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# Effects of Candesartan Compared With Amlodipine in Hypertensive Patients With High Cardiovascular Risks

## Candesartan Antihypertensive Survival Evaluation in Japan Trial

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*Abstract*—The Candesartan Antihypertensive Survival Evaluation in Japan Trial was designed to compare the long-term effects of the angiotensin II receptor blocker candesartan and the calcium channel blocker amlodipine on the incidence of cardiovascular events, represented as a composite of sudden death and cerebrovascular, cardiac, renal, and vascular events in high-risk Japanese hypertensive patients. We conducted a prospective, randomized, open-label study with blinded assessment of the end point in 4728 Japanese hypertensive patients (mean age: 63.8 years; mean body mass index: 24.6 kg/m$^2$). Patients were followed for an average of 3.2 years. Blood pressure was well controlled with both treatment-based regimens (systolic blood pressure/diastolic blood pressure: 136.1/77.3 mm Hg for candesartan-based regimens and 134.4/76.7 mm Hg for amlodipine-based regimens after 3 years). Primary cardiovascular events occurred in 134 patients with both the candesartan- and amlodipine-based regimens. The 2 treatment-based regimens produced no significant differences in cardiovascular morbidity or mortality in the high-risk Japanese hypertensive patients (hazard ratio: 1.01; 95% CI: 0.79 to 1.28; $P$=0.969). In each primary end point category, there was no significant difference between the 2 treatment-based regimens. New-onset diabetes occurred in fewer patients taking candesartan (8.7/1000 person-years) than in those taking amlodipine (13.6/1000 person-years), which resulted in a 36% relative risk reduction (hazard ratio: 0.64; 95% CI: 0.43 to 0.97; $P$=0.033). We disclosed that candesartan-based and amlodipine-based regimens produced no statistical differences in terms of the primary cardiovascular end point, whereas candesartan prevented new-onset diabetes more effectively than amlodipine. **(*Hypertension*. 2008;51:393-398.)**

**Key Words:** antihypertensive therapy ■ hypertension ■ cardiovascular diseases ■ angiotensin II ■ calcium channel blockers ■ clinical trials

Angiotensin II receptor blockers (ARBs) and calcium channel blockers (CCBs) have proven to be important advances for the treatment of hypertension.[1,2] These agents have been shown to be as effective or sometimes better than other antihypertensive drugs in terms of cardiovascular morbidity and mortality and associated adverse events.[3–5] Clinical trials have shown significant effects from treatment with CCBs or angiotensin-converting enzyme inhibitors for preventing cardiovascular morbidity and mortality in high-risk populations.[2,6,7] In the Valsartan Antihypertensive Long-term Use Evaluation (VALUE) Trial, the ARB valsartan was compared with the CCB amlodipine in Europe and the United States.[8] The VALUE Trial concluded that the main outcome (cardiac disease) did not differ between the groups, whereas unequal reductions in blood pressure may have accounted for the observed differences between the groups in the cause-specific outcomes. Thus, it is still unclear whether there are differences in the efficacies of ARBs and CCBs.

The event rates of cardiovascular disease in Japan differ from those in Europe and the United States. Mortality from ischemic heart disease in Japan is one third of that in the United States, and mortality from cerebrovascular disease in Japan is ≈1.5 times higher than that in the United States.[9] These differences may be partly explained by differences in the lifestyles of Japanese and Western populations, which are reflected in body mass index (BMI) (mean BMI: 23 to 25 kg/m$^2$ and 28 to 30 kg/m$^2$, respectively).[10,11] In this context, the Candesartan Antihypertensive Survival Evaluation in Japan (CASE-J) Trial was designed to evaluate the efficacies of the ARB candesartan cilexetil and the CCB amlodipine besylate for reducing the incidences of cardiovascular morbidity and mortality (primary and secondary end points), as

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This trial has been registered at www.clinicaltrials.gov (identifier NCT00125463).

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well as new-onset diabetes (prespecified end point) in high-risk Japanese hypertensive patients.

## Methods

### Study Design

The CASE-J Trial was a prospective, multicenter, randomized, open-label, active-controlled, 2-arm parallel-group comparison in Japan with a response-dependent dose titration and blinded assessment of the end points in high-risk hypertensive patients. The random assignment, data collection, and analyses were performed by the EBM Research Center of Kyoto University. The rationale and complete design of CASE-J Trial have been published elsewhere.[12] In addition, the end point of new-onset diabetes was prespecified at the 28th Annual Meeting of the Japanese Society of Hypertension on September 17, 2005.

