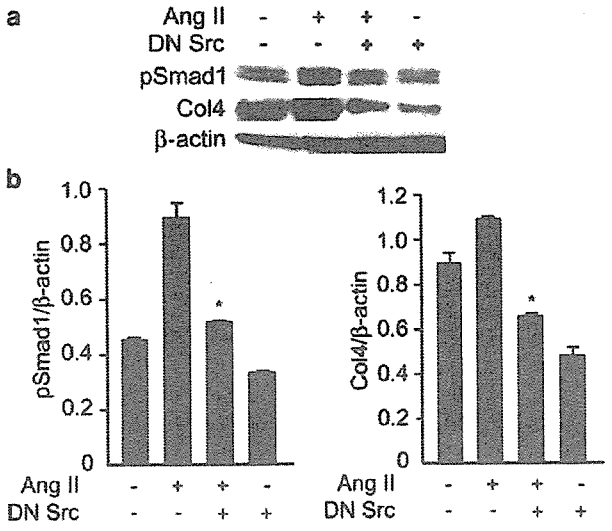

Figure 7 Effects of dominant negative Src on Ang II-mediated signaling in mesangial cells. (**a**) Effect of dominant negative Src on pSmad1 and Col4 in mesangial cells. After mesangial cells were transfected with dominant negative Src or mock vector, cells were starved and stimulated with Ang II (0.1 μM, 15 min). A representative data from three independent experiments is shown. (**b**) Optical densitometry of pSmad1 and Col4 in Western blotting. *$P<0.05$ *vs* mock vector with Ang II stimulation.

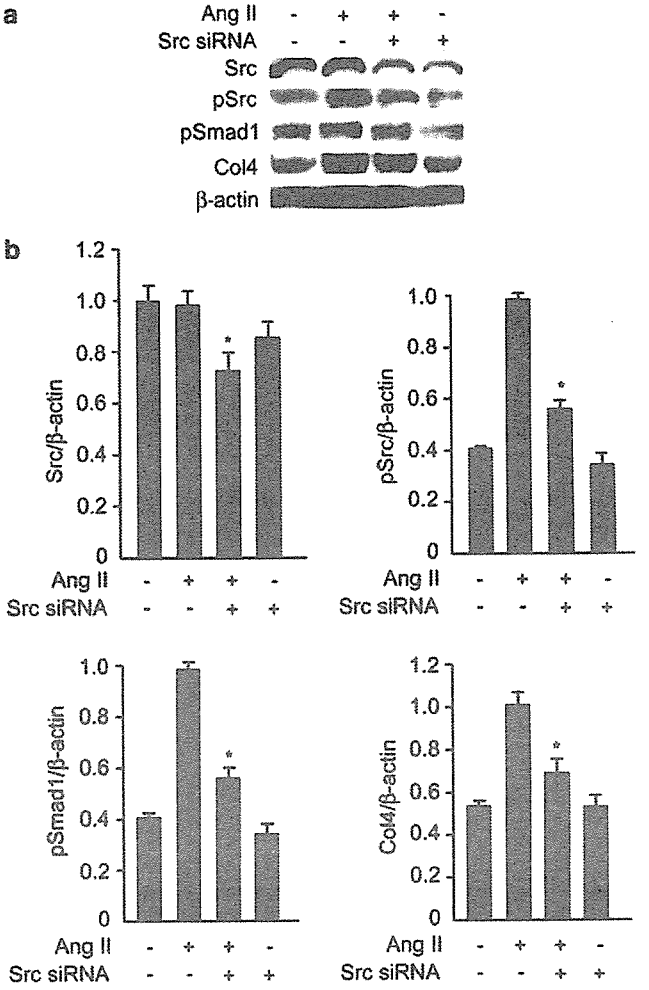

Figure 8 Effects of siRNA against Src on of Ang II-mediated signaling in mesangial cells. (**a**) Effect of siRNA knockdown of Src on Src, pSrc, pSmad1, and Col4 in mesangial cells. After mesangial cells were transfected with control siRNA or Src-specific siRNA, cells were starved and stimulated with Ang II (0.1 μM, 15 min). A representative data from three independent experiments is shown. (**b**) Optical densitometry of Src, pSrc, pSmad1, and Col4 in Western blotting. *$P<0.05$ *vs* control siRNA with Ang II stimulation.

the inhibition of the Smad1- and Src-mediated pathway. We also demonstrated that Ang II could stimulate Col4 production and induce a mesangial phenotypic change through Src and Smad1. This is the first demonstration showing the molecular mechanism of Ang II and AT1 through the Src/Smad1 pathway on mesangial matrix expansion in diabetic nephropathy.

In mesangial matrix expansion, mesangial cells undergo a phenotypic change with markedly upregulated expression of Col4 and α-SMA.[34,37] It is well known that Ang II plays an important role in the development of mesangial matrix expansion.[14,38] Previous studies have reported that transforming growth factor-β (TGF-β) and its downstream signaling as a critical factor in mediating mesangial matrix expansion in diabetes and that one mechanism whereby Ang II promotes diabetic mesangial matrix expansion is by stimulating TGF-β production.[39,40] However, TGF-β is not sufficient for the induction of mesangial matrix expansion.[41] Therefore, another signaling pathway might present to activate Smad1, inducing mesangial matrix expansion in the early phase of diabetic nephropathy. In this regard, we propose here that Ang II can also activate Smad1 via an Src-dependent pathway in the development of mesangial matrix expansion in the early phase of diabetic nephropathy. Therefore, we tried to dissect the role of RAS in the development of mesangial matrix expansion in the early phase of diabetic nephropathy in STZ diabetic models. These models are acceptable, because they can mimic those occurred in the early phase of human diabetic nephropathy, in terms of the association between functional and structural changes. However, they show only minimal glomerular structural changes even after 1 year of diabetes,[42] and STZ itself has virulence and carciogenecity. In this study, we demonstrated that one of the mechanisms of AT1 antagonists to improve in the early phase of diabetic nephropathy is mesangial matrix expansion through the inhibitory effect on the expression of Col4 and α-SMA possibly through the Src/Smad1 pathway in STZ rats. This conclusion is based on our following observations. Our *in vitro* study demonstrated that Ang II could induce rapid phosphorylation of Smad1 and Src in cultured mesangial cells and this phosphorylation was blocked by AT1 antagonists. We also showed that Ang II induced Col4 expression, which was also blocked by AT1 antagonists. Further, PP2[43] significantly inhibited not only Ang

II-induced phosphorylation of Src, but also of Smad1. The experiments by dominant negative and siRNA also confirmed the presence of interaction of Src and Smad1. Treatment of STZ rats with olmesartan clearly inhibited the phosphorylation of Src and Smad1 in the glomerulus. Thus our data indicate a novel interaction of Ang II, Src, and Smad1. So far, there is one report showing the possibility of interaction between Ang II and Smad7 in diabetic mouse.[44] To our knowledge, however, there is no report showing that Src is involved in diabetic nephropathy. Therefore, our study highlights the importance of Src in diabetic nephropathy. However, a direct molecular mechanism between Src and Smad1 is still unclear. Therefore, further study is needed to delineate the signaling mechanism in details.

In this study, we treated rats with a low dose of olmesartan and found no significant difference in systolic blood pressure among the four groups studied. In addition, olmesartan attenuated diabetic mesangial matrix expansion, independent of its effects on glucose metabolism. Indeed, in this study, olmesartan-treated rats showed a slight decline in creatinine clearance and albuminuria. There was a statistical difference between the groups, but are not so large. Therefore, the effect of AT1 antagonists in this study might be relatively small. Previous studies show that cyclic stretching of cultured mesangial cells that may occur in response to glomerular hypertension leads to increased synthesis of matrix proteins and TGF-β.[45,46]

Recently, mesangial cells have been shown to produce TGF-β when exposed to advanced glycation end products (AGEs).[47] We also showed that chronic exposure of AGEs induces the increase of Smad1 gene activation and expression, leading to Col4 overproduction via a TGF-β-dependent pathway.[18] Fukami *et al*[48] showed that AGEs can induce reactive oxygen species generation, which activates TGF-β-Smad2 signaling and subsequently induces mesangial cell hypertrophy and fibronectin synthesis by autocrine production of Ang II. Others indicate that intrarenal generation of Ang II is elevated in diabetes, which subsequently leads to TGF-β secretion.[40,49,50] These studies explained the interaction between Ang II and TGF-β in diabetic nephropathy. Therefore, it is intriguing whether these effects of Ang II in our study are mediated through TGF-β. However, the phosphorylation of Smad1 occurred very rapidly in mesangial cells. Further, TGF-β-neutralizing antibody did not affect the phosphorylation of Smad1 induced by Ang II (data not shown). Thus, our data indicate that the Ang II-Src/Smad1 pathway is independent of TGF-β activation in mesangial cells, supported by the observation that TGF-β is not sufficient for the induction of mesangial matrix expansion.[41]

Therapeutic approaches to downregulate TGF-β signaling under diabetic conditions provide one strategy for the treatment of diabetic nephropathy. In experimental animals, the endogeneous proteoglycan decorin, a natural inhibitor of TGF-β,[51,52] and the use of a neutralizing TGF-β antibody[53,54] have been shown to prevent the development of diabetic nephropathy. Our study indicates that the inhibition of Src may be a useful treatment for diabetic nephropathy. However, these experimental therapeutic approaches have not been practical in diabetic patients yet. Therefore, the administration of AT1 antagonists, which can downregulate the signaling of Src/Smad1 should be a first-line therapy for diabetic nephropathy.

Ang II binding to AT1 receptor triggers the activation of Ca^{2+} signaling and protein kinase C. The signal is then transmitted to tyrosine (Src) phosphorylation, mitogen-activated protein kinase leading to proliferation.[55] In this study, we have shown the direct link between Src and Smad1 activation, and subsequent increase of Col4 synthesis in mesangial cells. However, the phosphorylation of Smad1 is independent of p44/42 MAP kinase activation in mesangial cells. Therefore, the Ang II-dependent Src-Smad1 pathway is specifically responsible for the development of diabetic mesangial matrix expansion.

In conclusion, our study indicates that Ang II can modulate Smad1-mediated signaling in diabetic mesangial matrix expansion via an Src-dependent pathway. Our study also implies that AT1 antagonists can prevent the glomerular structural changes in the early phase of diabetic nephropathy through the inhibition of Src and Smad1.

Acknowledgements

We thank Hideo Uchiyama (Taigenkai Hospital), Maki Watanabe, and Ayumi Hosotani (Kyoto University) for excellent technical assistance. This study was supported by Grants-in Aid from the Ministry of Education, Culture, Science, Sports, and Technology of Japan; Center of Excellence grants from the Ministry of Education, Culture, Science, Sports, and Technology of Japan (12CE2006); a research grant for health sciences from the Japanese Ministry of Health, Labor, and Welfare; and a grant from Takeda Medical Research Foundation.

References

1 Striker GE, Schainuck LI, Cutler RE, *et al.* Structural–functional correlations in renal disease. I. A method for assaying and classifying histopathologic changes in renal disease. Hum Pathol 1970;1:615–630.
2 Alpers CE, Hudkins KL, Gown AM, *et al.* Enhanced expression of 'muscle-specific' actin in glomerulonephritis. Kidney Int 1992;41:1134–1142.
3 Ziyadeh FN. The extracellular matrix in diabetic nephropathy. Am J Kidney Dis 1993;22:736–744.
4 Fogo AB. Diabetic nephropathy: it's in the numbers. Kidney Int 2002;61:2274–2275.

