoing vitreous

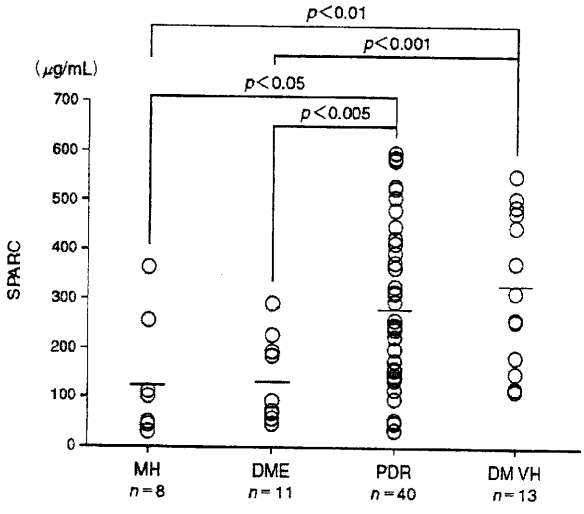


**Fig. 1.** Measurement of SPARC in patients with proliferative diabetic retinopathy.

Human SPARC-specific ELISA was performed in vitreous samples from diabetic patients with DME ($n=11$), DM VH ($n=13$), PDR ($n=40$). MH of control vitreous samples ($n=8$) was obtained from nondiabetic patients being treated for other ailments.
The vitreous fluid concentration of SPARC was significantly elevated in patients with PDR (285.3 ng/mL [38.2-602.1 ng/mL]) and DM VH (326.1 ng/mL [116.7-543.0 ng/mL]).

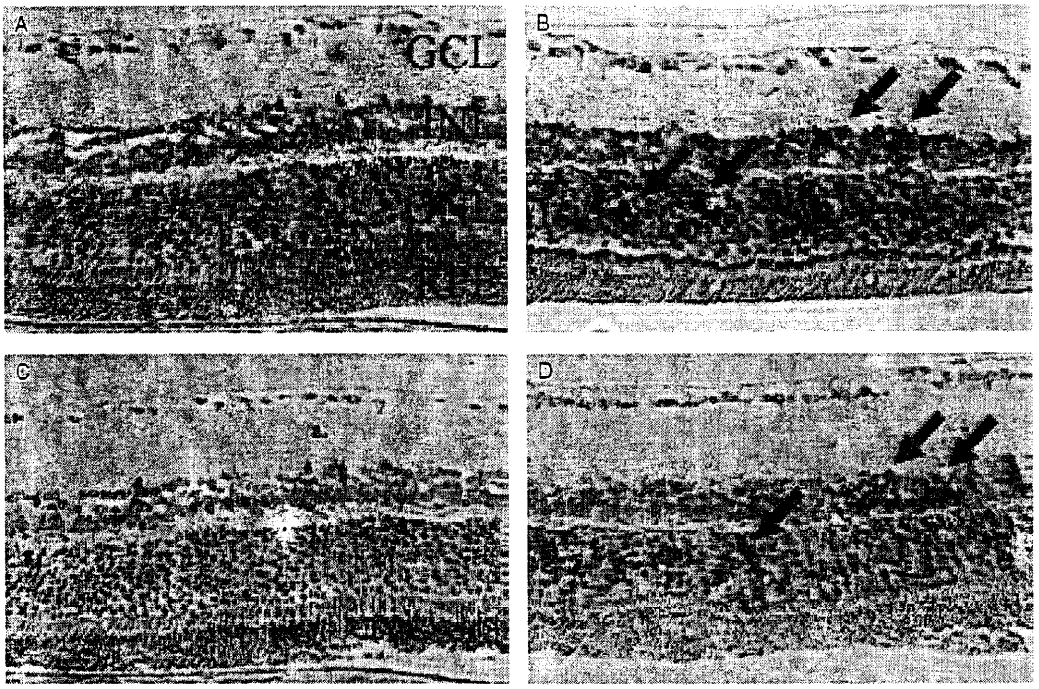


Fig. 2. Localization of SPARC mRNA in STZ rats.

A; Weak SPARC mRNA expression was observed in cells the ONL. B; Strong SPARC mRNA expression was observed in cells in INL and ONL. C; Intensity of VEGF mRNA expression was unchanged between sense and antisense probe. D: Weak VEGF mRNA expression was observed in cells in INL and ONL. A and C: the retinas of saline injected non-diabetic control rats. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (containing photoreceptor cells); RPE, retinal pigment epithelium. B and D: the retinas of STZ induced diabetic rats. Signals for SPARC or VEGF mRNA appeared as dark blue or purple precipitates (arrows).

surgery (40 with PDR, 13 with DM VH, and 11 with DME), and 8 nondiabetic patients who received vitrectomy for MH were analyzed. As expected, SPARC was significantly increased in patients with PDR, compared to patients with MH or DME. SPARC concentration was also shown to be increased in the vitreous of DM VH compared to MH or DME. There were no significant differences between the vitreous of MH and DME. These results indicated that SPARC is highly expressed in the vitreous of patients with proliferative tissue.

To investigate whether high levels of blood glucose may cause an increase in SPARC expression in the retina, we investigated the expression of SPARC in the retina of STZ-induced diabetic rats. As expected, the expression level of SPARC was markedly increased in the retina of STZ-induced diabetic rats compared to normal rats, suggesting that prolonged high levels of blood glucose may cause increased SPARC expression. Furthermore, subretinal injection of recombinant adenovirus expressing SPARC was shown to induce preretinal neovascularization, proliferation of RPE and the growth of blood vessels from the retinal vasculature into inner plexiform layers in the rat eye. These results suggest that in the retina of diabetic rats, a high blood glucose level increases the expression of SPARC, which in turn may directly induce neovascularization. In addition, simultaneous injection of recombinant adenoviruses expressing SPARC and VEGF was shown to have an additive effect on the induction of permeability and proliferation of blood vessels, suggesting that both SPARC and VEGF may be involved in the progression of DR. These findings strongly suggested that SPARC may be a major player involved in the development of diabetic retinopathy.