The Ethics Committee at the Kyoto University Graduate School of Medicine approved the CASE-J Trial protocol according to the principles of the Helsinki Declaration. After obtaining informed consent, the patients were randomly assigned to the treatment groups. Enrolled patients were given 1 of 2 medications: candesartan cilexetil or amlodipine besylate. The former was administered orally at a dose of 4 to 8 mg/d. When the patient's blood pressure (BP) did not reach the targets for controlled BP, the dose was increased to 12 mg/d. The latter was administered orally at a dose of 2.5 to 5.0 mg/d and was increased to 10.0 mg/d when necessary. Once a patient was given the assigned medication, the use of other ARBs, CCBs, and all of the angiotensin-converting enzyme inhibitors was prohibited. Patients already being treated with diuretics, $\alpha$-blockers, $\beta$-blockers, or $\alpha$- and $\beta$-blockers before enrollment were allowed to continue taking these medications. According to the guideline proposed by Japanese Society of Hypertension, $\geq$2 consecutive BP measurements were taken from each patient in a sitting position at a clinic.[13] The targets for the control of BP were as follows: <60 years old, systolic BP (SBP)/diastolic BP (DBP) <130/85 mm Hg; 60 to 69 years old, SBP/DBP <140/90 mm Hg; 70 to 79 years old, SBP/DBP <150/90 mm Hg; and $\geq$80 years old, SBP/DBP <160/90 mm Hg.[13]

### Population and Treatment

Patients with high-risk hypertension (SBP $\geq$140 mm Hg or DBP $\geq$90 mm Hg in patients <70 years old or SBP $\geq$160 mm Hg or DBP $\geq$90 mm Hg in patients $\geq$70 years old) were enrolled in the study. As reported previously,[12] high-risk patients were defined by the presence of any of the following factors: (1) severe hypertension (SBP $\geq$180 mm Hg or DBP $\geq$110 mm Hg); (2) type 2 diabetes mellitus; (3) a history of stroke or transient ischemic attack >6 months before the screening; (4) left ventricular hypertrophy, which was defined by the thickness of the left ventricular posterior wall or the interventricular septum wall $\geq$12 mm on echocardiography or Sv1+Rv5 $\geq$35 mm on electrocardiography, angina pectoris, or a history of myocardial infarction >6 months before the screening; (5) proteinuria or a serum creatinine concentration $\geq$1.3 mg/dL; or (6) arteriosclerotic peripheral artery obstruction. The exclusion criteria have also been reported elsewhere.[12] The event evaluation was performed independently by the event evaluation committee, which was blinded to the assigned treatment groups and adjudicated according to the protocol criteria. Adverse events and prespecified safety parameters were monitored by the data and safety monitoring board. The CASE-J Trial was closed on January 1, 2006.

### Outcome Measures

The primary end point, which was the first fatal/nonfatal cardiovascular event, the secondary end points, and the prespecified end point are listed in Table 1. For the analysis of new-onset diabetes, we excluded all of the patients with type 2 diabetes mellitus at baseline from the analysis. Individual case report forms and adverse-event databases were monitored for any information reporting that the patients began to use antidiabetic drugs and/or for newly apparent cases of type 2 diabetes.

**Table 1. Outcome Measures**

| |
|---|
| Primary end points (composite of the following events) |
| Sudden death: unexpected death that happened within 24 hours without external causes |
| Cerebrovascular events: stroke or transient ischemic attack |
| Cardiac events: heart failure, angina pectoris, or acute myocardial infarction |
| Renal events: serum creatinine concentration $\geq$4.0 mg/dL, doubling of the serum creatinine concentration (however, creatinine $\leq$2.0 mg/dL is not regarded as an event), or end-stage renal disease |
| Vascular events: dissecting aortic aneurysm or arteriosclerotic occlusion of a peripheral artery |
| Secondary and prespecified end points |
| All-cause deaths |
| New-onset diabetes |
| Discontinuance of treatment because of adverse events |

### Statistical Methods

Based on previous results from studies of CCBs,[2,14,15] the CASE-J Trial was designed to detect a 40% relative risk reduction in the cardiovascular incidence rate in patients taking candesartan-based regimens with a 2-sided $\alpha$ level of 0.05 and 90% power.[16] Assuming a 20% loss to follow-up, we required a minimum of 3200 patients in total, and each patient was enrolled during a 1.5-year period and was followed for $\geq$3 consecutive years. An interim analysis was conducted 1 year after the completion of enrollment (December 2003). An O'Brien-Fleming spending function was used to adjust the $\alpha$ level.[17]