5 Lewis EJ, Hunsicker LG, Bain RP, *et al.* The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group. N Engl J Med 1993;329:1456–1462.
6 Brenner BM, Cooper ME, de Zeeuw D, *et al.* Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. N Engl J Med 2001;345:861–869.
7 Lewis EJ, Hunsicker LG, Clarke WR, *et al.* Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. N Engl J Med 2001;345:851–860.
8 Ruggenenti P, Perna A, Gherardi G, *et al.* Renal function and requirement for dialysis in chronic nephropathy patients on long-term ramipril: REIN follow-up trial. Gruppo Italiano di Studi Epidemiologici in Nefrologia (GISEN). Ramipril Efficacy in Nephropathy. Lancet 1998;352:1252–1256.
9 Mimran A, Alfaro V. Candesartan: nephroprotective effects and treatment of diabetic nephropathy. Drugs Today (Barc) 2003;39:439–450.
10 Anderson S, Jung FF, Ingelfinger JR. Renal renin–angiotensin system in diabetes: functional, immunohistochemical, and molecular biological correlations. Am J Physiol 1993;265:F477–F486.
11 Anderson S. Role of local and systemic angiotensin in diabetic renal disease. Kidney Int Suppl 1997;63: S107–S110.
12 Ichikawa I, Brenner BM. Glomerular actions of angiotensin II. Am J Med 1984;76:43–49.
13 Anderson S, Rennke HG, Brenner BM. Therapeutic advantage of converting enzyme inhibitors in arresting progressive renal disease associated with systemic hypertension in the rat. J Clin Invest 1986;77:1993–2000.
14 Fogo AB. The role of angiotensin II and plasminogen activator inhibitor-1 in progressive glomerulosclerosis. Am J Kidney Dis 2000;35:179–188.
15 Striker LJ, Doi T, Elliot S, *et al.* The contribution of glomerular mesangial cells to progressive glomerulosclerosis. Semin Nephrol 1989;9:318–328.
16 Striker LJ, Peten EP, Elliot SJ, *et al.* Mesangial cell turnover: effect of heparin and peptide growth factors. Lab Invest 1991;64:446–456.
17 Stokes MB, Hudkins KL, Zaharia V, *et al.* Up-regulation of extracellular matrix proteoglycans and collagen type I in human crescentic glomerulonephritis. Kidney Int 2001;59:532–542.
18 Abe H, Matsubara T, Iehara N, *et al.* Type IV collagen is transcriptionally regulated by Smad1 under advanced glycation end product (AGE) stimulation. J Biol Chem 2004;279:14201–14206.
19 Matsubara T, Abe H, Arai H, *et al.* Expression of Smad1 is directly associated with mesangial matrix expansion in rat diabetic nephropathy. Lab Invest 2006;86: 357–368.
20 Brown MT, Cooper JA. Regulation, substrates and functions of src. Biochim Biophys Acta 1996;1287: 121–149.
21 Fessart D, Simaan M, Laporte SA. c-Src regulates clathrin adapter protein 2 interaction with beta-arrestin and the angiotensin II type 1 receptor during clathrin-mediated internalization. Mol Endocrinol 2005;19:491–503.
22 Waters CM, Connell MC, Pyne S, *et al.* c-Src is involved in regulating signal transmission from PDGFbeta receptor-GPCR(s) complexes in mammalian cells. Cell Signal 2005;17:263–277.
23 Kansra S, Stoll SW, Johnson JL, *et al.* Src family kinase inhibitors block amphiregulin-mediated autocrine ErbB signaling in normal human keratinocytes. Mol Pharmacol 2005;67:1145–1157.
24 Liu J, Huang C, Zhan X. Src is required for cell migration and shape changes induced by fibroblast growth factor 1. Oncogene 1999;18:6700–6706.
25 Taneda S, Hudkins KL, Topouzis S, *et al.* Obstructive uropathy in mice and humans: potential role for PDGF-D in the progression of tubulointerstitial injury. J Am Soc Nephrol 2003;14:2544–2555.
26 Hudkins KL, Gilbertson DG, Carling M, *et al.* Exogenous PDGF-D is a potent mesangial cell mitogen and causes a severe mesangial proliferative glomerulopathy. J Am Soc Nephrol 2004;15:286–298.
27 Takahashi T, Abe H, Arai H, *et al.* Activation of STAT3/Smad1 is a key signaling pathway for progression to glomerulosclerosis in experimental glomerulonephritis. J Biol Chem 2005;280:7100–7106.
28 Flyvbjerg A, Bennett WF, Rasch R, *et al.* Inhibitory effect of a growth hormone receptor antagonist (G120K-PEG) on renal enlargement, glomerular hypertrophy, and urinary albumin excretion in experimental diabetes in mice. Diabetes 1999;48:377–382.
29 Nangaku M, Miyata T, Sada T, *et al.* Anti-hypertensive agents inhibit *in vivo* the formation of advanced glycation end products and improve renal damage in a type 2 diabetic nephropathy rat model. J Am Soc Nephrol 2003;14:1212–1222.
30 Makibayashi K, Tatematsu M, Hirata M, *et al.* A vitamin D analog ameliorates glomerular injury on rat glomerulonephritis. Am J Pathol 2001;158:1733–1741.
31 Yamamoto Y, Kato I, Doi T, *et al.* Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. J Clin Invest 2001;108:261–268.
32 Nagai K, Matsubara T, Mima A, *et al.* Gas6 induces Akt/mTOR-mediated mesangial hypertrophy in diabetic nephropathy. Kidney Int 2005;68:552–561.
33 Pippin JW, Qu Q, Meijer L, *et al.* Direct *in vivo* inhibition of the nuclear cell cycle cascade in experimental mesangial proliferative glomerulonephritis with Roscovitine, a novel cyclin-dependent kinase antagonist. J Clin Invest 1997;100:2512–2520.
34 Takeoka H, Iehara N, Uematsu-Yanagita M, *et al.* A multifunctional transcription factor (A1p145) regulates the smooth muscle phenotype in mesangial cells. Biochem Biophys Res Commun 1998;252: 290–295.
35 Nagai K, Arai H, Yanagita M, *et al.* Growth arrest-specific gene 6 is involved in glomerular hypertrophy in the early stage of diabetic nephropathy. J Biol Chem 2003;278:18229–18234.
36 Stockelman MG, Lorenz JN, Smith FN, *et al.* Chronic renal failure in a mouse model of human adenine phosphoribosyltransferase deficiency. Am J Physiol 1998;275:F154–F163.
37 Abe H, Iehara N, Utsunomiya K, *et al.* A vitamin D analog regulates mesangial cell smooth muscle phenotypes in a transforming growth factor-beta type II receptor-mediated manner. J Biol Chem 1999;274: 20874–20878.
38 Fogo AB. Mechanisms in nephrosclerosis and hypertension-beyond hemodynamics. J Nephrol 2001; 14(Suppl 4):S63–S69.

39 Border WA, Noble NA. Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis. Hypertension 1998;31:181–188.
40 Weigert C, Brodbeck K, Klopfer K, *et al.* Angiotensin II induces human TGF-beta 1 promoter activation: similarity to hyperglycaemia. Diabetologia 2002;45: 890–898.
41 Yang CW, Striker GE, Chen WY, *et al.* Differential expression of glomerular extracellular matrix and growth factor mRNA in rapid and slowly progressive glomerulosclerosis: studies in mice transgenic for native or mutated growth hormone. Lab Invest 1997;76:467–476.
42 Zatz R, Meyer TW, Rennke HG, *et al.* Predominance of hemodynamic rather than metabolic factors in the pathogenesis of diabetic glomerulopathy. Proc Natl Acad Sci USA 1985;82:5963–5967.
43 Hanke JH, Gardner JP, Dow RL, *et al.* Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J Biol Chem 1996;271:695–701.
44 Liao J, Kobayashi M, Kanamuru Y, *et al.* Effects of candesartan, an angiotensin II type 1 receptor blocker, on diabetic nephropathy in KK/Ta mice. J Nephrol 2003;16:841–849.
45 Riser BL, Cortes P, Zhao X, *et al.* Intraglomerular pressure and mesangial stretching stimulate extracellular matrix formation in the rat. J Clin Invest 1992;90:1932–1943.
46 Riser BL, Cortes P, Heilig C, *et al.* Cyclic stretching force selectively up-regulates transforming growth factor-beta isoforms in cultured rat mesangial cells. Am J Pathol 1996;148:1915–1923.
47 Throckmorton DC, Brogden AP, Min B, *et al.* PDGF and TGF-beta mediate collagen production by mesangial cells exposed to advanced glycosylation end products. Kidney Int 1995;48:111–117.
48 Fukami K, Ueda S, Yamagishi S, *et al.* AGEs activate mesangial TGF-beta-Smad signaling via an angiotensin II type I receptor interaction. Kidney Int 2004;66: 2137–2147.
49 Nagai Y, Yao L, Kobori H, *et al.* Temporary angiotensin II blockade at the prediabetic stage attenuates the development of renal injury in type 2 diabetic rats. J Am Soc Nephrol 2005;16:703–711.
50 Singh R, Alavi N, Singh AK, *et al.* Role of angiotensin II in glucose-induced inhibition of mesangial matrix degradation. Diabetes 1999;48:2066–2073.
51 Border WA. Transforming growth factor-beta and the pathogenesis of glomerular diseases. Curr Opin Nephrol Hypertens 1994;3:54–58.
52 Isaka Y, Brees DK, Ikegaya K, *et al.* Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney. Nat Med 1996;2: 418–423.
53 Ziyadeh FN, Hoffman BB, Han DC, *et al.* Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal anti-transforming growth factor-beta antibody in db/db diabetic mice. Proc Natl Acad Sci USA 2000;97: 8015–8020.
54 Chen S, Iglesias-de la Cruz MC, Jim B, *et al.* Reversibility of established diabetic glomerulopathy by anti-TGF-beta antibodies in db/db mice. Biochem Biophys Res Commun 2003;300:16–22.
55 Sabri A, Govindarajan G, Griffin TM, *et al.* Calcium- and protein kinase C-dependent activation of the tyrosine kinase PYK2 by angiotensin II in vascular smooth muscle. Circ Res 1998;83:841–851.

Gender Difference in Coronary Events in Relation to Risk Factors in Japanese Hypercholesterolemic Patients Treated With Low-Dose Simvastatin

Jun Sasaki, MD; Toru Kita, MD*; Hiroshi Mabuchi, MD**; Masunori Matsuzaki, MD†; Yuji Matsuzawa, MD††; Noriaki Nakaya, MD‡; Shinichi Oikawa, MD‡‡; Yasushi Saito, MD§; Kazuaki Shimamoto, MD§§; Suminori Kono, MD¶; Hiroshige Itakura, MD¶¶,a; the J-LIT Study Group

Background Gender differences between the risk factors for coronary heart disease and coronary events were examined in the Japan Lipid Intervention Trial, a 6-year observational study.

Methods and Results Men (12,575) and women (27,013) were analyzed for risk of coronary events (acute myocardial infarction and sudden cardiac death). Simvastatin reduced serum low-density lipoprotein cholesterol (LDL-C) by 27% in both genders, and increased serum high-density lipoprotein cholesterol (HDL-C) in men (5%) and women (4%). The incidence of coronary events was lower in women (0.64/1,000 patient-years) than in men (1.57/1,000 patient-years). The risk of coronary events increased by 18% in men and 21% in women with each 10 mg/dl elevation of LDL-C, and decreased by 39% in men and 33% in women with each 10 mg/dl elevation of HDL-C. The risk increased proportionally with aging in women, but not in men. Diabetes mellitus (DM) was more strongly related to the risk of coronary events for women (relative risk 3.07) than for men (relative risk 1.58).

Conclusions The incidence of coronary events is lower in women. Serum LDL-C is related to an increased risk of coronary events to the same extent in both genders. DM seems to be a more important risk factor in women, trading off the lower risk of coronary events among them. (*Circ J* 2006; **70:** 810–814)

Key Words: Coronary events; Hyperlipidemia; Risk factors; Serum cholesterol; Sex differences

Coronary heart disease (CHD), including myocardial infarction and cardiac sudden death, is one of the leading causes of death in Japan[1] The risk of developing CHD is known to be markedly different between men and women:[2,3] CHD incidence is 2 to 5 times higher among middle-aged men than women. In the Japan Lipid Intervention Trial (J-LIT),[4–7] we previously reported that serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) concentrations were positively and serum high-density lipoprotein cholesterol (HDL-C) concentration was inversely related to CHD or cerebrovascular disease risk in patients under treatment for hypercholesterolemia. The role of coronary risk factors in the development of CHD has been studied extensively in men[8–10] but relatively few studies have investigated women[2,11]

(Received January 16, 2006; revised manuscript received March 13, 2006; accepted March 29, 2006)

*International University of Health and Welfare Graduate School of Clinical Trial Management, Fukuoka, *Kyoto University Graduate School of Medicine, Kyoto, **Kanazawa University Graduate School of Medicine, Kanazawa, †Yamaguchi University Graduate School of Medicine, Ube, ††Sumitomo Hospital, Osaka, ‡Nakaya Clinic, ‡‡Nippon Medical School, Tokyo, §Chiba University Graduate School of Medicine, Chiba, §§Sapporo Medical University School of Medicine, Sapporo, ¶Kyushu University Faculty of Medical Sciences, Fukuoka and ¶¶Ibaraki Christian University, Hitachi, Japan*

[a]Hiroshige Itakura, MD, was a Chairman of the Central Committee.

Mailing address: Jun Sasaki, MD, International University of Health and Welfare Graduate School of Clinical Trial Management, 1-3-1 Nagahama, Chuo-ku, Fukuoka 810-0072, Japan. E-mail: jsas@nifty.com

This study aimed to assess gender differences in the association of risk factors with CHD in the J-LIT data. The J-LIT is a nationwide cohort study of 52,421 hypercholesterolemic patients treated with open-labeled low-dose simvastatin (5–10 mg/day)[4,5] The J-LIT included a large number of female patients, and we were able to investigate the gender difference in the role of risk factors in the occurrence of coronary events.

Methods

Study Design

The design of the J-LIT study has been previously described[12] Briefly, study patients with serum TC concentration ≥220 mg/dl, men aged 35–70 years and postmenopausal women aged 70 years or less, were treated with 5–10 mg/day of simvastatin. Body weight, serum lipid concentrations (TC, LDL-C, HDL-C, and triglyceride (TG)) were measured at baseline, and patients were interviewed as regards family history of CHD, number of cigarettes smoked, and the amount of alcohol ingestion. Serum lipid concentrations and CHD-related events (acute myocardial infarction and cardiac sudden death) were monitored every 6 months for 6 years in all patients, including those who discontinued simvastatin. Serum lipid concentrations were determined in each study institution, and the serum LDL-C concentration was calculated using the Friedewald formula for patients with TG concentration ≤400 mg/dl[13] Study physicians recommended dietary and exercise-therapy for hyperlipidemia to all patients. Additional lipid-lowering

agents were allowed only when an adequate response in serum TC concentration was not gained by simvastatin monotherapy. Each patient was informed of the purpose and method of the study, drug efficacy and the need for long-term treatment and they gave verbal, not written, informed consent.

Subjects

Patients who had been previously treated with a lipid-lowering agent were screened for eligibility after a washout period of at least 4 weeks. For patients previously treated with probucol, the washout period was at least 12 weeks. The exclusion criteria were the occurrence of acute myocardial infarction or stroke within the past month, concurrent uncontrolled diabetes mellitus (DM), serious hepatic or renal disease, secondary hypercholesterolemia, cancer or any other illness with potentially poor survival.

Of the 52,421 patients enrolled, 5,127 were excluded because of a history of CHD, 4,934 for lack of follow-up data, and 2,772 for missing data of the covariates. Therefore, data from 39,588 patients (12,575 men, 27,013 women) were used in the present study.

Endpoints

The primary endpoints were major coronary events, defined as nonfatal and fatal myocardial infarction and sudden cardiac death. Incidence of myocardial infarction or death was counted once for each patient during the treatment, and the follow-up data thereafter were excluded from the analysis. The events were reviewed and determined by the Endpoint Classification Committee.