SPARC is a secreted glycoprotein with a molecular mass of 32 kDa that is associated with development, remodeling, angiogenesis modulates cell adhesion, migration, and proliferation[13]. It has been reported that the generation of proteolytic fragments from SPARC was localized to the tips of growing vessels and the fragment of (K) GHK motif was shown to be released from SPARC by plasmin[14], MMP-3[15], and peptides containing this sequence induced angio-

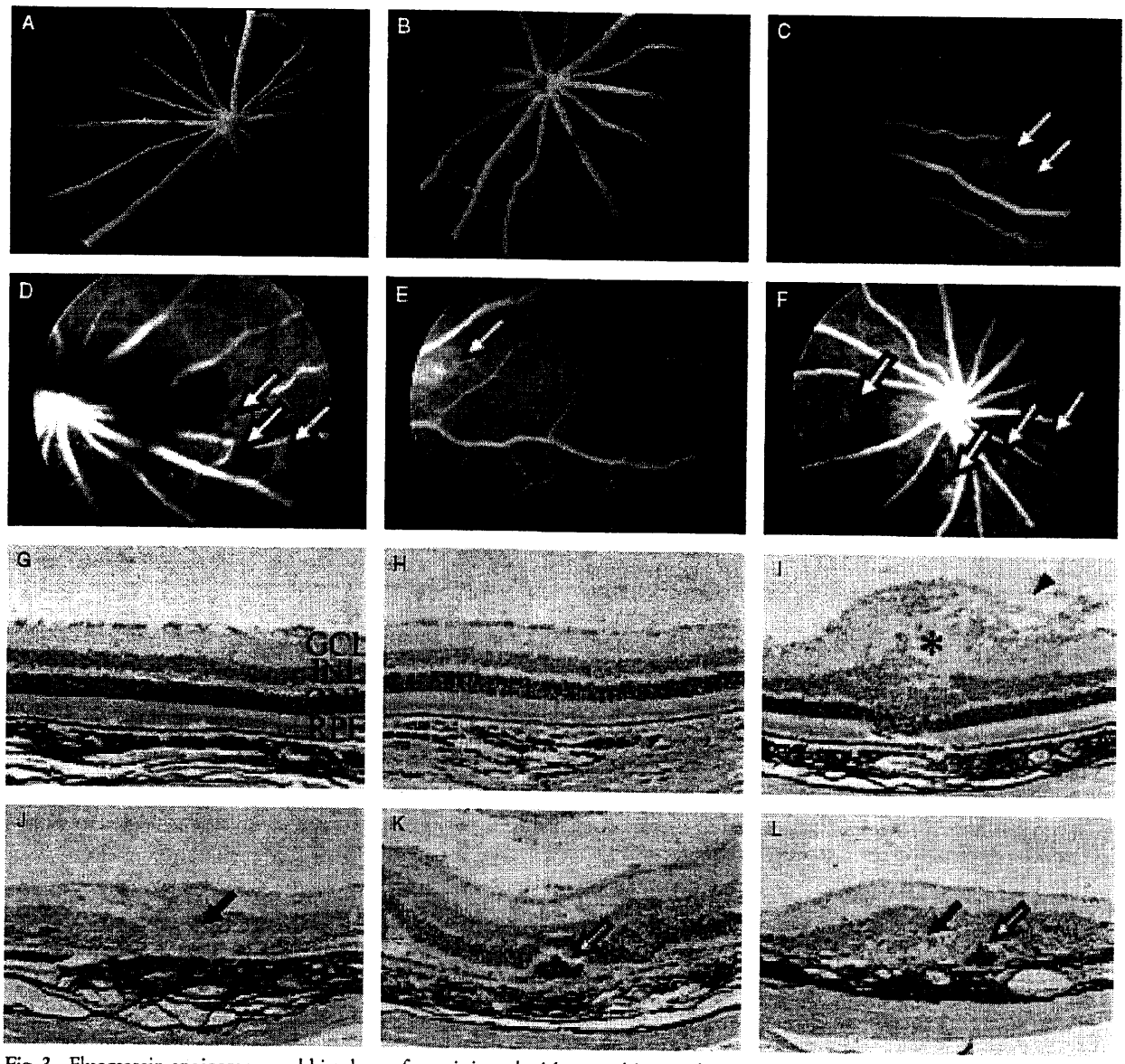


**Fig. 3.** Fluorescein angiograms and histology of eyes injected with recombinant adenovirus.

A to F: Fluorescein angiograms were observed 4 weeks post-subretinal injection with recombinant adenovirus. A: Control PBS injection. B: AdCMV.GFP. C and D: AdCMV.SPARC. E: AdCMV.VEGF. F: Combined AdCMV.SPARC and AdCMV.VEGF. Arrows indicate areas of vascular leakage.

G to L: Histology of eyes was observed 30 days post-subretinal injection with recombinant adenovirus. Five-micron sections of paraffin-embedded eyes were stained with Periodic acid-Schiff. G: Normal rat retina. H: AdCMV.GFP. I and J: AdCMV.SPARC. K: AdCMV.VEGF. L: combined AdCMV.SPARC and AdCMV.VEGF.

Dark arrowhead show preretinal neovascularization. Asterisk shows retinal vasculature in inner plexiform layers. Dark arrows show the proliferation of RPE. White arrows show development of CNV.

genesis *in vivo* and *in vitro*[13, 15, 16]. Several studies have suggested a correlation between SPARC and common diseases. It has been reported that chronic diabetic nephropathy was less severe in SPARC-null mice compared to WT mice, when induced to diabetes by STZ[17]. Plasma concentration of SPARC was shown to be increased in patients with coronary artery disease[18]. The migration of retinal pigment cells in proliferative vitreoretinopathy is modulated by SPARC[19]. Interestingly, the levels of SPARC expression in human

microvascular endothelium were shown to be increased by the addition of VEGF[20], which may suggest that SPARC is also involved in regulation by VEGF.

Our results have shown that SPARC may be directly involved in the progression of PDR. The potential of SPARC as a therapeutic target of PDR, as well as other diabetic complications, is an interesting challenge that should be investigated.

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# An Increase in Serum Retinol-Binding Protein 4 in the Type 2 Diabetic Subjects with Nephropathy

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**Abstract.** The present study was undertaken to determine retinol-binding protein 4 (RBP4) levels in subjects with diabetic nephropathy. A total of 149 type 2 diabetic subjects and 19 control subjects were enrolled. Serum levels of RBP4 were measured by a method of ELISA. Serum RBP4 levels were significantly greater in the subjects with type 2 diabetes mellitus than the controls (70.5 ± 35.3 vs. 40.1 ± 13.0 μg/ml, mean ± SD, $p<0.01$). Serum RBP4 levels were gradually increased according to the progression of diabetic nephropathy (p value in trend test: <0.001). Its elevation was significantly greater in the diabetic subjects with stages 1, 3B and 4 than the control subjects (Stage 1: 64.6 ± 29.7, Stage 3B: 123.3 ± 71.8, Stage 4: 91.4 ± 33.8 vs. Control: 40.1 ± 13.0 μg/ml, $p<0.01$). Similar results were obtained in the subjects based on the amount of albuminuria (Normo-: 64.6 ± 29.7, Micro-: 63.7 ± 29.4, and Marcoalbuminuria: 90.3 ± 44.6 μg/ml, $p <0.001$). Serum RBP4 levels had a positive correlation with serum creatinine levels($r = 0.377$, $p<0.001$), and a negative correlation with 1/creatinine ($r = -0.420$, $p<0.001$). Also, there was a negative correlation between serum RBP4 and the estimated glomerular filtration rate($r = -0.436$, $p<0.001$). Multiple regression analysis showed that estimated glomerular filtration rate was an independent determinant for increased serum RBP4 levels. There was no difference in serum RBP4 levels between the advanced nephropathy with and without macrovascular diseases. These results indicate an increase in serum RBP4 levels in the type 2 diabetic subjects, particularly complicated with advanced renal impairment.