The incidence proportions were calculated using the Kaplan–Meier method and were compared with a log-rank test stratified by diabetic status at baseline. The hazard ratio (HR) and 95% CI were also estimated using Cox regression analysis. The $P$ value and CI were adjusted for sequential testing of the results of the primary end point. These analyses were performed based on the intention-to-treat principle. If there were inequalities in BP levels during the follow-up, the imbalance in the BPs was adjusted using Cox regression analysis with SBP or DBP as the time-dependent covariate.

Exploratory subgroup analyses were prespecified to assess the primary, secondary, and prespecified results corrected for the baseline characteristics (diabetes; sex; age; SBP and DBP; systolic hypertension; BMI; CCB, angiotensin-converting enzyme inhibitor or ARB use before starting the CASE-J Trial; creatinine clearance; and history of cerebrovascular events, cardiac events, or renal events). Cox regression analysis was used to identify the treatment effect in these subgroups. Cox regression analysis was also used to identify the clinically relevant interactions between the treatment and these subgroups.

The safety population was grouped according to the treatment actually received. Differences in the frequency of adverse events were analyzed with the $\chi^2$ test. All of the statistical tests were 2-sided with an $\alpha$ level of 0.05 and were performed using SAS version 9.1 (SAS Institute) and East 4.1 (Cytel).

## Results

### Study Profile and Baseline Characteristics

Between September 2001 and December 2002, 4728 patients with a mean age of 63.8 years and a mean BMI of 24.6 kg/m$^2$ were assigned to the 2 treatment-based regimens. As shown in Figure 1, 4703 randomly assigned patients were included in the analysis, and 136 patients (2.9%) were lost to follow-up. Table 2 summarizes the characteristics of the patients at baseline. There was a statistical difference between the sex ratios for the 2 treatment-based regimens (46.4% and 43.2% of the subjects were female for the candesartan-based regimens and

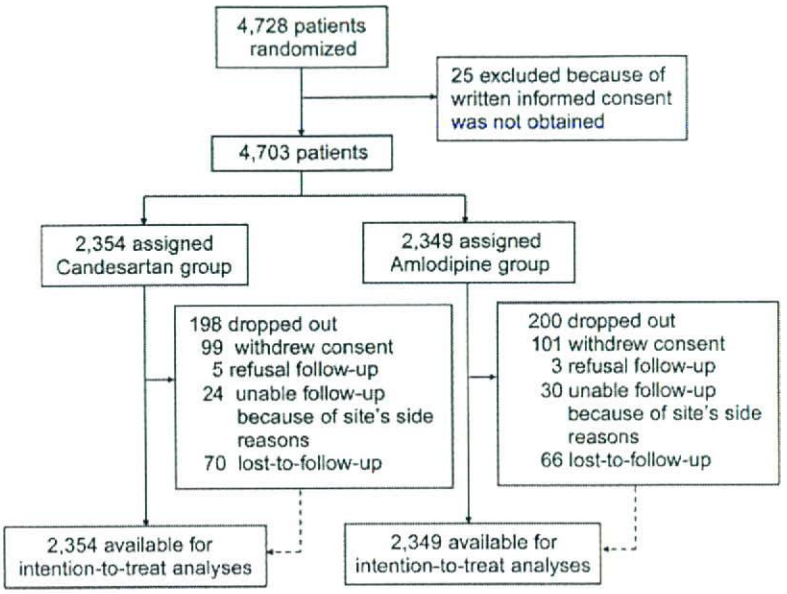


**Figure 1.** Trial profile of the CASE-J Trial.

amlodipine-based regimens, respectively), whereas there were no differences in terms of the other clinical parameters.

## Duration of Follow-Up and Adherence to the Treatment

For both treatment-based regimens, the mean follow-up periods were 3.2 years, and the median values were 3.4 years. The fifth to 95th percentile interval of the follow-up periods was 0.9 to 4.1 years for the candesartan-based regimens and 1.0 to 4.2 years for the amlodipine-based regimens. The study accumulated 15 175 person-years of follow-up (7563 person-years and 7612 person-years for the candesartan- and amlodipine-based regimens, respectively). The percentages of patients who received >80% of the allocated drugs during the follow-up were 96.5% and 96.0% in the candesartan- and amlodipine-based regimens, respectively. The percentage of the candesartan-treated patients who received other antihypertensive drugs was larger than that of the amlodipine-treated patients (54.5% and 42.7%, respectively; $P<0.001$; Table 3). After 3 years, the mean number of antihypertensive drugs used, including the allocated drugs, was 1.54 for patients treated with candesartan-based regimens and 1.37 for those treated with amlodipine-based regimens.