Statistical Analysis

The mean lipid concentrations were calculated using data available at the follow-up points in time during the treatment period. The data of lipid concentrations after the onset of events were excluded. Data during the treatment period after discontinuation of simvastatin were also included for analysis. Mean values for serum lipid concentrations and age were tested with unpaired t-test, and the prevalence of baseline characteristics were tested with the chi-square test for comparison between men and women. Patients in each sex were categorized into 5–6 groups according to the mean lipid concentrations of treatment period for TC, TG, LDL-C and HDL-C with intervals of 20, 50, 20, 10 mg/dl, respectively, and for the LDL-C/HDL-C ratio with an interval of 0.5. The reference category for the relative risk was set on the group with the lowest lipid concentrations and the lowest value of LDL-C/HDL-C ratio. Relative risks and the 95% confidence intervals (CI) were calculated using the Cox proportional hazards model with adjustment for baseline characteristics such as sex, age, hypertension, DM, body mass index (BMI), ECG abnormality, family history of CHD, alcohol ingestion and cigarette smoking. Heterogeneity between men and women was evaluated by the likelihood ratio test. Two-sided p-value <0.05 was considered statistically significant. All the statistical calculations were performed using SAS software (version 8.02, SAS Institute, Inc, Cary, NC, USA).

Results

Serum Lipids and Other Risk Factors

There were no significant difference as regards the prevalence of obesity (BMI ≥25.0 kg/m^2), hypertension, ECG abnormality, and family history of CHD between men and women (Table 1). In men, the prevalence of DM was higher ($p<0.001$), and cigarette smoking and alcohol ingestion were much more frequent ($p<0.001$).

Table 1 Baseline Characteristics of the Subjects

	Men (n=12,575)	*Women (n=27,013)*
Age (years)	*54.0 (9.1)*	*59.5 (6.5)*
Obesity (%)[a]	*36.7*	*32.2*
Hypertension (%)[b]	*45.4*	*46.3*
Diabetes mellitus (%)[c]	*20.0*	*13.9*
ECG abnormality (%)[d]	*13.4*	*12.9*
Family history of CHD (%)[e]	*5.1*	*4.8*
Cigarette smoking (%)[e]	*43.8*	*4.1*
Alcohol use (%)[e]	*73.4*	*8.7*
Lipid profiles		
Baseline (mg/dl)		
TC	*268 (41)*	*271 (31)*
LDL-C	*178 (34)*	*184 (33)*
TG	*250 (241)*	*169 (111)*
HDL-C	*49 (15)*	*55 (15)*
During the treatment (mg/dl)		
TC	*218 (31)*	*221 (29)*
LDL-C	*130 (31)*	*135 (28)*
TG	*198 (133)*	*148 (77)*
HDL-C	*51 (13)*	*57 (14)*

Figs are mean ± SD unless otherwise specified.
CHD, coronary heart disease; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol.
[a] Body mass index ≥25 kg/m^2. [b] Systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg or medication for hypertension. [c] Fasting plasma glucose ≥140 mg/dl or medication. [d] Study physician's diagnosis. [e] Self-reported information.

Lipid profiles at baseline and during the treatment period are shown for men and women in Table 1. Men had higher concentrations of serum TG and lower concentrations of serum HDL-C at baseline and during the treatment in comparison with women. Mean percent changes in the TC, LDL-C, TG, and HDL-C concentrations from baseline to during the treatment in men were −18.8% ($p<0.001$), −27.2% ($p<0.001$), −20.9% ($p<0.001$), and +4.7% ($p<0.001$), respectively, and the corresponding values in women were −18.2% ($p<0.001$), −26.6% ($p<0.001$), −12.8% ($p<0.001$) and +4.4% ($p<0.001$), respectively.

Incidence of Coronary Events

The incidence of coronary events was greater (105/12,575) in men than in women (93/27,013) during the treatment period. Incidence rates of coronary events per 1,000 patient-years were 1.57 in men and 0.64 in women. The age-adjusted relative risk of coronary events for men vs women was 2.81 (95% CI 2.10–3.76, $p<0.001$).

Serum Lipid Concentrations During the Treatment Period and Risk of Coronary Events

The risk of coronary events in relation to serum lipid concentrations is shown in Table 2. Increased risk for coronary events was evident at TC ≥240 mg/dl and LDL-C ≥160 mg/dl in both men and women. An increased risk of CHD associated with elevated concentration of TG (≥250 mg/dl) was noted in women but not in men. In men, the relationship between TG and CHD risk was not measurable. A lower risk of coronary events associated with elevation in HDL-C was seen in both sexes, but the protec-

Table 2 Relative Risk of Coronary Events According to Serum Lipid Concentrations During Treatment[a)]

	Men					Women				
	N	*Event*	*RR*	*95%CI*	*p value*	*N*	*Event*	*RR*	*95%CI*	*p value*
TC (mg/dl)										
<200	*3,442*	*24*	*1.00*	*(Referent)*		*5,833*	*22*	*1.00*	*(Referent)*	
200–219	*3,643*	*23*	*0.99*	*(0.56–1.77)*	*0.984*	*8,194*	*14*	*0.52*	*(0.27–1.02)*	*0.057*
220–239	*3,029*	*25*	*1.46*	*(0.83–2.56)*	*0.192*	*7,070*	*18*	*0.88*	*(0.47–1.64)*	*0.687*
240–259	*1,431*	*15*	*2.01*	*(1.05–3.88)*	*0.036*	*3,668*	*22*	*2.19*	*(1.21–3.98)*	*0.010*
260–	*1,030*	*18*	*3.48*	*(1.86–6.52)*	*<0.001*	*2,248*	*17*	*2.82*	*(1.48–5.36)*	*0.002*
LDL-C (mg/dl)										
<120	*4,680*	*27*	*1.00*	*(Referent)*		*8,050*	*22*	*1.00*	*(Referent)*	
120–139	*3,542*	*23*	*1.24*	*(0.71–2.16)*	*0.456*	*8,418*	*17*	*0.83*	*(0.44–1.57)*	*0.566*
140–159	*2,406*	*21*	*1.84*	*(1.03–3.26)*	*0.038*	*6,185*	*19*	*1.42*	*(0.77–2.64)*	*0.263*
160–179	*1,057*	*12*	*2.60*	*(1.31–5.17)*	*0.006*	*2,673*	*17*	*3.29*	*(1.74–6.23)*	*<0.001*
180–	*648*	*17*	*6.58*	*(3.53–12.25)*	*<0.001*	*1,564*	*17*	*5.78*	*(3.03–11.00)*	*<0.001*
TG (mg/dl)										
<100	*1,521*	*11*	*1.00*	*(Referent)*		*6,337*	*18*	*1.00*	*(Referent)*	
100–149	*3,663*	*22*	*0.84*	*(0.41–1.74)*	*0.634*	*10,444*	*32*	*0.98*	*(0.55–1.76)*	*0.946*
150–199	*3,127*	*33*	*1.51*	*(0.76–3.02)*	*0.243*	*5,861*	*17*	*0.87*	*(0.44–1.71)*	*0.684*
200–249	*1,768*	*18*	*1.46*	*(0.68–3.15)*	*0.330*	*2,429*	*9*	*1.12*	*(0.50–2.53)*	*0.783*
250–	*2,494*	*21*	*1.24*	*(0.58–2.65)*	*0.572*	*1,921*	*17*	*2.62*	*(1.32–5.21)*	*0.006*
HDL-C (mg/dl)										
<40	*2,198*	*36*	*1.00*	*(Referent)*		*1,758*	*10*	*1.00*	*(Referent)*	
40–44	*2,133*	*23*	*0.64*	*(0.38–1.09)*	*0.099*	*2,794*	*17*	*1.12*	*(0.51–2.45)*	*0.776*
45–49	*2,207*	*17*	*0.44*	*(0.25–0.80)*	*0.006*	*4,101*	*24*	*1.09*	*(0.52–2.28)*	*0.819*
50–54	*1,956*	*13*	*0.39*	*(0.21–0.74)*	*0.004*	*4,440*	*13*	*0.57*	*(0.25–1.30)*	*0.179*
55–59	*1,402*	*8*	*0.33*	*(0.15–0.72)*	*0.005*	*4,053*	*13*	*0.66*	*(0.29–1.51)*	*0.324*
60–	*2,679*	*8*	*0.17*	*(0.08–0.36)*	*<0.001*	*9,867*	*16*	*0.33*	*(0.15–0.73)*	*0.006*
LDL-C/HDL-C										
<2.0	*2,851*	*11*	*1.00*	*(Referent)*		*7,426*	*11*	*1.00*	*(Referent)*	
2.0–2.4	*2,719*	*11*	*1.10*	*(0.48–2.55)*	*0.817*	*6,909*	*19*	*1.95*	*(0.92–4.10)*	*0.080*
2.5–2.9	*2,598*	*17*	*1.91*	*(0.89–4.10)*	*0.095*	*5,884*	*14*	*1.68*	*(0.76–3.72)*	*0.199*
3.0–3.4	*1,889*	*20*	*3.21*	*(1.53–6.74)*	*0.002*	*3,545*	*21*	*4.57*	*(2.19–9.54)*	*<0.001*
3.5–4.0	*1,082*	*13*	*3.87*	*(1.72–8.72)*	*0.001*	*1,728*	*12*	*5.04*	*(2.21–11.49)*	*<0.001*
4.0–	*1,194*	*28*	*8.06*	*(3.95–16.44)*	*<0.001*	*1,398*	*15*	*8.56*	*(3.88–18.88)*	*<0.001*

RR, relative risk; CI, confidence interval. Other abbreviations see in Table 1.

[a)] Coronary events included acute myocardial infarction and sudden cardiac death. Adjustment for age, hypertension, diabetes mellitus, body mass index, ECG abnormality, family history of CHD, cigarette smoking, and alcohol use.

Table 3 Relative Risk of Coronary Events and Baseline Characteristics[a)]

	Men					Women					*Heterogeneity p value[b)]*
	N	*Event*	*RR*	*95%CI*	*p value*	*N*	*Event*	*RR*	*95%CI*	*p value*	
Age (years)											
<55	*6,281*	*49*	*1.00*	*(Referent)*		*6,137*	*8*	*1.00*	*(Referent)*		*0.008*
55–59	*2,182*	*14*	*0.74*	*(0.41–1.34)*	*0.320*	*6,488*	*15*	*1.82*	*(0.77–4.29)*	*0.174*	
60–64	*2,164*	*17*	*0.87*	*(0.50–1.53)*	*0.627*	*7,112*	*29*	*3.02*	*(1.38–6.62)*	*0.006*	
≥65	*1,948*	*25*	*1.42*	*(0.86–2.34)*	*0.168*	*7,276*	*41*	*4.11*	*(1.92–8.82)*	*<0.001*	
Obesity[c)]	*4,621*	*40*	*0.99*	*(0.66–1.48)*	*0.956*	*8,700*	*32*	*0.91*	*(0.59–1.40)*	*0.663*	*0.676*
Hypertension[d)]	*5,705*	*68*	*2.15*	*(1.42–3.26)*	*<0.001*	*12,511*	*62*	*2.05*	*(1.32–3.18)*	*0.001*	*0.864*
Diabetes mellitus[e)]	*2,513*	*29*	*1.58*	*(1.03–2.43)*	*0.037*	*3,747*	*31*	*3.07*	*(1.99–4.74)*	*<0.001*	*0.019*
ECG abnormality[f)]	*1,681*	*26*	*1.86*	*(1.18–2.91)*	*0.007*	*3,473*	*23*	*1.67*	*(1.04–2.70)*	*0.035*	*0.972*
Family history of CHD[g)]	*637*	*10*	*2.00*	*(1.04–3.84)*	*0.038*	*1,289*	*13*	*3.34*	*(1.85–6.04)*	*<0.001*	*0.317*
Cigarette smoking[g)]	*5,506*	*52*	*1.46*	*(0.98–2.17)*	*0.063*	*1,105*	*9*	*2.94*	*(1.43–6.02)*	*0.003*	*0.148*
Alcohol use[g)]	*9,224*	*70*	*0.63*	*(0.41–0.96)*	*0.031*	*2,337*	*6*	*0.61*	*(0.26–1.45)*	*0.266*	*0.933*

Abbreviations see in Tables 1,2.

[a)] Coronary events included acute myocardial infarction and sudden cardiac death. Adjustment for age, hypertension, diabetes mellitus, body mass index, ECG abnormality, family history of CHD, cigarette smoking, and alcohol use. [b)] Heterogeneity between men and women, based on the likelihood ratio test. [c)] Body mass index ≥25 kg/m^2. [d)] Systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg or medication for hypertension. [e)] Fasting plasma glucose ≥140 mg/dl or medication. [f)] Study physician's diagnosis. [g)] Self-reported information.

tive association was more evident in men. The relative risk for coronary events was substantially increased in patients with LDL-C/HDL-C ≥3.0 in both men and women.