*Key words*: RBP4, Diabetes mellitus, Diabetic nephropathy, Albuminuria, estimated GFR

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**RETINOL-BINDING** protein 4 (RBP4) is a small protein (molecular weight approximately 21 kDa), which is synthesized mainly in hepatocytes. RBP4 is also expressed in skeletal muscles and white adipose tissues, that are sensitive to insulin [1]. RBP4 binds to retinol and transthyretin, and delivers to the tissues [2, 3]. It is reported that RBP4 is increased in plasma of subjects with obesity, impaired glucose tolerance and diabetes mellitus [4, 5]. Yang *et al.* [6] showed that RBP4 is closely linked to insulin resistance in obesity and type 2 diabetes. Alterations in RBP4-transthyretin binding contribute to elevated serum RBP4 levels in insulin-resistant states [7]. However, there are several controversial results regarding insulin resistance [8–13]. In diabetic nephropathy plasma RBP4 levels are further elevated in diabetic subjects with microalbuminuria [14–16].

Diabetic nephropathy is the major microvascular complication of diabetes mellitus, which could progressively develop renal impairment. Endothelial dysfunction may be responsible for accelerated atherosclerosis in chronic kidney diseases. Damaged endothelial cells secrete cytokines and growth factors, and they accumulate into the subendothelial space of the injured region, thus promoting atherogenic change [17–22]. Adipose tissues also produce and secrete many adipokines, including adiponectin, leptin, tumor necrosis factor α, RBP4 and others. Among them, they group into 2 types regarding inflammatory and atherogenic effects. It is well known that tumor necro-

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sis factor α has an inflammatory and atherogenic property, whereas adiponectin has an anti-atherogenic and anti-inflammatory adipokine. Because RBP4 may be linked to insulin resistance, it could affect vascular deterioration.

In the present study we determined serum RBP4 levels in the subjects with diabetic nephropathy. Furthermore, we would clarify clinical feature of RBP4 in diabetic nephropathy and related atherosclerotic disorders.

## Subjects and Methods

*Subjects*

One hundred forty nine subjects with type 2 diabetes mellitus were enrolled in the present study between March 2005 and January 2007. They were admitted to Jichi Medical University Saitama Medical Center for 2 weeks to learn how and why to treat diabetes mellitus, and also to control their plasma glucose levels practically. They were 78 males and 71 females, with the ages of 63.9 ± 11.7 years (mean ± SD) ranging from 18 to 88 years. Ninety-one subjects had hypertension, 90 had hyperlipidemia, and 60 had obesity. Twenty-six subjects had ischemic heart disease, 18 subjects had cerebral vascular disease, and 6 had arteriosclerosis obliterans. According to the progression of nephropathy, the diabetic subjects were divided into stages 1–4 based on the classification of diabetic nephropathy by Research Committee of the Japanese Ministry of Health, Labor and Welfare for Disorders of diabetes mellitus [23]. Namely, stage 1, no microalbuminuria; stage 2, microalbuminuria (>30 mg/g creatinine, <300 mg/g creatinine); stage 3A, macroalbuminuria (>300 mg/g creatinine) or positive urinary protein analysis (<1 g urinary protein/g creatinine/day); stage 3B, proteinuria (>1 g urinary protein/g creatinine/day) and normal serum creatinine level; stage 4, elevated serum creatinine level; and stage 5, under dialysis treatment. The numbers of subjects taking medication for diabetes mellitus, hypertension and hyperlipidemia were summarized in Table 1. Also, 19 age- and gender-matched control subjects were collected from the subjects examining their medical status. They were 11 males and 8 females, with the ages of 59.2 ± 8.5 years (mean ± SD) ranging from 46 to 72 years. Eight subjects had hypertension, 11 had hyperlipidemia and 1 had obesity. Also, 3 subjects had ischemic heart disease, 5 subjects had cerebrovascular diseases, and 1 subject had arteriosclerosis obliterans. Blood samples