**Table 2. Baseline Characteristics of Trial Participants**

| Baseline Characteristics | Candesartan (n=2354) | Amlodipine (n=2349) |
|---|---|---|
| Women | 1092 (46.4) | 1014 (43.2) |
| Age | 63.8±10.5 | 63.9±10.6 |
| BMI, kg/m$^2$ | 24.6±3.7 | 24.5±3.6 |
| SBP, mm Hg | 162.5±14.2 | 163.2±14.2 |
| DBP, mm Hg | 91.6±11.0 | 91.8±11.4 |
| Current smokers | 489 (20.8) | 536 (22.8) |
| Severe hypertension: SBP ≥180 mm Hg or DBP ≥110 mm Hg | 454 (19.3) | 493 (21.0) |
| Type 2 diabetes mellitus* | 1011 (42.9) | 1007 (42.9) |
| History of cerebrovascular events† | 248 (10.5) | 225 (9.6) |
| History of cardiac events‡ | 1007 (42.8) | 1023 (43.6) |
| History of renal events§ | 572 (24.3) | 543 (23.1) |
| Arteriosclerotic peripheral arterial obstruction | 29 (1.2) | 24 (1.0) |

Data are shown as the No. of patients (%) or the mean±SD.

*Type 2 diabetes mellitus was defined by fasting blood glucose levels ≥126 mg/dL, casual blood glucose levels ≥200 mg/dL, HbA1c ≥6.5%, 2-hour blood glucose levels in the 75-g oral glucose tolerance test ≥200 mg/dL, or current treatment with a hypoglycemic agent at baseline.

†History of cerebrovascular events includes cerebral hemorrhage, cerebral infarction, and transient ischemic attack.

‡History of cardiac events includes left ventricular hypertrophy, angina pectoris, and myocardial infarction.

§History of renal events includes proteinuria and serum creatinine levels ≥1.3 mg/dL.

**Table 3. No. of Patients Using Additional Drugs Throughout the Follow-Up Period**

| Additional Drugs | Candesartan (n=2354), n (%) | Amlodipine (n=2349), n (%) | $P$* |
|---|---|---|---|
| Antihypertensive drugs | 1282 (54.5) | 1003 (42.7) | <0.001 |
| Diuretics | 580 (24.6) | 323 (13.8) | <0.001 |
| α-Blockers | 610 (25.9) | 391 (16.6) | <0.001 |
| β-Blockers | 524 (22.3) | 397 (16.9) | <0.001 |
| α- and β-Blockers | 193 (8.2) | 146 (6.2) | 0.009 |
| Others | 100 (4.2) | 47 (2.0) | <0.001 |
| Antihyperlipidemics | 1050 (44.6) | 1032 (43.9) | 0.644 |
| Antidiabetics (including insulin) | 874 (37.1) | 900 (38.3) | 0.402 |
| Antithrombotics | 652 (27.7) | 620 (26.4) | 0.314 |
| Antianginal | 264 (11.2) | 280 (11.9) | 0.450 |
| Antiarrhythmic | 113 (4.8) | 122 (5.2) | 0.536 |

*$P$ values were obtained using $\chi^2$ tests.