The increase in the risk of coronary events for each 10 mg/dl elevation of LDL-C concentration during the treatment period was 18% (95% CI 12–24%) in men and 21% (95% CI 15–27%) in women, and the decrease in CHD risk associated with each 10 mg/dl elevation of HDL-C concentration was 39% in men and 33% in women. The relationships of coronary events with baseline LDL-C and HDL-C concentrations were also examined, but were much weaker than those observed during the treatment period. With each 10 mg/dl elevation of LDL-C concentration at baseline, the increase in the relative risk was 7% for men

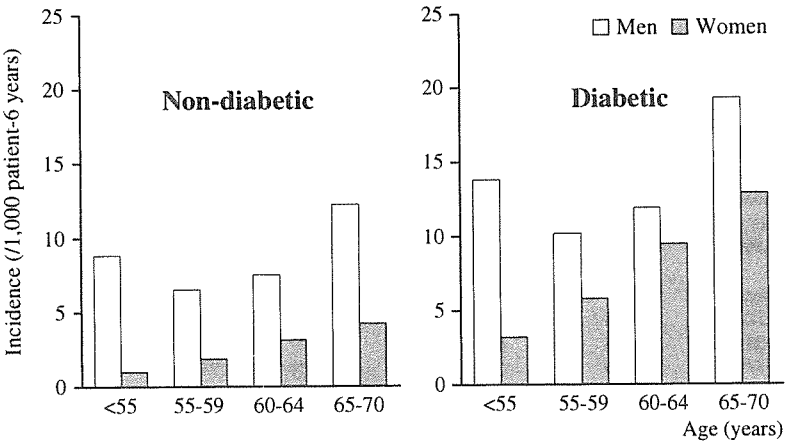

Fig 1. Estimated rates of coronary events according to age in men and women with and without diabetes mellitus (DM). Incidence rates were calculated from coronary heart disease (CHD) relative risks and the proportion of patients in each age category, for men and women separately, using Cox proportional hazards model, in which adjustment was made for age, hypertension, DM, body mass index, ECG abnormality, family history of CHD, cigarette smoking, and alcohol use.

and 9% for women and the decrease in risk with each 10 mg/dl elevation of HDL-C at baseline was 20% in both men and women.

Patient Baseline Characteristics and Risk of Coronary Events

The effect of age on the risk of coronary events was seen in women, but not in men (Table 3). Hypertension, DM, ECG abnormalities and a family history of CHD were also risk factors for coronary events in both men and women, but increased risks associated with DM and a family history of CHD were more marked for women than for men; the relative risk with DM was 1.58 in men and 3.07 in women, and the corresponding values for a family history of CHD were 2.00 in men and 3.34 in women. Obesity was unrelated to coronary events in either men or women. Although alcohol ingestion was protective in both men and women to the same extent, cigarette smoking was more strongly related to an increased risk of coronary events in women.

Discussion

This report addresses the gender differences in the relationship of serum lipid concentrations and other risk factors to CHD risk in Japanese patients under long-term treatment for hypercholesterolemia. Although serum TC and LDL-C concentrations were very similarly related to CHD risk in men and women, there was a difference between men and women in the relationship to serum TG and HDL-C concentrations. An inverse relationship of HDL-C to CHD risk was seen in men and women, but the HDL-C concentration showing a decreased risk of CHD differed by sex. The risk was significantly decreased at HDL-C ≥45 mg/dl in men and at HDL-C ≥60 mg/dl in women. The findings agree with observations published in the United States and Europe[2,3] and further indicate that the criterion of "low HDL-C" must be differential for men and women. An increased risk was observed only in women with an extremely high concentration of TG (≥250 mg/dl). Interpretation of this finding is difficult, and we do not have a clear idea about the implication of the present finding on serum TG.

In the present study, men did not show a clear increase in the risk of coronary events with increasing age, whereas there was a progressive increase in the risk with advancing age in women. The latter finding could be a reflection of the increase in serum TC and LDL-C concentrations with increasing age after menopause. The lack of an increasing trend in the association between age and coronary events in men is an unexpected finding, and may have been due to unknown characteristics of the male participants in the present study.

Whereas DM was related to increased CHD risk in both men and women, the increased risk was much greater in women, as indicated by a statistically significant interaction (p=0.019). These results did not change when further adjusted for TC or LDL-C. However, the risk difference between men and women for DM was not unique to the J-LIT patients. In a meta-analysis of 10 prospective studies, Lee et al showed that the effect of DM on the CHD risk was greater in women than in men![14] They showed that the relative risk of coronary death for DM patients vs non-DM patients was 2.58 (95% CI 2.05–3.26) in women and 1.85 (95% CI 1.47–2.33) in men (interaction p=0.045)![14] It was further noted in a later study that DM diminished the female advantage for lower CHD incidence![15] That DM is a stronger CHD risk factor in women may be related to the lower concentrations of HDL-C. Walden suggested that lower HDL-C concentrations in diabetic women as compared with men might be relevant to a stronger association between DM and CHD in women![16] In the present study, mean HDL-C concentrations in female diabetic patients were lower than those of non-diabetic patients (55.5 vs 57.5 mg/dl, p<0.001), but there was no difference in the HDL-C concentrations between the 2 groups in men (50.8 vs 51.3 mg/dl, p=0.09). The relative risk for DM was unchanged with adjustment for HDL-C. When the predicted rates of CHD incidence according to age were examined in men and women with and without DM (Fig 1), the increase in CHD incidence with aging was augmented in the presence of DM. Notably, DM diminished the women's advantage of having a lower CHD incidence in older patients.

Both cigarette smoking and family history of CHD were related to a greater increase in the risk of coronary events in women than in men. These differential increases in men and women may have been caused by random variation, as indicated by the lack of statistical significance for the interaction. As regards the effect of cigarette smoking, some studies suggest that smoking is a stronger risk factor in women than in men[2,17] but others have failed to find such a finding![18]

Finally, the present study results indicated that hypertension was an important risk factor in men and women equally, and that alcohol ingestion was protective in both sexes. These findings are in agreement with observations reported elsewhere![19–21]

In conclusion, the incidence of coronary events was 60% lower in women than in men among the J-LIT participants. Although the relationship of serum TC and LDL-C concentrations to coronary events was similar in men and women, the HDL-C concentration associated with a decreased risk of coronary events was slightly higher in women. DM was a stronger risk factor in women, and traded off the women's advantage of having a lower risk of coronary events, especially in aged patients.

Acknowledgment

This study was in part supported by a grant from Banyu Pharmaceutical Co, Ltd, Tokyo, Japan.

References

1. Ministry of Health, Labour and Welfare. Vital Statistics. Tokyo: the Ministry, 2001.
2. Njolstad I, Arnesen E, Lund-Larsen PG. Smoking, serum lipids, blood pressure, and sex differences in myocardial infarction: A 12-year follow-up of the Finnmark Study. *Circulation* 1996; **93:** 450–456.
3. Barrett-Connor E. Sex differences in coronary heart disease: Why are women so superior? The 1995 Ancel Keys lecture. *Circulation* 1997; **95:** 252–264.
4. Matsuzaki M, Kita T, Mabuchi H, Matsuzawa Y, Nakaya N, Oikawa S, et al and the J-LIT Study Group. Large scale cohort study of the relationship between serum cholesterol concentration and coronary events with low-dose simvastatin therapy in Japanese patients with hypercholesterolemia: Primary prevention cohort study of the Japan Lipid Intervention Trial (J-LIT). *Circ J* 2002; **66:** 1087–1095.
5. Mabuchi H, Kita T, Matsuzaki M, Matsuzawa Y, Nakaya N, Oikawa S, et al and the J-LIT Study Group. Large scale cohort study of the relationship between serum cholesterol concentration and coronary events with low-dose simvastatin therapy in Japanese patients with hypercholesterolemia and coronary heart disease: Secondary prevention cohort study of the Japan Lipid Intervention Trial (J-LIT). *Circ J* 2002; **66:** 1096–1100.
6. Matsuzawa Y, Kita T, Mabuchi H, Matsuzaki M, Nakaya N, Oikawa S, et al and the J-LIT Study Group. Sustained reduction of serum cholesterol in low-dose 6-year simvastatin treatment with minimum side effects in 51,321 Japanese hypercholesterolemic patients: Implication of the J-LIT study, a large scale nationwide cohort study. *Circ J* 2003; **67:** 287–294.
7. Nakaya N, Kita T, Mabuchi H, Matsuzaki M, Matsuzawa Y, Oikawa S, et al and the J-LIT Study Group. Large-scale cohort study on the relationship between serum lipid concentrations and risk of cerebrovascular disease under low-dose simvastatin in Japanese patients with hypercholesterolemia: Sub-analysis of the Japan Lipid Intervention Trial (J-LIT). *Circ J* 2005; **69:** 1016–1021.
8. Neaton JD, Wentworth D. Serum cholesterol, blood pressure, cigarette smoking, and death from coronary heart disease: Overall findings and differences by age for 316,099 white men: Multiple Risk Factor Intervention Trial Research Group. *Arch Intern Med* 1992; **152:** 56–64.
9. NIH Consensus Conference. Triglyceride, high-density lipoprotein, and coronary heart disease: NIH Consensus Development Panel on Triglyceride, High-Density Lipoprotein, and Coronary Heart Disease. *JAMA* 1993; **269:** 505–510.
10. Iwashita M, Matsushita Y, Sasaki J, Arakawa K, Kono S and the Kyushu Lipid Intervention Study (KLIS) Group. Relation of serum total cholesterol and other risk factors to risk of coronary events in middle-aged and elderly Japanese men with hypercholesterolemia: The Kyushu Lipid Intervention Study. *Circ J* 2004; **68:** 405–409.
11. Rich-Edwards JW, Manson JE, Hennekens CH, Buring JE. The primary prevention of coronary heart disease in women. *N Engl J Med* 1995; **332:** 1758–1766.
12. Matsuzawa Y, Itakura H, Kita T, Mabuchi H, Matsuzaki M, Nakaya N, et al and J-LIT Study Group. Design and baseline characteristics of a cohort study in Japanese patients with hypercholesterolemia: The Japan Lipid Intervention Trial (J-LIT). *Curr Ther Res* 2000; **61:** 219–243.
13. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972; **18:** 499–502.
14. Lee WL, Cheung AM, Cape D, Zinman B. Impact of diabetes on coronary artery disease in women and men: A meta-analysis of prospective studies. *Diabetes Care* 2000; **23:** 962–968.
15. Juutilainen A, Kortelainen S, Lehto S, Ronnemaa T, Pyorala K, Laakso M. Gender difference in the impact of type 2 diabetes on coronary heart disease risk. *Diabetes Care* 2004; **27:** 2898–2904.
16. Walden CE, Knopp RH, Wahl PW, Beach KW, Strandness E Jr. Sex differences in the effect of diabetes mellitus on lipoprotein triglyceride and cholesterol concentrations. *N Engl J Med* 1984; **311:** 953–959.
17. Asia Pacific Cohort Study Collaboration. Smoking, quitting, and the risk of cardiovascular disease among women and men in the Asia-Pacific region. *Int J Epidemiol* 2005; **24:** 1–10.
18. Ueshima H, Choudhury SR, Okayama A, Hayakawa T, Kita Y, Kadowaki T, et al. Cigarette smoking as a risk factor for stroke death in Japan: NIPPON DATA80. *Stroke* 2004; **35:** 1836–1841.
19. Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, et al and the INTERHEART Study Investigators. Effects of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): Case-control study. *Lancet* 2004; **364:** 937–952.
20. D'Agostino RB, Grundy S, Sullivan LM, Wilson P and the CHD Risk Prediction Group. Validation of the Framingham coronary heart disease prediction scores: Results of a multiple ethnic groups investigation. *JAMA* 2001; **286:** 180–187.
21. Thun MJ, Peto R, Lopez AD, Monaco JH, Henley J, Heath CW Jr, et al. Alcohol consumption and mortality among middle-aged and elderly U.S. adults. *N Engl J Med* 1997; **337:** 1705–1714.

Word List Recall in the current study was the presence of neuropathy or nephropathy. Several mechanisms are involved in the pathogenesis of diabetic neuropathy, including vascular dysfunction, polyol pathway, and advanced glycation end-product accumulations.[10] A common mechanism may be involved in DM-related central nervous system dysfunction and peripheral neuropathy.

The current analysis demonstrates that the specific factors associated with decline were different in two different tests, suggesting that multiple factors may cause diabetes-related cognitive decline.

Mari Suzuki, MD
Hiroyuki Umegaki, MD, PhD
Satsuki Ieda, MS
Nanaka Mogi, PhD
Akihisa Iguchi, MD, PhD
Department of Geriatrics
Nagoya University Graduate School of Medicine
Nagoya, Aichi, Japan

ACKNOWLEDGMENTS

Financial Disclosure: This work was supported by a Grant-in-Aid for Longevity Scientific Research H17-Cyouju-013 from the Ministry of Health, Labour and Welfare, Japan.

Author Contributions: All authors had active roles in study concept and design, acquisition of data, analysis and interpretation of data, and preparation of manuscript.

Sponsor's Role: None.

REFERENCES

1. Strachen MW, Ewing FM, Deary IJ et al. Is type 2 diabetes associated with an increased risk of cognitive dysfunction? A critical review of published studies. Diabetes Care 1997;20:438–445.
2. Mogi N, Umegaki H, Hattori A et al. Cognitive function in Japanese elderly with type 2 diabetes mellitus. J Diabetes Complict 2004;18:42–46.
3. Yaffe K, Lindquist K, Penninx BW et al. Harris inflammatory markers and cognition in well-functioning African-American and white elders. Neurology 2003;61:76–80.
4. Engelhart MJ, Geerlings MI, Meijer J et al. Inflammatory proteins in plasma and the risk of dementia: The Rotterdam Study. Arch Neurol 2004;61:668–672.
5. Mohs RC, Rosen WG, Davis KL. The Alzheimer's disease assessment scale: An instrument for assessing treatment efficacy. Psychopharmacology 1983;19: 448–450.
6. Rifai N, Russel PT, Ridker PM. Clinical efficacy of an automated high-sensitivity C-reactive protein assay. Clin Chem 1999;45:2136–2141.
7. Campbell IL, Abraham CR, Masliah E et al. Neurological disease induced in transgenic mice by cerebral overexpression of interleukin 6. Proc Natl Acad Sci U S A 1993;90:10061–10065.
8. Young JL, Libby P, Schonbeck U. Cytokines in the pathogenesis of atherosclerosis. Thromb Haemat 2002;88:554–567.
9. Hoshi T, Kitagawa K, Yamagami H et al. Relations of serum high-sensitivity C-reactive protein and interleukin-6 levels with silent brain infarction. Stroke 2005;36:768–772.
10. Gispen WH, Biessels GJ. Cognition and synaptic plasticity in diabetes mellitus. Trends Neurosci 2000;11:542–549.