**Table 1.** Clinical and laboratory findings in the diabetic patients at the hospitalization

| | Control subjects | Diabetic patients | | | | | P value |
|---|---|---|---|---|---|---|---|
| | | Stage 1 | Stage 2 | Stage 3A | Stage 3B | Stage 4 | |
| N | 19 | 85 | 28 | 10 | 7 | 19 | |
| Sex (male/female) | 11/8 | 42/43 | 12/16 | 9/1 | 3/4 | 12/7 | |
| Age (years) | 59.2 ± 8.5 | 61.4 ± 11.5 | 65.5 ± 11.8 | 59.7 ± 14.3 | 69.1 ± 6.0 | 72.5 ± 6.9 | 0.0006 |
| BMI | 23.3 ± 1.2 | 24.4 ± 4.4 | 25.7 ± 4.2 | 24.3 ± 4.3 | 24.6 ± 3.0 | 25.3 ± 2.7 | 0.39 |
| Systolic blood pressure (mmHg) | 132.9 ± 11.0 | 132.2 ± 18.0 | 138.1 ± 16.9 | 147.0 ± 20.3 | 139.3 ± 16.8 | 135.4 ± 19.7 | 0.14 |
| Diastolic blood pressure (mmHg) | 72.8 ± 8.6 | 75.1 ± 10.5 | 77.6 ± 9.9 | 78.8 ± 12.2 | 70.1 ± 8.8 | 69.6 ± 10.1 | 0.063 |
| Total cholesterol (mg/dl) | 203.6 ± 41.8 | 209.2 ± 68.8 | 203.8 ± 43.6 | 203.1 ± 39.3 | 235.9 ± 30.1 | 180.3 ± 47.0 | 0.31 |
| HDL cholesterol (mg/dl) | 47.3 ± 7.5 | 46.5 ± 13.2 | 50.1 ± 11.5 | 46.9 ± 11.0 | 48.6 ± 16.1 | 44.5 ± 14.5 | 0.74 |
| Triglyceride (mg/dl) | 130.9 ± 47.6 | 227.1 ± 427.7 | 157.6 ± 103.2 | 141.3 ± 58.9 | 181.1 ± 84.3 | 119.8 ± 46.3 | 0.67 |
| Fasting plasma glucose (mg/dl) | 109.3 ± 10.9 | 162.3 ± 56.8 | 179.1 ± 54.1 | 159.3 ± 62.9 | 153.0 ± 65.1 | 129.7 ± 50.9 | 0.0002 |
| HbA1c (%) | 5.6 ± 0.1 | 8.9 ± 1.6 | 9.3 ± 2.1 | 9.2 ± 1.5 | 8.9 ± 1.3 | 8.1 ± 1.7 | 0.0067 |
| Creatinine (mg/dl) | 0.70 ± 0.10 | 0.65 ± 0.15 | 0.64 ± 0.16 | 0.77 ± 0.12 | 1.00 ± 0.27 | 1.25 ± 0.28 | <0.0001 |
| 1/creatinine | 1.46 ± 0.19 | 1.62 ± 0.41 | 1.66 ± 0.43 | 1.32 ± 0.18 | 1.06 ± 0.26 | 0.84 ± 0.18 | <0.0001 |
| Estimated GFR (ml/min/1.73m2) | 87.8 ± 14.6 | 84.5 ± 21.0 | 84.2 ± 22.3 | 76.7 ± 13.7 | 48.6 ± 8.6 | 40.0 ± 9.0 | <0.0001 |
| Diabetes mellitus (n) (diet only/drug/insulin) | 0 | 5/61/19 | 0/15/13 | 2/5/3 | 0/5/3 | 1/9/9 | 0.074 |
| Hypertension (n) (%) | 8 (42.1) | 38 (44.7) | 19 (67.9) | 9 (90) | 7 (100) | 18 (94.7) | <0.0001 |
| Hyperlipidemia (n) (%) | 11 (57.9) | 55 (64.7) | 15 (53.6) | 3 (30) | 6 (85.7) | 11 (57.9) | 0.22 |
| Ischemic heart disease (IHD) (n) (%) | 3 (15.8) | 11 (12.9) | 4 (14.3) | 1 (10) | 2 (28.5) | 8 (42.1) | 0.062 |
| Cerebral vascular disease (CVD) (n) (%) | 5 (26.3) | 7 (8.2) | 3 (10.7) | 3 (30) | 1 (14.2) | 4 (21.1) | 0.154 |
| Arteriosclerosis obliterans (ASO) (n) (%) | 1 (5.3) | 1 (1.2) | 1 (3.6) | 0 | 0 | 4 (21.1) | 0.006 |

Values are mean ± SD. Values are analyzed by one-way ANOVA or chi-square for independence test.

were collected from the subjects in the supine position after an overnight fast to determine fasting plasma glucose, hemoglobin A1c, total cholesterol, high-density lipoprotein cholesterol, triglyceride, and serum RBP4 at the time of hospitalization or visiting to the outpatient clinic. Risk factors for atherosclerosis were defined as follows: Dyslipidemia was defined as a total cholesterol of greater than 220 mg/dl, a high-density lipoprotein cholesterol level of less than 40 mg/dl, and a triglyceride level of greater than 150 mg/dl, or the subject's having taken either statins or fibrates. Hypertension was defined as systolic blood pressure of greater than 140 mmHg, diastolic pressure of greater than 90 mmHg, or the subject's having taken antihypertensive agents. The present study was approved by the ethical committee of Jichi Medical University for human studies. We obtained informed consents from the subjects who joined the present protocol.

*Measurements*

Blood samples were collected into tubes and centrifuged at 3,000 rpm at 4°C for 15 minutes. The supernatants were decanted and frozen at −80°C until assayed. RBP4 was measured by the method of ELISA using Human RBP4 ELISA kits (AdipoGen, Seoul, Korea). Fasting plasma glucose, hemoglobin A1c, total cholesterol, HDL-cholesterol, triglyceride and creatinine were determined by standard laboratory methods. Urine samples were collected in the morning and 24-hour urine collection was made. Renal function was calculated as the estimated glomerular filtration rate (eGFR) by the Modification of Diet in Renal Disease equation (MDRD) revised for Japanese by the Japan Society of Nephrology: eGFR (ml/min/1.73 $m^2$) = $0.741 \times 175 \times age^{-0.203} \times (\text{serum creatinine (mg/dl)})^{-1.154} \times (0.742, \text{in female})$.

*Statistical analysis*

All values are expressed as means ± SD. The values were analyzed by one-way ANOVA to compare the difference among the groups. Categorical data were analyzed by the $\chi^2$ test. Simple regression analysis was performed to evaluate correlation between the parameters. Also, we checked the independence of parameter by multiple regression analysis. The statistical packages of Statcel Statistical Software (Second Edition, OMS Publishing Inc., Japan) and Mulcel Statistical Software (First Edition, OMS Publishing Inc., Japan) were employed for the present analysis. A p values less than 0.05 was considered significant.

## Results

We compared clinical features in the diabetic subjects and control subjects (Table 1). Fasting plasma glucose and hemoglobin A1c were elevated in the diabetic subjects as compared to the control subjects (p value for trend test: 0.0002 and 0.0067). The number of hypertensive subjects was increased according to the progression of diabetic nephropathy (p value for trend test <0.0001).

Serum RBP4 levels were increased to 70.5 ± 35.3 μg/ml in all the subjects with type 2 diabetes mellitus, a value significantly greater than that of 40.1 ± 13.0 μg/ml in the control subjects ($p<0.01$). Initially, we analyzed serum RBP4 levels in the diabetic subjects according to the amount of albuminuria. Serum RBP4 was significantly increased in the subjects with macroalbuminuria as compared to that in the subjects having normoalbuminuria (Normo-: 64.6 ± 29.7, Micro-: 63.7 ± 29.4, and Macroalbuminuria: 90.3 ± 44.6 μg/ml, p value for trend test <0.01).

Fig. 1 shows serum RBP4 levels in the diabetic subjects following the stage of nephropathy. According to the progression of diabetic nephropathy, serum RBP4 levels were gradually elevated in the diabetic subjects (p value for trend test <0.001). Its elevation was significantly greater in the diabetic subjects with stages 1, 3B and 4 than the control subjects (Control: 40.1 ±

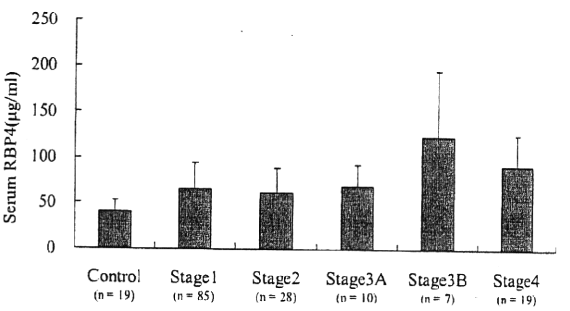


**Fig. 1.** Serum RBP4 levels in the diabetic patients on the classification of nephropathy and the controls. Serum RBP4 levels in the patients with stages 1, 3B and 4 were significantly elevated than the control subjects (p-value for trend test <0.001). Values are means ± SD.