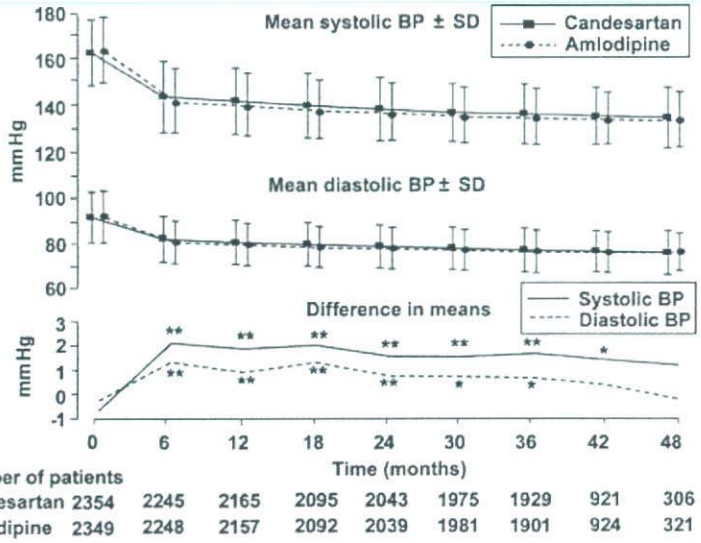


**Figure 2.** Changes in the SBP and DBP, as well as differences (candesartan−amlodipine) during the follow-up period. Mean SBP and mean DBP measured in the treatment groups and differences between the means. $^{**}P<0.01$; $^{*}P<0.05$.

## Effects on BP

The SBP and DBP were well controlled in the CASE-J Trial. SBP/DBP was 162.5/91.6 mm Hg (SD: 14.2/11.0) at baseline and 136.1/77.3 mm Hg (SD: 12.9/9.6) after 3 years for candesartan-based regimens. SBP/DBP was 163.2/91.8 mm Hg (SD: 14.2/11.4) at baseline and 134.4/76.7 mm Hg (SD: 12.1/9.3) after 3 years for amlodipine-based regimens (Figure 2). Both the SBP and DBP were significantly lower in amlodipine-treated patients compared with candesartan-treated patients; after 3 years, the SBP and DBP were 1.7 mm Hg ($P<0.001$) and 0.6 mm Hg ($P=0.028$) lower in the amlodipine-treated patients, respectively.

## Primary Outcome

Primary cardiovascular events occurred in 134 patients with both the candesartan- and amlodipine-based regimens. The 2 treatment-based regimens produced no significant difference in cardiovascular morbidity or mortality in the high-risk hypertensive patients (HR: 1.01; 95% CI: 0.79 to 1.28; $P=0.969$; Figure 3). In each primary end point category, there was no significant difference between the 2 treatment-based regimens (Figure 4). The HR for primary composite end point after an adjustment for the baseline characteristics (sex, age, CCB use, angiotensin-converting enzyme inhibitor or ARB use, creatinine clearance rate, and history of cerebrovascular, cardiac, and renal events) was 1.00 (95% CI: 0.78 to 1.27), and HRs after an adjustment using Cox regression analysis with SBP and DBP as the time-dependent covariates were 0.98 and 1.02 (95% CI: 0.77 to 1.25 and 0.80 to 1.30), respectively. The primary result did not change after these adjustments. In addition, we also evaluated the time-specific interval risk ratios of cardiovascular events every 6 months. There were no statistically significant time-specific interval risk ratios between the 2 treatment-based regimens.

## Secondary and Prespecified Outcomes

For the secondary end points, 73 candesartan-treated patients (9.4/1000 person-years) and 86 amlodipine-treated patients (11.1/1000 person-years) died during the follow-up period. Neither the all-cause death rates nor the death rates because of cardiovascular events differed significantly between the 2 regimens. At baseline, 1343 candesartan-treated patients (mean age:

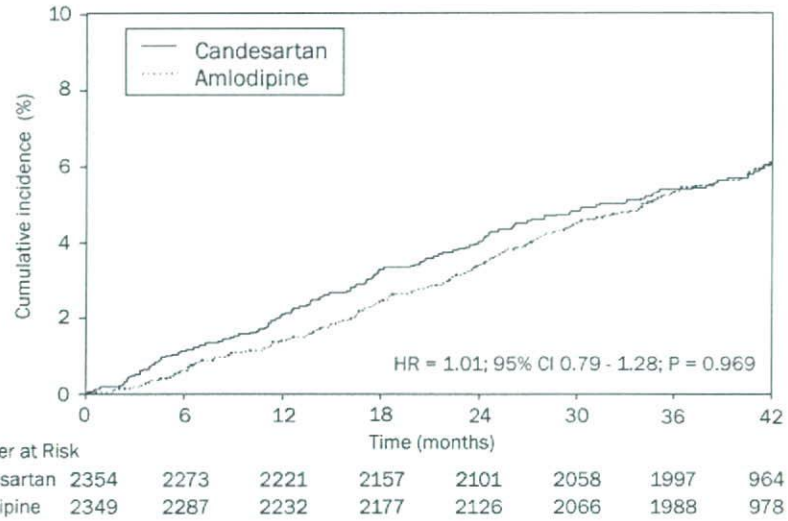


**Figure 3.** Kaplan–Meier curves for the primary composite end point. The primary end point was the time to the first cardiovascular event.