AGE, SOCIAL STRATUM, AND OBESITY IN LATIN AMERICA: SIMILARITIES IN THE INEQUALITIES FOR OLDER PEOPLE

To the Editor: In the recent years, there has been accumulating evidence about the increasing prevalence of obesity in developing countries—in children and adults.[1,2]

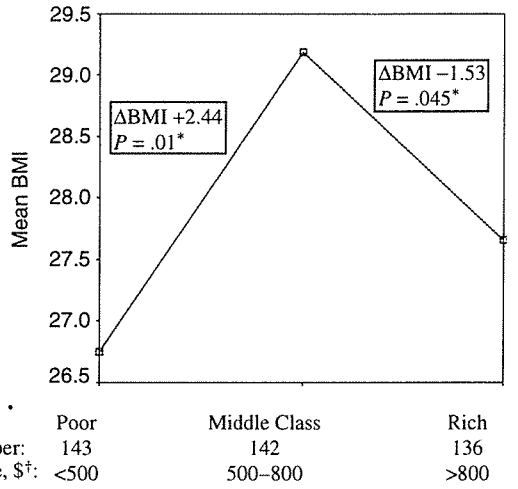

Figure 1. Mean body mass index (kg/m^2) according to the social stratum. Analysis of variance, $P = .01$. *Post hoc analysis. †International dollars.

In a recent *Lancet* article, Silveira argues that "it is intriguing that in these social layers (poor and rich), low birthweight and obesity are becoming more frequent."[3] In addition, she makes some interesting considerations about the importance of this phenomenon to pediatrics.

Whereas increasing prevalence of obesity among the poor is being reported in Brazil, as well as in many other developing regions of the world,[1] it does not seem to be the case for the upper social stratum, at least in Brazil. Indeed, some Brazilian studies have demonstrated that the prevalence of obesity in the upper class, although high, is stabilizing or declining.[4] This phenomenon might also be the case in older people.

In a recent community research, in which 421 older people living in the medium-sized city of Porto Alegre, Brazil, were evaluated, an intriguing significantly ($P = .01$) higher body mass index (29.2) was found in the middle class (monthly income $300–500) than in the poor (26.7) or rich (27.7) strata (Figure 1).

In addition, education was lower in the middle than the upper class ($P = .002$), and the former tended to eat more beef ($P = .03$) than the other two social strata.

The Bambuí project, a Brazilian longitudinal study on aging, has also found obesity to be more common in nonpoor than in poor older people.[5] In this Brazilian sample, malnutrition was found to be associated with lower family income ($P = .05$). The Bambuí study also reported that underweight in older people increased inversely with family income.

Malnutrition may lead to immunodeficient status, which in the tropical developing countries, means a higher risk of developing tropical diseases. Indeed, the same study reported higher prevalence of *Trypanosoma cruzi* in undernourished older people.[5]

Here is where pediatrics and geriatrics come together, because the two opposite extremes of age are more dependent upon the environment. The possibility that developing economies have been affecting the prevalence of obesity and malnutrition in children and older people in a different fashion is puzzling. It is possible that an increasing prevalence of obesity in the less-privileged stratum and decreasing frequency in the richer class may help to explain

the higher mean body mass index found in this sample's middle class.

In the last decade, Brazilian economic policies have reduced the once 100% retirement pensions for the middle class. This study also found that the middle class tended to keep working more after retirement ($P = .002$) and to exercise less than the other two strata ($P = .001$). It is possible that a combination of actively working after retirement, having less time to exercise, being sedentary, and being under stress may create a milieu for obesity.

Whereas redistributive economic policies in Latin America are mandatory, governments should not put the onus excessively upon the middle-class aged.

Matheus Roriz-Cruz, MD
Department of Geriatrics
Kyoto University
Kyoto, Japan
Department of Clinical Medicine
Division of Geriatrics
Universidade Federal de Sergipe
Sergipe, Brazil

Idiane Rosset, NP, MPH
Department of Geriatrics
Masayuki Ishine, MD
Teiji Sakagami, MD
Department of Field Medicine
Kyoto University
Kyoto, Japan

Jarbas S. Roriz, MD
Departments of Geriatrics and Gerontological Nursing
Universidade de São Paulo
São Paulo, Brazil

Ademar Chiez, Jr, MD
Institute of Geriatrics
Universidade Católica do Rio Grande do Sul
Rio Grande do Sul, Brazil

Rosalina Partezani-Rodrigues, BN, PhD
Departments of Geriatrics and Gerontological Nursing
Universidade de São Paulo
São Paulo, Brazil

Antonio C. De Souza, MD, PhD
Institute of Geriatrics
Universidade Católica do Rio Grande do Sul
Rio Grande do Sul, Brazil

Toru Kita, MD, PhD
Department of Cardiology
Kozo Matsubayashi, MD, PhD
Department of Field Medicine
Kyoto University
Kyoto, Japan

ACKNOWLEDGMENTS

We are thankful to Nieves Godinez for reviewing the manuscript.

Financial Disclosure: This study was supported by the Japanese Ministry of Education. No other disclosures to report.

Author Contributions: This projected was conceived and designed by Matheus Roriz-Cruz, Idiane Rosset, Prof. Toru Kita, and Prof. Kozo Matsubayashi. All the authors except Toru Kita participated in field research and data collection. Jarbas S. Roriz, Ademar Chiez, Jr., and Antonio C. De Souza were responsible for doing the epidemiological survey and elderly recruitment (in a censored basis) in the communities—in addition to collaborating via e-mail in the analysis and discussion process. Analysis and interpretation of data were done through weekly group discussions, including all Japanese members plus the first two authors, which special help from Toru Kita and Kozo Matsubayashi. The preparation of the manuscript was centralized in the first author, with suggestions and corrections offered by the entire group.

Sponsor's Role: No other role was performed by the Japanese Ministry of Education.

REFERENCES

1. Popkin BM. The nutrition transition and obesity in the developing world. J Nutr 2001;131:871S–873S.
2. Fraser B. Latin America's urbanization is boosting obesity. Lancet 2005;365:1995–1996.
3. Silveira PP, Portella AK, Goldani MZ. Obesity in Latin America: Similarities in the inequalities. Lancet 2005;366:451–452.
4. Monteiro CA, D'A Benicio MH, Conde WL et al. Shifting obesity trends in Brazil. Eur J Clin Nutr 2000;54:342–346.
5. Barreto SM, Passos VMA, Lima-Costa MFF. Obesity and underweight among Brazilian elderly. The Bambui Health and Aging Study. Cad Saúde Pública 2003;19:605–612.

PURPLE URINE BAG SYNDROME IN GERIATRIC PATIENTS

To the Editor: Purple urine bag syndrome (PUBS) is an uncommon but interesting condition that has been encountered in geriatric wards. Two patients with PUBS are described below, followed by a brief discussion of this condition.

Patient 1 was a 70-year-old bedridden man who suffered from progressive paraplegia as a result of tuberculous meningitis and arachnoiditis. He required long-term urinary catheterization for urinary retention and had repeated urinary tract infections. Patient's condition was further complicated with a urethrocutaneous fistula that healed poorly, because he refused suprapubic catheterization to facilitate healing. He was chronically constipated and required habitual use of laxatives. After staying in a chronic care ward for 4 years, his urine bag, together with the drainage catheter, was noted to have purple discoloration for the first time (Figure 1). Patient was afebrile, and all vital signs were stable. His indwelling urinary catheter and drainage bag were changed, but the purple color appeared again shortly afterward. He developed fever 3 days later, and a sepsis examination was performed. Bedside urine Multistix revealed urine pH of 8.5, protein of 100 mg/dL, and was negative for leukocyte, red cells, nitrite, and glucose. Urine microscopy revealed moderate numbers (10,000–100,000 cells/mL) of leukocytes and grew *Providencia* species (>100, 000 colony forming units (CFU)/mL), whereas blood culture per-

Laboratory Investigation (2006) 86, 357–368

www.laboratoryinvestigation.org

Expression of Smad1 is directly associated with mesangial matrix expansion in rat diabetic nephropathy

Takeshi Matsubara[1], Hideharu Abe[2], Hidenori Arai[3], Kojiro Nagai[1], Akira Mima[1], Hiroshi Kanamori[1], Eriko Sumi[1], Toshikazu Takahashi[2], Motokazu Matsuura[2], Noriyuki Iehara[1], Atsushi Fukatsu[1], Toru Kita[4] and Toshio Doi[2]

[1]*Department of Nephrology, Kyoto University Graduate School of Medicine, Kyoto, Japan;* [2]*Department of Clinical Biology and Medicine, Tokushima University School of Medicine, Tokushima, Japan;* [3]*Department of Geriatric Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan and* [4]*Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan*

Diabetic nephropathy is the leading cause of end-stage renal disease, and glomerular mesangial matrix expansion is the hallmark in diabetic nephropathy. However, the precise mechanism for the development of mesangial matrix expansion has remained unknown. The key component involved in mesangial matrix expansion is type IV collagen (Col4). Recently, we have reported that Smad1 transcriptionally regulates expression of Col4 under diabetic conditions *in vitro*. Here we show that this direct regulator of Col4 also plays a crucial role for mesangial matrix expansion *in vivo*. Streptozotocin-induced diabetic rats are the model of incipient diabetic nephropathy, and showed various levels of mesangial matrix expansion at 24 weeks. The glomerular expression of Smad1 was significantly increased in diabetic rats with more mesangial matrix expansion by Western blot and immunohistochemical analysis. Furthermore, the glomerular expression of Smad1 was closely correlated with the glomerular expression of Col4 and smooth muscle alpha actin (α-SMA), while albuminuria or glomerular filtration rate was not correlated with mesangial matrix expansion. We also found that urinary excretion of Smad1 was closely associated with the severity of mesangial matrix expansion. In cultured mesangial cells expression of Smad1 upregulated the transcriptional activity of key molecules in mesangial matrix expansion, such as Col4 and α-SMA. These data indicate the critical involvement of Smad1 in mesangial matrix expansion in the early phase of diabetic nephropathy. Our data imply that urinary Smad1 might be a representative diagnostic marker for mesangial matrix expansion in diabetic nephropathy.
Laboratory Investigation (2006) **86,** 357–368. doi:10.1038/labinvest.3700400; published online 13 February 2006

Keywords: diabetic nephropathy; mesangial matrix expansion; Smad1; smooth muscle alpha actin; type IV collagen

Diabetic nephropathy is the leading cause of end-stage renal disease in the United States, Japan, and so on. The major morphological changes are characterized by thickening of glomerular basement membrane (GBM) and mesangial matrix expansion. The latter is more important, because there is widespread agreement that they are generally accompanied by various tubulointerstitial damages followed by nephron loss to end-stage renal failure.[1,2] The decline of glomerular filtration rate (GFR) is associated with the increasing number of obsolescent glomeruli[3] and the severity of mesangial matrix expansion in type I diabetes. On the other hand, the thickening of GBM has little or no correlation with the decline of GFR.[4] Therefore, mesangial matrix expansion is the hallmark from the point of pathological and clinical events in diabetic nephropathy. Glomerulosclerosis is defined as the segmental or global collapse or closure of capillary loops with associated mesangial matrix expansion. However, the early stage of diabetic nephropathy is associated with glomerular hyperfiltration and glomerular hypertrophy, but not collapse of glomerular capillaries. Therefore, mesangial matrix expansion is one of the most important pathological findings in the early phase of diabetic nephropathy.

Correspondence: Dr H Arai, MD, PhD, Department of Geriatric Medicine, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail: harai@kuhp.kyoto-u.ac.jp
Received 18 September 2005; revised and accepted 13 January 2006; published online 13 February 2006

Albuminuria is one of the most characteristic functional changes in the early phase of diabetic nephropathy. Recent study suggests that the percentage of microalbuminuric patients progressing to proteinuria over ~10 years is only 30–45%.[5] Furthermore, extensive studies of the glomerular structure in diabetic patients with or without microalbuminuria failed to find a significant difference in the glomerular structural changes such as mesangial matrix expansion between the two groups in the absence of a raised blood pressure or reduced creatinine (Cre) clearance.[6,7] These reports show that albuminuria might not be a definite marker for mesangial matrix expansion in the early phase of diabetic nephropathy, although albuminuria correlates with and predicts the late development of diabetic nephropathy with decreased GFR and glomerulosclerosis.

Mesangial matrix expansion is characterized by increased amounts of extracellular matrix (ECM), including alpha 1/alpha 2 type IV collagen (Col4), laminin, type I and III collagens, heparan sulfate proteoglycan, and fibronectin.[8] One of the key components in the pathophysiology is Col4. Various peptides of growth factors are shown to mediate the regulation of this key component.[9] However, the direct regulation of Col4 had not been proved until our recent report that Smad1 can directly bind to the promoter of Col4, and can upregulate its transcriptional activity.[10] We also found that advanced glycation endproducts (AGE) can induce the phosphorylation of Smad1 in cultured mesangial cells, and that Smad1 is highly expressed in human advanced diabetic nephropathy.[10] Thus, Smad1 is the first molecule shown to regulate the expression of Col4 directly under diabetic condition *in vitro*. However, these findings are obtained only in cultured mesangial cells, and the precise mechanism for the development of mesangial matrix expansion *in vivo* remains unknown. Therefore, the aim of this study is to investigate the role of Smad1 in diabetic nephropathy *in vivo* using experimental diabetic animals.

The streptozotocin-induced diabetic (STZ) rat, a type I diabetic model, is known to have glomerular functional changes including albuminuria and the increasing of GFR as seen in the early phase of diabetic nephropathy.[11] However, STZ rats show only minimal glomerular structural changes even after 1 year of diabetes.[12] We have induced various levels of mesangial matrix expansion in this model at 24 weeks by feeding special breeding chows. The glomerular structural characteristics in these rats were similar to human early glomerular structural changes, which are a useful model for analyzing mesangial matrix expansion in the early phase of diabetic nephropathy.