**Table 2.** Linear regression analysis of serum RBP4 levels with varying parameters in the diabetic patients

| | Parameters | r | p-value |
|---|---|---|---|
| RBP4 vs. | Age | 0.199 | 0.015 |
| | BMI | 0.023 | 0.775 |
| | Systolic blood pressure | 0.008 | 0.927 |
| | Diastolic blood pressure | –0.128 | 0.12 |
| | Total cholesterol | 0.088 | 0.285 |
| | HDL-cholesterol | –0.062 | 0.45 |
| | Triglyceride | –0.011 | 0.89 |
| | Fasting plasma glucose | –0.092 | 0.264 |
| | HbA1c | –0.113 | 0.17 |
| | HOMA-R | –0.064 | 0.528 |
| | Fasting serum insulin | –0.086 | 0.398 |
| | Creatinine | 0.377 | <0.001 |
| | 1/Creatinine | –0.42 | <0.001 |
| | Estimated GFR | –0.436 | <0.001 |
| | Log (albuminuria) | 0.211 | 0.015 |

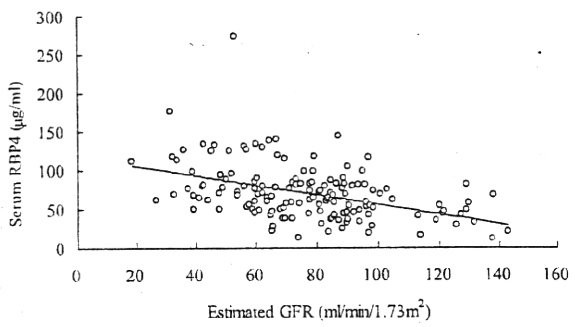


**Fig. 2.** The relationship of serum RBP4 levels with estimated GFR in the diabetic patients. Serum RBP4 (μg/ml) = 117.6 – 0.62 × estimated GFR, r = –0.436, p<0.001.

13.0 μg/ml vs. Stage 1: 64.6 ± 29.7, p<0.05, Stage 3B: 123.3 ± 71.8, p <0.01, and Stage 4: 91.4 ± 33.8 μg/ml, p<0.01).

Table 2 shows the correlation between serum RBP4 levels and varying parameters in the diabetic subjects with nephropathy. Serum RBP4 levels had a positive correlation with serum creatinine levels (r = 0.377, p<0.001), and a negative correlation with 1/creatinine (r = –0.420, p<0.001). Fig. 2 depicts the relationship between the estimated glomerular filtration rate (GFR) and serum RBP4 levels in the diabetic subjects. Serum RBP4 levels had a negative correlation with the estimated GFR (r = –0.436, p<0.001). In addition, serum RBP4 levels positively correlated with log-transformed albuminuria. Multiple regression analysis was performed using the following parameters, which were associated with serum RBP4 levels at the p<0.1 levels in the simple linear regression analysis (Table 2). Estimated GFR was an independent determinant for increased serum RBP4 levels (standardized coefficient = –0.466, p<0.001).

## Discussion

The present study demonstrated that serum RBP4 levels were elevated in the diabetic subjects with nephropathy. eGFR was equivalent in the control subjects and the diabetic subjects with stage 1, but serum RBP4 levels were significantly increased in the stage 1 diabetic subjects compared with the controls. The marked difference was only the deterioration of plasma glucose control between the stage 1 diabetic subjects and the control. Though it is still not clear that plasma glucose could alter RBP4 level, impaired control of plasma glucose may affect the elevation of serum RBP4 in the diabetic subjects. We analyzed several parameters to clarify the association of serum RBP4 with renal impairment. A significant increase in serum RBP4 was obtained in the subjects with macroalbuminuria, but not in those with normo- and microalbuminuria. Similar study was reported by Raila *et al.* [14]. They noted that serum RBP4 was increased in patients with microalbuminuria, an early stage of diabetic nephropathy. Similarly, serum RBP4 levels were elevated according to the progression of clinical stages of diabetic nephropathy. They were further elevated in the diabetic subjects with stages 3B and 4. Furthermore, serum RBP4 levels had a negative correlation with the estimated GFR in the diabetic subjects. There was a possibility that the discrepancy of the findings from those of previous reports may result from the analysis using different assay kit of RBP4. Ziegelmeier *et al.* [24] reported a fourfold increase in serum RBP4 levels in patients with chronic hemodialysis compared with control subjects. Their patients included both diabetic and non-diabetic patients. As in the present study the diabetic subjects were admitted to learn how and why control plasma glucose, end-stage kidney disease was not contained in the analysis. Nevertheless, the finding was in concert with that of Ziegelmeier *et al.* in the end-stage kidney disease. These results indicate an increase in serum RBP4 levels in advanced renal impairment of the diabetic subjects.

The previous study has suggested that RBP4 is related to insulin resistance in diabetic subjects [4, 5]. We analyzed linear regression of serum RBP4 with other variables as shown in Table 2. Serum RBP4 did not correlate with either of fasting serum insulin levels and HOMA-R, a finding distinct from the study of Graham *et al.* [4]. The discrepancy may come from the blood samplings, that is, the blood was collected in a steady state of glucose homeostasis in the non-hospitalized subjects in the study of Graham *et al.* [4], and it was collected in the hospitalized, poorly controlled subjects in the present study. No relationship of serum RBP4 levels with insulin resistance was also reported by several investigators [8–13]. There was no difference in serum RBP4 levels between the subjects with and without macrovascular diseases. In the subjects with advanced diabetic nephropathy (stages 3 and 4) no difference in serum RBP4 was between the presence and absence of macrovascular diseases. Taken together, there was no evidence that elevation of serum RBP4 is associated with insulin resistance and atherosclerotic changes in the diabetic subjects. However, study limitation of the present analysis was come from the small number of the subjects with atherosclerotic diseases. Further study will be necessary to elucidate the exact relation of RBP4 with atherosclerotic diseases.