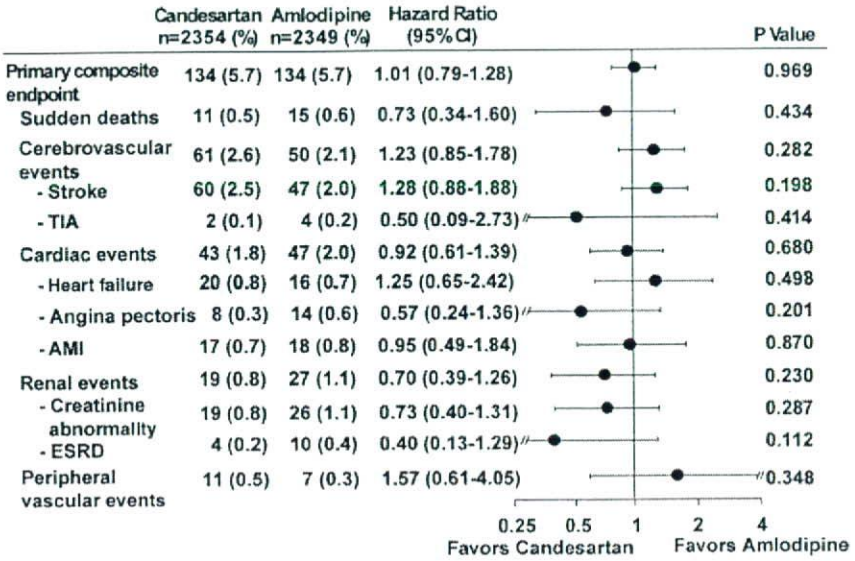


**Figure 4.** Comparisons of the primary composite end point and each cardiovascular event. The first event for each category was counted, including the number of each event, HRs and the corresponding 95% CIs, and *P* values. TIA indicates transient ischemic attack; AMI, acute myocardial infarction; ESRD, end-stage renal disease. Creatinine abnormality was defined as a serum creatinine concentration ≥4.0 mg/dL or doubling of the serum creatinine concentration. Any creatinine concentration ≤2.0 mg/dL, however, was not regarded as an event.

63.6; mean BMI: 24.2 kg/m$^2$) and 1342 amlodipine-treated patients (mean age: 63.8; mean BMI: 24.0 kg/m$^2$) did not have diabetes. With the exception of a small imbalance in the sex ratios, there were no significant differences between the 2 treatment groups for the other clinical parameters. New-onset diabetes occurred in significantly fewer patients treated with candesartan-based regimens (8.7/1000 person-years) than in those treated with amlodipine-based regimens (13.6/1000 person-years). A 36% relative risk reduction was observed for the incidence of new-onset diabetes with the candesartan-based regimens (HR: 0.64; 95% CI: 0.43 to 0.97; $P$=0.033). The number needed to treat to prevent an additional new-onset diabetic patient with the candesartan-based regimens compared with the amlodipine-based regimens was 63 patients during the average 3.2-year follow-up.

### Exploratory Subgroup Analyses

We assessed the prespecified subgroup analysis of the primary end point. There were no significant interactions between the 2 treatment-based regimens for each of the baseline characteristics. In the prespecified subgroup analyses of all-cause death, candesartan-based regimens were favorable for the subgroup with BMI ≥27.5 kg/m$^2$ (HR: 0.33; 95% CI: 0.14 to 0.79). In addition, a 48% (HR: 0.52; 95% CI: 0.29 to 0.95) relative risk reduction of new-onset diabetes was observed in candesartan-based regimens compared with amlodipine-based regimens in the subgroup with BMI ≥25.0 kg/m$^2$ (mean BMI: 27.7 kg/m$^2$).

### Safety Parameters

A total of 125 patients treated with candesartan-based regimens (5.4%) and 134 patients treated with amlodipine-based regimens (5.8%) discontinued treatment because of adverse events. Hyperkalemia was noted more often in candesartan-treated patients (1.0% for candesartan-based regimens versus 0.3% for amlodipine-based regimens), and flushing was observed more frequently in amlodipine-treated patients (0.0% versus 0.2%). In addition, pneumonia was more frequently reported with amlodipine-based regimens (0.1% versus 0.5%) (Table S1, available at http://hyper.ahajournals.org).