In this study, we have examined the role of Smad1 for the development of mesangial matrix expansion *in vivo*, and also studied whether urinary excretion of Smad1 could be a new representative maker for detecting severity of mesangial matrix expansion. *In vitro*, we examined whether Smad1 can regulate the transcriptional activity of Col4 and smooth muscle alpha actin (α-SMA) in mesangial cells.

Materials and methods

Animals and Cell Culture

Male Sprague–Dawley rats were purchased from Shimizu Laboratory Animal center (Hamamatsu, Japan). All of animal experiments were performed in accordance with institutional guidelines, and the Review Board of Kyoto University granted ethical permission to this study. The glomerular mesangial primary culture was established and maintained according to the method previously described.[13]

Male rats weighing 170–200 g were made diabetic by a single intravenous injection of STZ (Wako, Osaka, Japan) (55 mg/kg body weight (BW)) in 0.05 mol/l citrate buffer (pH 4.5). Rats receiving an injection of citrate buffer were used as controls. The levels of blood glucose were determined 2 days after injection of STZ or vehicle, and rats with blood glucose levels more than >16.7 mmol/l were used. To develop various severities of mesangial matrix expansion in these rats, we used special chows suitable for breeding, containing slightly more protein, and less fat. (LABO H STANDARD, Nosan Corporation, Yokohama, Japan) At 24 weeks after STZ injection, the rats were weighted and killed.

Renal Histology and Morphometric Analyses

Renal tissue blocks were fixed in ethyl Carnoy's solution, and embedded in paraffin. Sections (2 μm) were stained with periodic acid-Schiff methenamine (PASM). To study the severity of mesangial matrix expansion, we quantified mesangial matrix area by measuring mesangial PASM-positive area using an image analyzer with a microscope (Image Processor of Analytical Pathology; IPAP: Sumitomo Chemical Co., Tokyo, Japan).[14,15] The mesangial matrix fraction (Mes Fx) was determined as percentage of mesangial PASM-positive area per total glomerular surface area. For each animal, 50 glomeruli were analyzed.

Immunohistochemistry

Kidney sections were processed for immunohistochemistry following standard procedures. Ethyl Carnoy's solution-fixed and paraffin-embedded tissue blocks were used. Kidney sections were rehydrated and treated with 0.3% H_2O_2 in methanol for 30 min. Sections were blocked with the appropriate preimmune serum, and then incubated with AvidinD and Biotin blocking solutions (Vector, CA, USA). Sections were incubated with the anti-Col4 antibody (PROGEN, Heidelberg, Germany), and

anti-Smad1 antibody (Santa Cruz, CA, USA) overnight at 4°C and then incubated with the appropriate biotinylated secondary antibodies followed by incubation with the avidin–biotin peroxidase complex (Vector). Peroxidase conjugates were subsequently localized using diamino-benzidine. To study α-SMA and AGE, the tissues were snap frozen in OCT compound (Sakura Finetechnical, Tokyo, Japan), and then cut in 4 μm-thick sections and fixed in acetone for 10 min, and treated with 0.3% H_2O_2 in methanol for 30 min. Sections were treated with anti-α-SMA antibody (clone 1A4, SIGMA), and anti-AGE antibody (clone 6D12, Transgenic Co. Ltd., Kumamoto, Japan). Sections were counterstained with hematoxylin solution.

Immunofluorescence Staining and Morphometric Analyses of Glomerular Col4 Expression

Immunofluorescence staining for Col4 was performed using frozen kidney sections. Sections (4 μm) were fixed in acetone, blocked with 10% donkey serum, and incubated with anti-Col4 antibody followed by incubation with FITC labeled secondary antibody. The immunoreactivity of Col4 was quantified by measuring Col4-positive area in glomeruli using Image-Pro PLUS (Media Cybernetics). The glomerular expression of Col4 was determined as percentage of Col4-positive area per total glomerular surface area. For each animal, 50 glomeruli were analyzed.

Isolation of Glomeruli

Glomeruli were isolated from renal cortices of rats using the differential sieving method.[16,17]

Western Blotting

Isolated glomeruli were suspended in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% SDS, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml of aprotinin) for 1 h at 4°C. After centrifugation, the supernatants were used as total glomerular lysates. Of each sample (40 μg) was applied to SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose filters (Schleicher & Schuell). The blots were subsequently incubated with rabbit anti-Smad1 (Upstate Biotechnology), anti-phospho Smad1/5/8 (Cell Signaling Technology), ALK-1 (Santa Cruz), TGF-β (Santa Cruz), and α-SMA antibody, following by incubation with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). The immunoreactive bands were visualized using the enhanced chemiluminescent system (Amersham Biosciences). Quantitative analysis of the band density was performed using Image Gauge (Fuji Photo Film Co., Ltd).

AGE Preparation

AGE-bovine serum albumin (BSA) was prepared by the regular methods as described previously.[10] Unmodified BSA was incubated under the same conditions without glucose 6-phosphate for control. Protein concentration was determined by the Bradford method using BSA as a standard. All AGE-protein-specific fluorescence determinations were performed by measuring emission at 440 nm upon excitation at 370 nm using a fluorescence spectrometer (Hitachi). AGE content was estimated by fluorescence intensity at a protein concentration of 1 mg/ml. AGE-BSA contained 89.9 AGE units per mg of protein, and unmodified BSA contained 3.71 AGE units per mg of protein.

Dot Blot Immunoassay

Dot blots were performed by applying 5 μg of glomerular lysates onto a nitrocellulose transfer membrane (PROTRAN). Standard solutions of AGE prepared at concentrations ranging from 0.5 to 32 arbitrary units/5 μg total protein with the dilution buffer, were also applied to the membrane, which was then blocked with 5% skim milk for 1 h. The membrane was then incubated with anti-AGE monoclonal antibody followed by incubation with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). The immunoreactive dots were visualized and quantitatively analyzed as described in Western blotting and ELISA procedure.

Blood and Urine Examination

Blood was collected at killing. Serum Cre, urinary Cre, and blood urea nitrogen were determined with a Hitachi Model 736 autoanalyzer.[18] HbA1c was measured using DCA 2000 analyzer (Bayer Medical, Tokyo, Japan). Urine volume was measured at 24 weeks by 24 h urine collection from rats housed in individual metabolic cages. The Cre clearance was calculated and normalized to BW. The urinary albumin concentration was measured by Nephrat (Exocell Inc.), and adjusted by urinary Cre concentration.

Urine Preparation

Urine samples were centrifuged for 15 min. The supernatants were stored at −80°C and rapidly thawed and centrifuged to remove any urates or phosphates before use in assays. Because Smad1 concentrations in urine are below the detection limits of our assays, the following methods for concentration were developed and tested.

A 2-ml urine sample was placed in a Centricon-10 filter (Amicon) pretreated with 0.1% Tween-20 used to limit adsorption to its polypropylene components. The extent of concentration achieved was

calculated from the exact retenate volume measured with a Hamilton pipet. This ranged from 25- to 60-fold, with a median of 55-fold concentration. This prepared urine concentrate was diluted to total volume of 110 μl with the dilution buffer as described below. Subsequently, 100 μl of the 110 μl total volumes was utilized in the following assays. The final urine concentration was 18-fold higher than the original sample. This was taken into consideration in the final calculation of the concentration of Smad1 in each urine sample.

ELISA Procedure

The following buffers were prepared with deionized water and used in ELISA. Coating buffer: 0.1 mol/l phosphate buffer, pH 7.4 (PB) containing 3% BSA (SIGMA). Dilution buffer: PB containing 3% BSA and 0.1% Tween-20. Washing buffer: PB containing 0.1% Tween-20.

All the ELISA assays were performed in 96-well microtiter plates (Nunc). Anti-Smad1 monoclonal antibody (Santa Cruz) was diluted with the coating buffer to obtain a final concentration of 2.5 μg/ml. Aliquots (250 ng/100 μl) were added to each well, and the plate was incubated overnight at 4°C. The wells were washed three times. In all 50 μl of the prepared urine samples and standard solutions of recombinant Smad1 prepared at concentrations ranging from 0.125 to 512 ng/ml with the dilution buffer were added to the plate and incubated overnight at 4°C. The plates were then washed three times. Of rabbit anti-Smad1 polyclonal antibody (Upstate), 100 μl was diluted with the diluting buffer to obtain a final concentration of 2.5 μg/ml, added to each well, and incubated overnight at 4°C. Horseradish peroxidase-conjugated anti-rabbit IgG polyclonal antibody (Amersham) was diluted 500-fold with the dilution buffer, and 50 μl were then dispensed into each well and the plate was further incubated at room temperature for 120 min. The plates were washed three times.

Of freshly prepared peroxidase substrate solution (DAKO), 50 μl were added to each well. The plates were kept at room temperature in the dark for 30 min, and the enzyme reaction was then stopped by adding 50 μl of 2N H_2SO_4 to each well. The optical density was measured at 492 nm using a microplate reader SPECTRA (TECAN, Austria). Each Smad1 determination was carried out in duplicate. Delta SOFT3™ (Biometallics) was used to obtain the calibration curve and the polynomial equation. The optical densities of all urine samples were then inserted in the equation in order to calculate the corresponding Smad1 concentration.

Plasmid Constructs

The wild-type Col4 promoter reporter plasmid (Col4-LacZ) was constructed as follows: A 0.8 Kb *Srfl–Xba*I fragment of the 5′ flanking region of *Col4α2* gene was subcloned into the β-gal Reporter Vector, pNASSβ (Clontech). The α-SMA promoter reporter plasmid (SMA-Luc) contains 1074 bp of the proximal 5′-flanking region plus 43 bp 5′-untranslated region of the *α-SMA* gene[19] that is subcloned into the luciferase reporter vector (Promega).

Reporter Assay

Mesangial cells (1.0×10^5) were seeded into 12-well plates (Nunc). After 6 h, the cells were transfected with 500 ng of mouse Col4-LacZ reporter construct, or 500 ng of mouse SMA-Luc along with either 500 ng of vector encoding wild type of Smad1 or the mock vector. Transfection was performed with FuGENE6 transfection reagent (Roche) according to the manufacture's instructions. After 48 h, the cells were harvested in reporter lysis buffer. β-Galactosidase and luciferase activity were measured using the luminescent β-galactosidase reporter system (BD Bioscience) and the luciferase reporter system from Promega. β-Galactosidase and luciferase activities were normalized to total protein (RC DC Protein Assay, Bio-Rad). Transfection experiments were quadruplicated, and repeated at least three times. The β-galactosidase and luciferase activity data were expressed as means ± s.d.

Statistical Analysis

Correlation was determined by Spearman's correlation analysis. Regression formulations were obtained by linear regression analysis. As a result of their skewed distribution, glomerular expression of α-SMA, and Smad1, and urinary excretion of Smad1 were logarithmically transformed before statistical analysis. All analyses were performed using StatView (SAS Institute, Cary, CA, USA). A P-value <0.05 was considered statistically significant. All values were expressed as the mean ± s.d., except for glomerular expression of α-SMA, and Smad1, and urinary excretion of Smad1, where geometric means were given, and analyzed by Mann–Whitney nonparametric analysis, or one-way analysis of variance with a modified t-test. Statistical significance was defined as $P<0.05$. Quantitation of immunohistochemistry was analyzed by one-way ANOVA followed by the *post hoc* test. P-values <0.05 were considered significant. Data are expressed as means ± s.d.

Results

STZ-Induced Diabetic Rats Show Various Severities of Mesangial Matrix Expansion

We first analyzed mesangial matrix expansion in rats after 24 weeks of STZ injection by measuring the Mes Fx (Figure 1). The Mes Fx was significantly

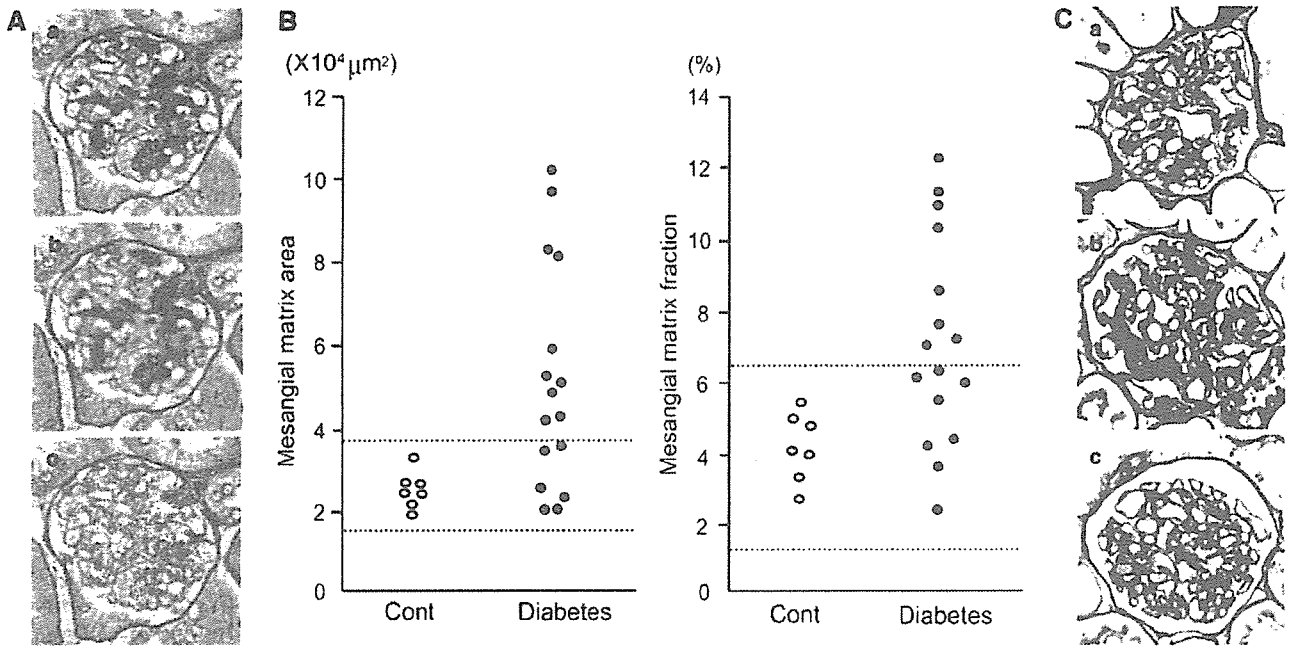

Figure 1 Mesangial matrix fraction in the study groups. (**A**) Representative glomerulus was shown whose glomerular surface area and mesangial PASM-positive area was determined by IPAP as described in Materials and methods. (a) Image of the PASM stained glomerulus. (b) The assessed glomerular area expressed in red color. (c) Image of the same glomerulus showing the PASM positive area in green color. Note that mesangial PASM-positive area was mainly detected by IPAP. (**B**) The mesangial matrix fraction was determined as percentage of mesangial matrix area per total glomerular surface area. The distribution of mesangial matrix area (left) and matrix fraction (right) in control and diabetic rats were shown. Each spot represents one rat. The area between two broken lines represents ± 2 s.d. in control. (**C**) Representative light microscopic appearance of glomeruli (PASM staining, $\times 400$ original magnification) for control rats (a), diabetic rats with more mesangial matrix expansion (b), and diabetic rats with less mesangial matrix expansion (c). Glomerular hypertrophy and mesangial sclerosis were noted in a glomerulus from a diabetic rat with more mesangial matrix expansion.