RBP4 is the primary carrier for vitamin A (retinol) in plasma and synthesized by the hepatocytes. RBP4 expression is also present in extrahepatic tissues including skeletal muscles and white adipose tissues [1]. It is known that kidney plays a role in maintenance of whole body retinol homeostasis [25, 26], which is regulated by glomerular filtration rate and subsequent reabsorption of RBP4 into proximal tubule. The present study showed a negative correlation of serum RBP4 with the estimated GFR, and an increase in serum RBP4 was apparently found in the advanced diabetic nephropathy. Though the clearance study was not performed, a decrease in GFR could accumulate RBP4 in the systemic circulation [27, 28]. Also, a protein complex of RBP4 bound to transthyretin homotetramer in the systemic circulation may reduce renal clearance of RBP4. Further study will have to be clarified whether kidney actually involves in plasma retinol homeostasis and whether renal-hepatic or renal-extrahepatic pathway stimulates the binding of retinol and RBP4.

As shown in Table 2, serum RBP4 levels had significant correlation with age, serum creatinine, 1/creatinine and estimated GFR. Also, the profound correlation of estimated GFR with serum RBP4 was proven by multiple regression analysis. Either of BMI, plasma glucose control, lipid metabolism and blood pressure did not affect serum RBP4 levels. Also, inflammatory alternations of CRP and IL-18 did not correlate with serum RBP4 levels (data not shown). Thus, metabolic and inflammatory changes did not affect serum RBP4 levels in the diabetic subjects with nephropathy.

In summary, the present study clarified that serum RBP4 levels were increased in the diabetic subjects. According to the progression of diabetic nephropathy, serum RBP4 was gradually elevated. There was a negative correlation between serum RBP4 levels and the estimated GFR. The alteration in serum RBP4 levels did not associate with insulin resistance and macrovascular diseases. The present study indicates an increase in serum RBP4 levels in the type 2 diabetic subjects, particularly complicated with advanced renal impairment.

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analysis showed a significant decrease in receptor expression at the plasma membrane ($Na^+/K^+$-ATPase) of treated cells compared to control (from 4.9±1.9 to 0.3±0.01%, $p<0.05$), with the receptor pool being retained in the Golgi apparatus (Gm130). Coherently with the impaired IRß trafficking to the plasma membrane, we also observed a 1,4 fold reduction in the transcriptional activity of the *insulin* promoter.
**Conclusion:** Our data suggest that the ß-secretes activity is needed for *insulin* expression in ß-cells and that the BACE2 is the enzyme involved. Thus, BACE2 is here presented as a potentially essential enzyme for ß-cell function.
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**Transgenic mice expressing an intestine-specific secretory protein, IBCAP, demonstrates pancreatic beta cell augmenting activity**
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**Background and aims:** Recent success with GLP-1 analogs and DPP IV inhibitory drugs in the clinical application for diabetic patients has highlighted the role of intestine as a hormone producing organ. Crucial roles of these hormones, including GLP-1, GIP and Ghrelin, in the control of energy metabolism and food intake, and their relation with the metabolic syndrome have been brought to worldwide attention. In the current studies, we aimed to search for secretory proteins expressed in the intestine.
**Materials and methods:** We have constructed and screened a mouse intestinal cDNA library to search for genes encoding secretory and membrane proteins, using the Oligo-cap Signal Sequence Trap (Oligo-cap SST) method developed in our laboratory. For CF266 functional analysis *in vivo*, we prepared the recombinant CF266 expressing adenovirus and we analyzed the phenotype of CF266 over-expression mice model.
**Results:** We have identified CF266 as a novel intestine-specific secretory protein using the Oligo-cap SST strategy. We demonstrated that CF266 had insulin secretion promoting effect, and furthermore, that adenovirus-mediated expression of CF266 in STZ-treated type 1 diabetes model mice improved the blood glucose level of the animal, and showed the increased pancreatic β-cells detected by the histological analysis. Here, we have developed transgenic (Tg) mice expressing CF266 under the control of CAG-promoter. Analyses of the Tg mice have shown marked increase of pancreatic islets, confirming our former findings with the STZ-induced diabetic mice treated with CF266 expressing adenovirus. Thus, we renamed CF266 as IBCAP; intestine-derived beta-cell augmenting promoter. Further analyses have shown that blood glucose concentration and OGTT of IBCAP Tg mice are relatively normal compared with the control mice. Therefore, IBCAP seems to have promoted the augmentation of islets which are functionally normal. We are now testing whether augmentation of the β-cell islets is due to inhibition of apoptosis or stimulation of proliferation.
**Conclusion:** Our findings will provide IBCAP as another potential therapeutic target for diabetes and pancreatic β-cell regeneration.
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# PS 35 Beta cell signal transduction II

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**Increased phosphorylation of FOXO1 during glucocorticoid excess is not mediated by SGK1 (Serum glucocorticoid inducible kinase 1) in insulin secreting cells**
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**Background and aims:** The essential role of insulin receptor signalling including PI3K and PKB activation for sufficient insulin disposal is well documented. Previously, we demonstrated that glucocorticoids induce the expression of serum- and glucocorticoid-inducible kinase 1 (SGK1), an enzyme with 54 % identity in the catalytic domain with PKB and stimulated by insulin. SGK1 in parallel to PKB exerts anti-apoptotic effects in a variety of cells. Indeed, unlike glucocorticoids, transfection with SGK1 did not augment apoptotic cell death of insulin secreting INS-1E cells. The aim of the present study was to examine whether FOXO1, a PKB substrate, is regulated by SGK1 in cells under glucocorticoid excess.
**Materials and methods:** Insulin secreting INS-1E cells were treated with dexamethasone (dexa, 100 nM) to induce the endogenous expression of SGK1. SGK1 activity was selectively inhibited with a specific inhibitor (GSK650394) or by transient transfection with siRNA against SGK1. Alternatively, cells were transiently transfected with constitutive active or dominant negative hSGK1. Expression and phosphorylation of proteins were analyzed by Western blotting. Cellular distribution of immunostained proteins was examined using confocal microscopy.
**Results:** Treatment of the cells with dexa reduced phosphorylation of PKB, but paradoxically increased phoshorylation of FOXO1. The inhibition of PKB by Akti-1/2 abolished phosphorylation of FOXO1 in control cells. In dexa-treated cells FOXO1 phosphorylation was inhibited by Akti-1/2 but not by SGK1 inhibitor (up to 10 µM). In parallel, Akti-1/2 promoted nuclear translocation of FOXO1 while SGK1 inhibitor did not. Pretreatment of cells with siRNA against SGK1 inhibited the induction of SGK1 by dexa by 66 % at the mRNA level and by 80 % at the protein level. Although SGK1 protein level was reduced dexa treatment still increased FOXO1 phosphorylation. Furthermore, in cells transfected with either constitutive active or dominant negative hSGK1 phosphorylation of FOXO1 was unchanged.
**Conclusion:** These data suggest that the increased phosphorylation of FOXO1 observed after dexa-treatment is sensitive to PKB inhibition but does not depend on SGK1 in INS-1E cells.
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**PLCζ expressing in islet beta cells: does it express in sperm specifically?**
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**Background and aims:** Phospholipase C (PLC) is considered to modulate insulin secretion stimulated by diverse factors such as nutrients, hormone, neurotransmitter and ions. There are six established families of PLC termed β, γ, δ, ε, ζ and η, however, the role of each isoform in stimulated insulin secretion remains obscure. In this study, several isoforms of PLC which could express in pancreatic beta cell were identified. And the expression level of each isoform after stimulated by different factors was quantified in order to reveal the function of each isoform in insulin secretion.
**Materials and methods:** The expression levels of the isoforms of PLC in INS-1 cells (a rat insulinoma cell line ) were semi-quantified by RT-PCR. In order to study on the role of each isoform in different factor-stimulated insulin secretion, glucose, L-aminoglutaminic acid (L-GLU), chloratum kalium (KCL), cholecystokinin-octopeptide (CCK8), and acerylcholine chloride were chosen as stimulating factors. INS-1cells were stimulated by the factors mentioned above respectively, and the mRNA level of each PLC isoform was semi-quantified by RT-PCR.
**Results:** Surprisingly, PLCζ, an isoform of PLC which was thought to express specifically in sperms was detected in INS-1 cell line and rat pancreatic tissue in our study. The expression of PLCδ, PLCβ, PLCγ and PLCη could also be detected in INS-1 cells except for PLCε. Many isoforms were induced when INS-1 cell line stimulated by different factors. PLCδ (1.97 folds), PLCβ and