## Discussion

The CASE-J Trial demonstrates no statistically significant difference between candesartan-based and amlodipine-based regimens in terms of the primary composite end point in high-risk Japanese hypertensive patients, although the BP level achieved with candesartan treatment was not as low as that achieved with amlodipine; the differences in SBP and DBP were 1.7 and 0.6 mm Hg after 3 years, respectively. Because BP is a crucial prognostic factor for cardiovascular events, the influence of BP differences on the primary composite end point is not negligible. When we adjusted for the imbalance in SBP or DBP levels using Cox regression analysis, we obtained similar results. Accordingly, it is likely that the failure to achieve similar levels of BP control did not influence the outcomes in the CASE-J Trial. Furthermore, it is notable that the BP levels achieved in the CASE-J Trial (<140/80 mm Hg) were lower than those reported in previous antihypertensive trials.[3,8,18] These findings suggest that strict BP control is important for the treatment of high-risk hypertensive patients.

The CASE-J Trial also shows that the incidence of new-onset diabetes was significantly lower in patients treated with candesartan-based regimens compared with patients treated with amlodipine-based regimens. The relative risk reduction for new-onset diabetes was 36% in the CASE-J Trial, although the incidence of new-onset diabetes in the amlodipine-treated patients in the CASE-J Trial (13.6/1000 person-years) was approximately one third of that in VALUE Trial (41.1/1000 person-years).[8] The mean BMI for patients without diabetes in the CASE-J Trial was 24.1 kg/m$^2$, whereas that in VALUE Trial was 28.0 kg/m$^2$.[19] In addition, the relative risk reduction of new-onset diabetes in the CASE-J Trial was 48% in the subgroup with BMI ≥25 kg/m$^2$, in which the mean BMI (27.7 kg/m$^2$) was similar to that in the VALUE Trial. The more favorable effect profile of candesartan in the CASE-J Trial compared with that of valsartan in the VALUE Trial may be explained by the smaller patient population taking additional diuretics in the CASE-J Trial, as well as the potentially beneficial effects of candesartan. Because the number of patients with diabetes and metabolic syndrome is increasing in Eastern coun-

tries as well as in Western countries, the beneficial effect of the ARB candesartan for the prevention of new-onset diabetes should prove to be valuable.

To evaluate the efficacy of drugs that are widely used all over the world, clinical trials should be designed to examine patient outcomes for various races in many countries. In the VALUE Trial, the largest percentage of the randomly assigned patients was from the United States and European countries, whereas only 3.5% of the patients in the VALUE Trial were from Asian countries.[8,20] The event rates of cardiovascular disease and the severity of obesity in Asian countries such as Japan (mean BMI in the CASE-J Trial: 24.6 kg/m$^2$) differ from those in Western countries (mean BMI in the VALUE Trial: 28.6 kg/m$^2$).[20] As far as we know, there is no published evidence about the efficacy of ARBs in mildly obese populations. The outcome of the CASE-J Trial provides useful information about Asian populations that have similar genetic predispositions and lifestyles as the Japanese population.

### Perspectives

The CASE-J Trial indicates that, with strict BP control, there is no significant difference between candesartan-based and amlodipine-based regimens in terms of the primary cardiovascular end point in high-risk hypertensive patients. Nevertheless, the ARB candesartan is more effective than the CCB amlodipine for the prevention of new-onset diabetes.

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## Disclosures

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# Overexpression of connective tissue growth factor in podocytes worsens diabetic nephropathy in mice

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**Connective tissue growth factor (CTGF) is a potent inducer of extracellular matrix accumulation. In diabetic nephropathy, CTGF expression is markedly upregulated both in podocytes and mesangial cells, and this may play an important role in its pathogenesis. We established podocyte-specific CTGF-transgenic mice, which were indistinguishable at baseline from their wild-type littermates. Twelve weeks after streptozotocin-induced diabetes, these transgenic mice showed a more severe proteinuria, mesangial expansion, and a decrease in matrix metalloproteinase-2 activity compared to diabetic wild-type mice. Furthermore, diabetic transgenic mice exhibited less podocin expression and a decreased number of diffusely vacuolated podocytes compared to diabetic wild-type mice. Importantly, induction of diabetes in CTGF-transgenic mice resulted in a further elevation of endogenous CTGF mRNA expression and protein in the glomerular mesangium. Our findings suggest that overexpression of CTGF in podocytes is sufficient to exacerbate proteinuria and mesangial expansion through a functional impairment and loss of podocytes.**