increased in diabetic rats ($4.0 \pm 1.1\%$ in control rats *vs* $7.0 \pm 2.9\%$ in diabetic rats, $P<0.05$). We also noticed that the value of Mes Fx was widely distributed in diabetic rats, and that the values in diabetic rats were overlapped with those in control rats. We then analyzed their physiological characteristics, and examined the relationship between these variables and mesangial matrix expansion. There was no difference in blood pressure between control rats and diabetic rats, or the correlation between blood pressure and the severity of mesangial matrix expansion. HbA1c and blood sugar were increased, and BW was decreased in diabetic rats compared with those in control rats. Serum Cre was decreased in diabetic rats compared with that in control rats (Table 1), reflecting the decrease of BW and the increase of GFR described below. However, all these variables were not correlated with the severity of Mes Fx (data not shown).

Table 1 Physiological characteristics in this study

	Control	*Diabetes*
N	6	16
Body weight (g)	668 ± 97	305 ± 72*
Blood pressure (mmHg)	131 ± 8	136 ± 6
Blood glucose (mg/dl)	112 ± 7.7	479 ± 78*
HbA1c (%)	2.8 ± 0.22	6.6 ± 1.0*
Cre (mg/dl)	0.35 ± 0.034	0.24 ± 0.051*

After 24 weeks of STZ injection, blood pressure (BP) was measured by the cuff-tailed method, and the rats were weighed (BW) and killed. Blood was taken to evaluate blood sugar, HbA1c, and creatinine (Cre). Data were represented as mean $\pm$ s.d.
*$P<0.05$ *vs* control rats.

Functional Changes do not Correlate with the Glomerular Structural Changes in Diabetic Rats

In the early phase of diabetic nephropathy, two major functional changes, albuminuria and increased GFR are observed. Therefore, we next measured albuminuria and GFR and examined the association of glomerular structural changes with these functional changes. Albuminuria and GFR were significantly increased in both diabetic rats compared with the control rats (control 0.039 ± 0.015 *vs* diabetes 0.33 ± 0.14 mg/mg · Cre for albuminuria, and control 8.5 ± 1.4 *vs* diabetes 21 ± 5.2 ml/day/100 gBW for GFR), but either was not correlated with the severity of mesangial matrix expansion (Figure 2). These data indicate that although albuminuria or GFR could be a sensitive indicator

of incipient diabetic nephropathy, either of them may not accurately reflect the severity of mesangial matrix expansion in STZ rats.

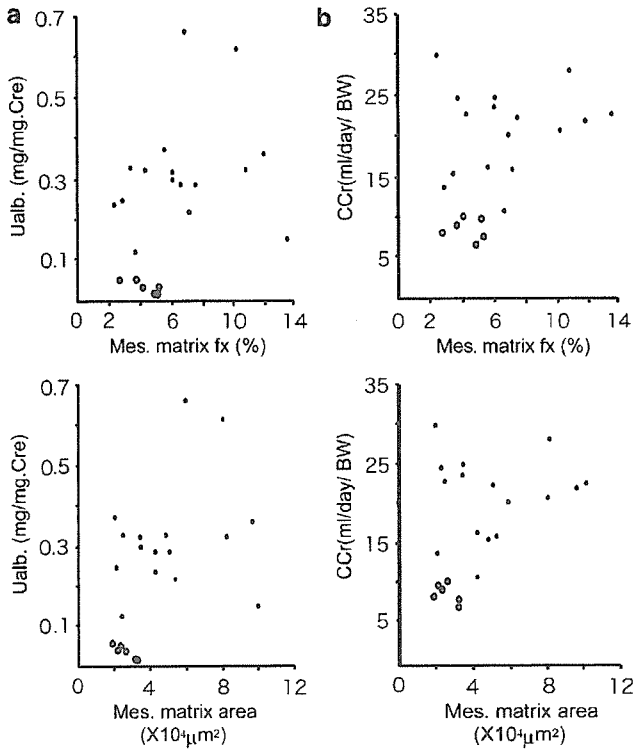

Figure 2 Functional changes in this experiment. The correlations between albuminuria (Ualb; **a**), or creatinine clearance (CCr; **b**), and mesangial matrix fraction, or mesangial matrix area were shown. There were no correlations between the two variables and mesangial matrix fraction (upper column) and mesangial matrix area (lower column). Albuminuria was measured by Nephrat, and creatinine clearance was calculated and normalized as described in Materials and methods. Open circles; control rats, filled circles; diabetic rats.

Glomerular Structural Changes are Closely Correlated with the Major Histological Changes in Diabetic Rats

In diabetic nephropathy, two major histological changes can be found in diabetic glomeruli with mesangial matrix expansion; one is an increase of Col4 in the mesangial area, and the other is an increase of α-SMA in mesangial cells. Therefore, to investigate these histological changes in STZ rats, we examined the expression of Col4 (Figure 3) and α-SMA (Figure 4) in control and diabetic rats. In diabetic rats, the glomerular expression of Col4 was increased (control 5.8±1.1% *vs* diabetes 8.8± 2.9%), and was mostly localized in the mesangial area by immunohistochemical analysis. We then examined glomerular expression of α-SMA by Western blotting. The glomerular expression of α-SMA was almost undetectable in control rats. On the other hand, the glomerular expression of α-SMA was significantly increased in diabetic rats with various severities, and its expression was also localized in the mesangial area by immunohistochemical analysis. Furthermore, both histological findings were significantly correlated with the severity of mesangial matrix expansion (Figures 3A and 4B).

Glomerular Expression of Smad1 and Phosphorylated Smads is Increased in Diabetic Rats with Greater Mesangial Matrix Expansion

We previously reported that Smad1 is a direct regulatory factor of Col4 under diabetic conditions *in vitro*. Therefore, to investigate the role of Smad1 in mesangial matrix expansion in diabetic nephropathy *in vivo*, we examined the glomerular expression of Smad1 along with phosphorylated Smads by Western blotting and immunohistochemistry (Figure 5). The

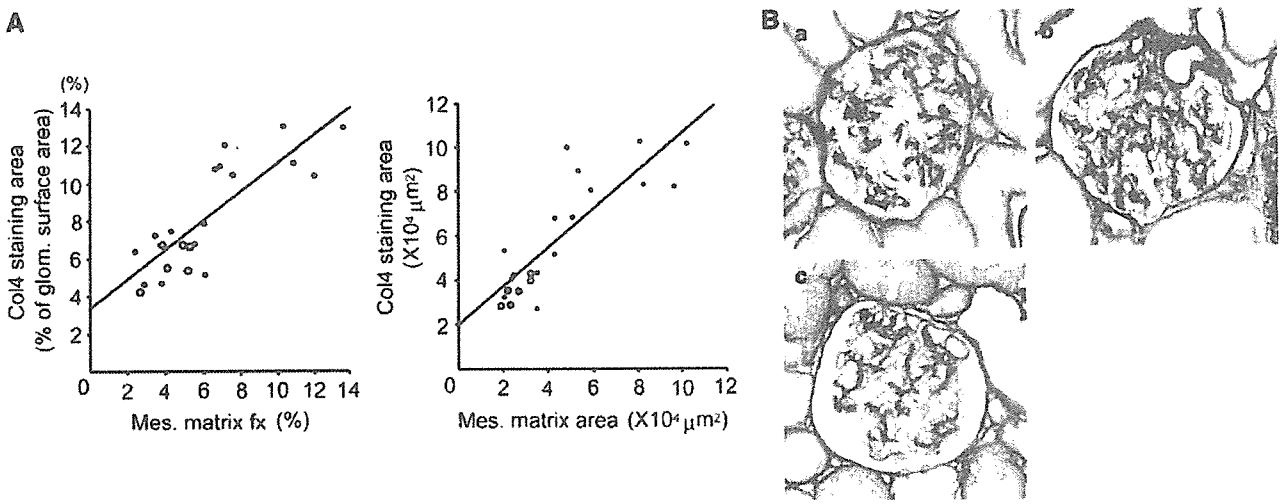

Figure 3 Glomerular expression of Col4. (**A**) The correlation between glomerular expression of Col4 and mesangial matrix fraction (left), or mesangial matrix area (right) was shown. $r=0.826$, $P<0.0001$, and $r=0.842$, $P<0.0001$, respectively. Open circles; control rats, filled circles; diabetic rats. Morphometric analysis of the glomerular expression of Col4 was performed as described in Materials and methods. (**B**) Immunohistochemistry of Col4 in control rats (a), diabetic rats with more mesangial matrix expansion (b), and diabetic rats with less mesangial matrix expansion (c). The original magnification was ×400.

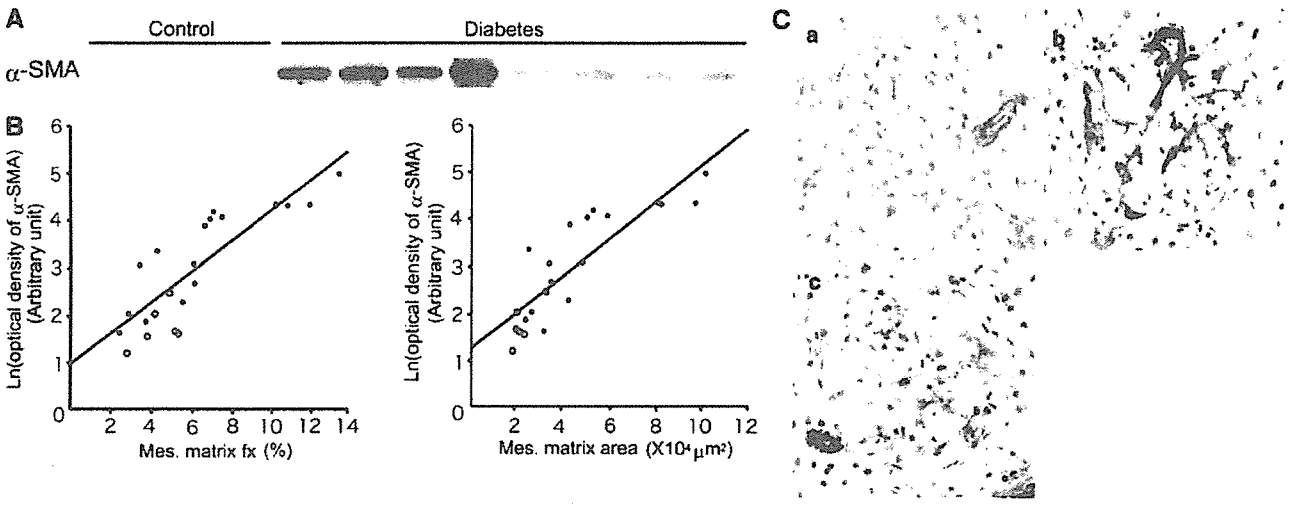

Figure 4 Glomerular expression of α-SMA. (**A**) Immunoblots for glomerular expression of α-SMA in control and diabetic rats are shown. Protein (40 μg) obtained from each glomerular lysates was loaded. Each lane represents a representative Western blot for the glomerular lysates from each rat. (**B**) The correlation between glomerular expression of α-SMA and mesangial matrix fraction (left) and mesangial matrix area (right) in this study. $r=0.843$, $P<0.0001$, and $r=0.862$, $P<0.0001$, respectively. Open circles; control rats, filled circles; diabetic rats. Optical densitometry of α-SMA was performed as described. (**C**) Immunohistochemistry of α-SMA in control rats (a), diabetic rats with more mesangial matrix expansion (b), and diabetic rats with less mesangial matrix expansion (c). Sections were counterstained with hematoxylin solution. Representative light microscopic appearance of glomerulus is shown. The original magnification was ×400.

glomerular expression of Smad1 and phosphorylated Smads was significantly increased with distinct varieties, while in control rats, the expression of both Smad1 and phosphorylated Smads was almost undetectable by Western blotting. We also found that increased Smad1 was localized mostly in the glomerular mesangial area. Furthermore, the glomerular expression of Smad1 was strongly correlated with all three major histological findings involved in diabetic glomeruli; the severity of mesangial matrix expansion, the extent of glomerular expression of Col4 and α-SMA (Figure 5C). These data strongly suggest that the glomerular expression of Smad1 is closely associated with mesangial matrix expansion in diabetic nephropathy *in vivo*.