Original Article

# Comparative Study of Atherosclerotic Parameters in Mongolian and Japanese Patients with Hypertension and Diabetes Mellitus

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***Aim***: Cardiovascular disease is becoming increasingly more problematic in Mongolia. The cardio-ankle vascular index (CAVI) and circulating C-reactive protein (CRP) are new atherosclerosis-related parameters, but no comparative studies of atherosclerotic parameters including CAVI and CRP are available between Mongolian and Japanese populations, such as disease populations of hypertension (HT) and diabetes mellitus (DM). Our study objective was to examine atherosclerotic profiles in HT and DM patients in both countries.

***Methods***: From a hospital-based population, 156 Mongolian outpatients with HT and DM (men: 46%, mean age: 57.1 years) and 156 age- and sex-matched Japanese outpatients with HT and DM (men: 46%, age: 57.7) were recruited. Body mass index (BMI), heart rate (HR), blood pressure (BP), pulse pressure (PP), ankle-brachial index (ABI), ultrasonographic carotid intima-media thickness (IMT), blood total cholesterol (T-Cho), glucose, insulin and homeostasis model assessment of insulin resistance (HOMA-IR) were measured, in addition to CAVI and CRP.

***Results***: The levels of BMI, HR, BP, PP, insulin and IMT were significantly higher and T-Cho and glucose were significantly lower in the Mongolian patients in comparison to the Japanese patients. Particularly, the levels of CAVI (mean ± SD) (8.1 ± 1.1 vs. 8.8 ± 1.2) and CRP (median [interquartile range]) (0.05 [0.03–0.12] vs. 0.19 [0.09–0.42] mg/dL) were significantly higher in Mongolian than Japanese patients. These significant differences remained unchanged, even after taking into account multiple variables, including BP and HOMA-IR. In addition, except for CAVI in the subgroup of DM, generally similar trends regarding atherosclerotic parameters were seen in the subgroup by sex and disease (HT, DM and HT plus DM).

***Conclusion***: These findings suggest that Mongolian patients with HT and DM may be at higher risk for cardiovascular disease than Japanese patients.

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**Key words;** Atherosclerosis, Cardio-ankle vascular index, C-reactive protein, Carotid intima-media thickness, Ethnicity

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## Introduction

Atherosclerosis is the leading pathology of cardiovascular disease (CVD) and is currently a major worldwide cause of mortality[1]. The World Health

Organization (WHO) statistical data in 2005 reported Mongolian people to have a 17-year shorter life expectancy than Japanese people[2]. CVD has been increasing and has become a more important public health problem than cancer in Mongolia[3]. From the data in 2006 among Mongolians (aged 15–64 years), the prevalence of hypertension (HT) was 28.1% and diabetes mellitus (DM) was 8.2%, respectively, thus suggesting that both are common diseases (the same has been said in Japan)[4]. Young and healthy Mongolian people could have a higher risk for CVD than Japanese people of a similar generation[5]. International comparative studies may give some insights to determine atherosclerotic traits among Mongolian and Japanese subjects, since the Japanese have a clearly higher average lifespan.

The cardio-ankle vascular index (CAVI) and ankle-brachial index (ABI) are both known to be indexes associated with atherosclerotic risk factors[6]. In particular, CAVI is a new index of arterial stiffness, and is more independent of systolic and diastolic blood pressure (SBP and DBP) than the measurement of pulse wave velocity (PWV)[6]. ABI is also useful for the diagnosis of low extremity peripheral arterial stenotic disease[7]; therefore, CAVI and ABI are considered to be associated with CVD occurrence[6, 8].

Atherosclerotic risk factors, such as age, male sex, smoking, lack of exercise, obesity, dyslipidemia, DM and HT, are usually considered in the pathophysiology of CVD[9, 10]. In addition, inflammatory processes have recently received a great deal of attention in atherogenesis, and C-reactive protein (CRP) is one of the representative markers[11]. However, there are few comparative studies on atherosclerosis between Mongolian and Japanese populations[5, 12], and there are no such studies examining CAVI and CRP in particular in patients with atherosclerosis-related diseases. Therefore, the objective of the present study was to compare the atherosclerotic traits, including CAVI and CRP, between Mongolian and Japanese patients with HT and DM.