To investigate the factors regulating the glomerular expression of Smad1, we then examined the upstream factors involved in the activation of Smad1. We have already shown that AGE can upregulate the expression and induce the phosphorylation of Smad1 in cultured mesangial cells.[10] Therefore, first we examined glomerular accumulation of AGE. AGE was detected in expanded mesangial area and GBM in diabetic rats by immunohistochemistry. We then quantified the content of glomerular accumulation of AGE using dot blot analysis of glomerular lysates. A significant correlation was found between glomerular deposition of AGE and glomerular expression of Smad1 ($r=0.494$, $P<0.05$). Furthermore, we checked glomerular expression of TGF-β and ALK-1, because these molecules are known to act as upstream factors of Smad1, being upregulated by AGE stimulation in cultured mesangial cells.[10] Compatible with our previous reports, the expression of these molecules was increased in diabetic glomeruli. However, we could not find the correlation between glomerular expression of Smad1 and that of TGF-β or ALK-1 (data not shown).

Urinary Excretion of Smad1 is Associated with the Mesangial Matrix Expansion in the Early Phase of Diabetic Nephropathy

Because the glomerular expression of Smad1 was significantly increased in diabetic rats with mesangial matrix expansion, we examined urinary excretion of Smad1. In our preliminary study, we could detect urinary Smad1 only in some of the urine samples from diabetic rats with mesangial matrix expansion, in proportionate to their glomerular expression of Smad1, by Western blotting. However, the concentration of Smad1 in urine samples was very low, and could not be quantified by Western blotting. Therefore, we developed ELISA to measure the concentration of urinary excretion of Smad1 as described in Materials and methods (Figure 6). In association with an increase of the glomerular expression of Smad1, urinary excretion of Smad1 was increased in diabetic rats by ELISA (control: 0.042 ng/mg·Cre *vs* diabetic 1.77 ng/mg·Cre). Furthermore, unlike albuminuria or GFR, urinary excretion of Smad1 was closely correlated with Mes Fx, and mesangial matrix area (Figure 6b left and right). These data suggest that urinary excretion of Smad1 is also closely associated with mesangial matrix expansion in the early phase of diabetic nephropathy.

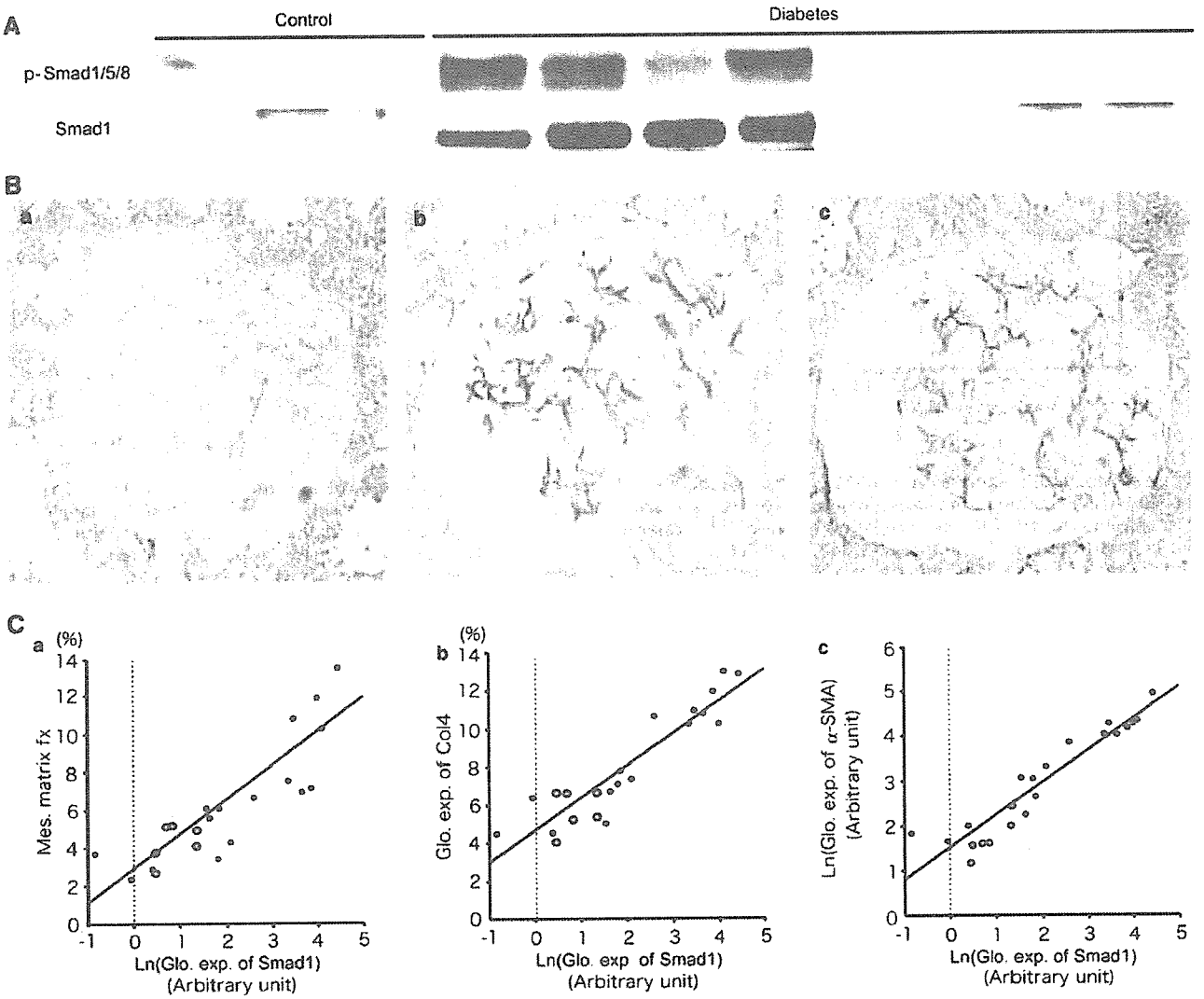

Figure 5 Glomerular expression of Smad1 and phospho-Smads. (A) Immunoblots for Smad1 and phospholylated Smad1/5/8 are shown. Glomerular lysates were prepared as described in Figure 4. Of each sample, (40 μg) was analyzed. Each lane represents a representative Western blot for the glomerular lysates from each rat. (B) Immunohistochemistry of Smad1 in control rats (a), diabetic rats with more mesangial matrix expansion (b), and diabetic rats with less mesangial matrix expansion (c). Representative light microscopic appearance of glomerulus is shown. The original magnification was $\times 400$. (C) The correlation between glomerular expression of Smad1 and mesangial matrix fraction (a; $r=0.850$, $P<0.0001$), glomerular expression of Col4 (b; $r=0.871$, $P<0.0001$), and α-SMA (c; $r=0.944$, $P<0.0001$) in this study. Optical densitometry of glomerular expression of Smad1 in Western blotting was performed as described.

Smad1 is Involved in the Expression not only of Col4, but also of α-SMA in Mesangial Cells

As described above, the glomerular expression of Col4 and α-SMA was significantly increased in parallel with the glomerular expression of Smad1. To investigate the relationship among Col4, α-SMA, and Smad1, we examined whether Smad1 can cause the increase of Col4 and α-SMA expression in mesangial cells *in vitro* (Figure 7). Transfection of a Smad1 expression vector resulted in an increase in the transcriptional activity of Col4 and α-SMA about by two- and four-folds in mouse mesangial cells, respectively. These data suggest that Smad1 is involved in the expression of both Col4 and α-SMA in mouse mesangial cells.

Discussion

Here we demonstrate that Smad1 is a key transcriptional factor by regulating the expression of Col4 and α-SMA, and plays an important role for the progression of mesangial matrix expansion in diabetic nephropathy *in vivo*. Our study also indicates that the amount of urinary Smad1 excretion would be a better diagnostic marker than albuminuria to assess mesangial matrix expansion in diabetic nephropathy.

This study showed the close relationship between mesangial matrix expansion and the glomerular expression of Smad1 in STZ rats. However, we could not show what causes the variety of mesangial matrix expansion and induces glomerular

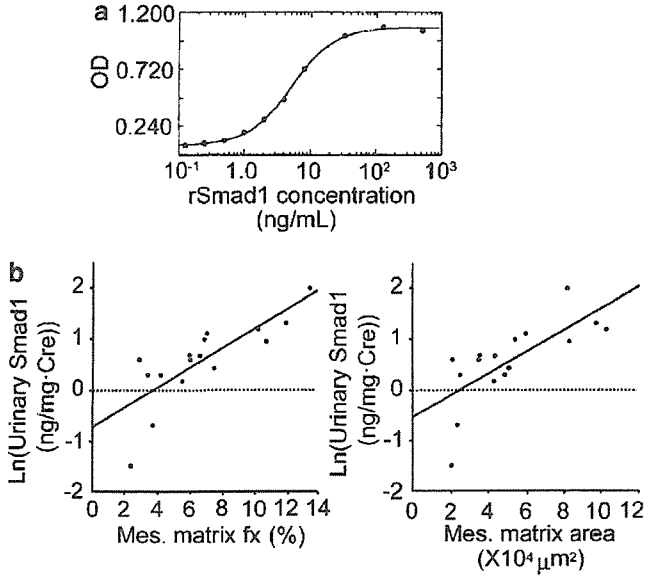

Figure 6 Urinary concentration of Smad1 (**a**) Standard curve of ELISA. The intra-assay and interassay coefficient variation for this assay were 3.8 and 17.5%, respectively. (**b**) The correlation between urinary excretion of Smad1 and mesangial matrix fraction (left), or mesangial matrix area (right) in diabetic rats. $r=0.733$, $P<0.01$, and $r=0.716$, $P<0.01$, respectively. Quantification of urinary concentration of Smad1 was performed as described in Materials and methods. The variables of control rats were scaled out of the ranges.

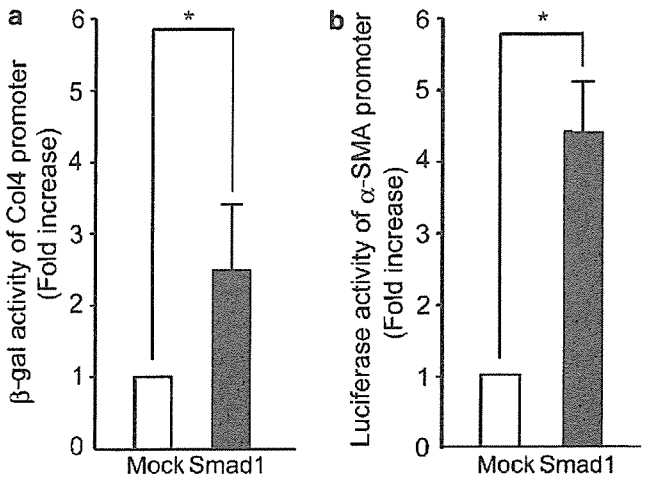

Figure 7 Effects of Smad1 on Col4 and α-SMA transcriptional activities. Mesangial cells were cotransfected with the mouse Col4 promoter-LacZ reporter construct, or the mouse α-SMA promoter luciferase reporter construct along with either the vector encoding wild type of Smad1 or the mock vector. After 48 h, β-gal activity for Col4 (**a**) and luciferase activity for α-SMA (**b**) were measured. Both activities were expressed as fold increase over mock vector cotransfection. Data represent the mean±s.d. of triplicated samples. $^*P<0.05$.

expression of Smad1 in STZ rats. There are many factors that can influence the severity of mesangial matrix expansion in diabetes; duration of diabetes, glycemic control, blood pressure, diets, and so on. In our experiment, however, we could not find any correlation between these variables and the severity of mesangial matrix expansion. According to our data in this and previous reports, the variety of glomerular structural changes could result from the variety of glomerular expression of Smad1. However, there are only a few reports about factors that could regulate the expression of Smad1, although many reports were published about the activation of Smad1.[20,21] Consistent with our previous report that AGE can induce Smad1 *in vitro*, here we have shown a positive correlation between AGE and Smad1 in diabetic glomeruli. We also showed that glomerular expression of TGF-β and ALK-1 was increased in diabetic rats. However, the direct correlation between glomerular expression of Smad1 and that of TGF-β or ALK-1 was not found. These results raised the possibility that TGF-β is not sufficient for the induction of glomerulosclerosis,[22] or that another signaling pathway could activate Smad1, leading to glomerulosclerosis. In this regard, we have already found that angiotensin II can also activate Smad1 via a Src-dependent pathway both in cultured mesangial cells and STZ rats (Mima A, Matsubara T, *et al*, unpublished observation). Therefore, we need further studies to elucidate the interaction between these two distinct signaling pathways in the development of mesangial matrix expansion in the early phase of diabetic nephropathy.

Genetic factors could also influence the severity of diabetic nephropathy[23,24] and could regulate the differential induction of Smad1. In this experiment, we used Sprague–Dawley rats, which were known to be one of the common outbred strains, and therefore each rat seemed to have, to some extent, a different genetic background. Thus, the variety of glomerular expression of Smad1 in this experiment could result from the genetic variety of the factors that can affect the expression of Smad1 under diabetic conditions.

We have already shown that Smad1 was a direct transcriptional regulator of *Col4* gene *in vivo* and *in vitro*. In this study, we have further extended our study and showed that overexpression of Smad1 resulted in an increase of *α-SMA* gene transcription *in vitro*. Evidence is accumulating which suggests that mesangial cells, during the process of glomerular injuries, including diabetes mellitus, can undergo a phenotypic modulation in which they markedly upregulate their expression of α-SMA, in addition to upregulating the expression of Col4.[25,26] Therefore, our report suggests that two major different events observed in the phenotypic modulation of mesangial cells under diabetic conditions might occur under the regulation of the common transcriptional factor, Smad1. There is no putative direct binding site for Smad1 in the α-SMA promoter sequence.[10] The α-SMA promoter has several consensus DNA sequences, which are involved in various transcription factors, some of which are known to interact with, or to be downstream factors of Smad1. Therefore, Smad1 might regulate α-SMA