## Subjects and Methods

The recruitment of patients for this study was carried out between 2006 and 2008 at the Hospital Number 2 and the State Mental Hospital of Mongolia (Ulaanbaatar city, Mongolia) for Mongolians and at the Jichi Medical University Hospital (Tochigi Prefecture, Japan) for Japanese, respectively. This study was approved by the Ethics Committee of Jichi Medical University and the Mongolian Ministry of Health, and each patient gave their informed consent. Initially, adult outpatients (≥20 years of age) with HT and DM, without a medical history of CVD and without serum levels of ≥220 mg/dL in total cholesterol (T-Cho) and ≥150 mg/dL in triglyceride (TG)[13], were consecutively recruited from a random invitation to participate in the present study. HT was defined as ≥140 mmHg systolic blood pressure (SBP) and/or ≥90 mmHg diastolic blood pressure (DBP) or the use of drugs against HT[14]. DM was defined as ≥126 mg/dL fasting plasma glucose or the use of drugs against DM[15]. Whether drugs for HT and/or DM were prescribed was confirmed by self-reports (the precise names of the drugs themselves were not required). During this initial recruitment, 199 Mongolian patients and 166 Japanese patients were enrolled, respectively. Of the 199 Mongolian patients, 21 patients, who were unsure of the indication for the drugs they used for HT and DM, were excluded. Next, considering the imbalanced distribution of age to compare the Mongolian and Japanese patient groups (the age range of Mongolian patients was relatively wide), from the initial Mongolian patients ($n$=178) and Japanese ($n$=166), age- and sex-matched data (1:1 ratio with ±1 year of age in respective Mongolian and Japanese patient groups) were selected in a further random manner. The age range of patients in this selection was 30 to 69 years. Thereafter, the final study cohort included 156 Mongolian patients with HT and DM (men: 71, women: 85; mean age: 57.1 years) and 156 Japanese patients with HT and DM (men: 71, women: 85; age: 57.7 years). The percentages of HT alone, DM alone and HT plus DM were 46.8% ($n$=73), 30.8% ($n$=48) and 22.4% ($n$=35) among Mongolian patients, and 48.1% ($n$=75), 28.2% ($n$=44) and 23.7% ($n$=37) among Japanese patients, respectively.

The patients self-reported their smoking habits, which were defined as current or former/never smokers. Body mass index (BMI) was calculated as the weight divided by the square of the body height while wearing light clothes. Blood samples were collected from the antecubital vein after a 12-hr overnight fast and serum/plasma samples were stored at −80℃. Blood measurements were performed at one laboratory facility in Japan. The serum T-Cho and plasma glucose were measured enzymatically and plasma insulin was measured by a sandwich enzyme immunoassay (TOSOH Co. Ltd., Tokyo, Japan). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated based on the plasma insulin and glucose levels, as described previously[16]. Serum CRP was measured using a latex agglutination immune assay (EIKEN Chemical, Co. Ltd., Tokyo, Japan).

**Table 1.** Comparison of atherosclerotic parameters between Japanese and Mongolian patients

| Parameters | Japanese $n=156$ | Mongolian $n=156$ |
|---|---|---|
| Age, years | 57.7 ± 8.4 | 57.1 ± 8.4 |
| Men, % | 45.5 | 45.5 |
| Smoking, % | 17.9 | 26.3** |
| BMI, kg/m$^2$ | 23.7 [21.5-26.3] | 27.5 [24.0-31.0]** |
| HR, bpm | 64.0 [58.0-71.0] | 67.5 [59.0-78.0]* |
| SBP, mmHg | 128.0 [119.0-138.0] | 147.0 [134.0-164.0]** |
| DBP, mmHg | 80.0 [75.0-88.3] | 92.0 [82.3-101.8]** |
| PP, mmHg | 47.0 [41.0-53.0] | 53.5 [44.0-65.8]** |
| T-Cho, mg/dL | 186.6 [172.0-199.5] | 180.0 [160.3-194.8] |
| Glucose, mg/dL | 107.5 [94.0-144.0] | 83.0 [75.3-113.3]** |
| Insulin, $\mu$U/mL | 6.1 [3.8-9.2] | 8.9 [5.7-14.9]** |
| HOMA-IR | 1.60 [1.01-2.81] | 2.11 [1.17-3.87] |
| CRP, mg/dL | 0.05 [0.03-0.12] | 0.19 [0.09-0.42]** |
| IMT, mm | 0.70 [0.60-0.89] | 0.84 [0.67-1.51]** |
| ABI | 1.16 [1.11-1.20] | 1.15 [1.10-1.20] |
| CAVI | 8.1 ± 1.1 | 8.8 ± 1.2** |
| Disease composition | | |
| HT, % | 48.1 | 46.8 |
| DM, % | 28.2 | 30.8 |
| HT plus DM, % | 23.7 | 22.4 |
| Medication | | |
| Non-pharmacological therapy, % | 7.1 | 13.3 |
| Anti-hypertensive drugs, % | 41.0 | 46.2 |
| Anti-diabetic drugs, % | 22.4 | 26.3 |

Age and CAVI were presented as the mean ± standard deviation. Other parameters are presented as the median [interquartile range]. BMI: body mass index, HR: heart rate, SBP: systolic blood pressure, DBP: diastolic blood pressure, PP: pulse pressure, T-Cho: total cholesterol, HOMA-IR: homeostasis model assessment of insulin resistance, CRP: C-reactive protein, IMT: intima-media thickness, ABI: ankle-brachial index, CAVI: cardio-ankle vascular index, HT: hypertension, DM: diabetes mellitus. Significance level: *$p<0.05$, **$p<0.01$ (sex, smoking and disease composition: $\chi^2$-test, other parameters: unpaired $t$-test after the log-transformation; smoking adjustment was performed).

CAVI was determined by oscillometry using the VaSera VS-1000 vascular screening system (Fukuda Denshi Co. Ltd., Tokyo, Japan). CAVI values are based on the stiffness parameter calculated using the following formula: $CAVI = a\ \{(2\rho/\Delta P) \times \ln(Ps\text{-}Pd)\ PWV^2\} + b$; that is, Ps: SBP, Pd: DBP, $\rho$: blood density, $\Delta P$: (Ps-Pd), PWV: pulse wave velocity, a and b: constants[6]. The values of SBP, DBP, heart rate (HR) and pulse pressure (PP: calculated as SBP minus DBP) were measured in a simultaneous setting. Next, after relaxing for 5 minutes in the supine position, the intima-media thickness (IMT) of the bilateral common carotid arteries of patients was measured by ultrasonography (EH54-9DR system; DIASUS, Scotland, UK). IMT levels were determined by the average values at points 1, 2 and 3 cm below the carotid bifurcation on each side of the artery.

**Statistical Analyses**

Data are presented as the mean ± standard deviation. Parameters with non-parametric distributions are shown as the median and interquartile range. In all analyses, parameters with non-parametric distributions were used after log-transformation. Comparisons between groups were examined using the unpaired $t$-test (for continuous variables) and the $\chi^2$-test (for categorical variables). Since the smoking percentage could differe between Mongolian and Japanese patients, smoking was usually considered to be a confounding factor. Comparisons with adjustments for smoking and other factors were analyzed using the general linear model, entering the adjusted variables as co-variables. All statistical analysies were performed with the Statistical Package for Social Science (SPSS) version 16.0 for Windows (SPSS Inc., Chicago, USA).