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Diabetic nephropathy is a major complication in diabetes and the leading cause of end-stage renal disease worldwide.[1] Diabetic nephropathy is characterized by microalbuminuria, renal and glomerular hypertrophy, mesangial expansion with increased thickness of the glomerular basement membrane (GBM), arteriolar hyalinosis, and global glomerular sclerosis, which ultimately cause the progression of proteinuria and renal failure.[2] Accumulation of the extracellular matrix (ECM) components in the mesangium, GBM, and tubulointerstitium plays an important role in its pathology.[3,4] Evidence has shown that such renal lesions are driven, in part, by transforming growth factor-β (TGF-β) in humans and experimental models.[4–6] Recently, functional and structural abnormalities in glomerular podocytes have become highlighted as one of the earliest events in the development of diabetic glomerular injury.[7] Podocyte loss and injury are found at very early stages in patients with diabetic nephropathy, being associated with the acceleration of glomerular structural abnormalities.[8] Causes and consequences of podocyte injury during early diabetic nephropathy, however, remain poorly understood.

Connective tissue growth factor (CTGF, also known as CCN2) belongs to a family of cysteine-rich growth factors, the CCN family, that consists of cysteine-rich protein 61 (Cyr61/CCN1), CTGF/CCN2, nephroblastoma overexpressed (Nov/CCN3), and Wnt-induced secreted proteins (WISP)-1, 2, and 3 (CCN4, 5, and 6, respectively).[9–11] Accumulating evidence has demonstrated that CTGF is crucially involved in the fibrogenic properties of TGF-β.[12–14] CTGF gene expression is strongly induced by TGF-β, and recombinant CTGF potently stimulates fibroblast proliferation and ECM protein synthesis.[12–16] TGF-β-induced collagen synthesis *in vitro* is shown to be CTGF dependent, which is shown by a neutralizing antibody or the antisense gene targeting CTGF.[17] Furthermore, we have demonstrated *in vivo* that knockdown of CTGF gene expression with antisense gene transfer into rat kidney ameliorates tubulointerstitial fibrosis in obstructive nephropathy.[18] CTGF has been proposed to be a crucial mediator for the development of diabetic glomerulosclerosis.[19–24] CTGF mRNA is mainly expressed at podocytes and detected in some parietal epithelial cells of glomeruli under normal conditions in

humans[24] and rats.[13] Under the diabetic milieu, CTGF expression is markedly upregulated in podocytes, mesangial cells, and tubulointerstitial cells, being associated with fibrotic lesions and epithelial-to-mesenchymal transformation.[19–24] CTGF may also enhance ECM accumulation through inhibition of matrix degradation.[25] Although these observations are consistent with CTGF as a key mediator of the progression of diabetic nephropathy, the precise role of CTGF in diabetic glomeruli, especially in podocytes, still remains elusive.

To explore the roles of CTGF in podocytes and in diabetic nephropathy, we generated a transgenic mouse model harboring the podocyte-specific expression of the CTGF gene under the control of the nephrin promoter. We demonstrated acceleration of diabetic nephropathy in CTGF-overexpressing mice, indicating that dysregulated expression of CTGF in podocytes may play a role in mesangial expansion and podocyte loss in diabetic nephropathy.

## RESULTS

### Generation of podocyte-specific CTGF-transgenic mice

We constructed a transgene using a fragment of the human nephrin gene (*Nphs1*),[26] rabbit β-globin intron, mouse CTGF cDNA, and polyA signal (Figure 1a). Transgenic founder lines carrying the human *Nphs1* promoter-mouse CTGF transgene were identified by Southern blot analysis. We obtained five founder CTGF-transgenic (CTGF-Tg) mice, and semiquantitative analysis showed 10 (line 30), 10 (line 68), 15 (line 56), 20 (line 52), and 5 (line 12) transgene copies in these mice (Figure 1b). Northern blot analyses for CTGF revealed that the transgene was expressed abundantly in the isolated glomeruli of CTGF-Tg mice, but not in other tissues, including aorta, brain, heart, lung, liver, spleen, stomach, and pancreas (Figure 1c). The size of transgene-derived CTGF mRNA (1.7 kb) was smaller than the endogenous one, because we used only CTGF coding region (Figure 1c). The glomerular CTGF protein level in CTGF-Tg mice (line 12) was five times higher than that in wild-type mice (Figure 1d).

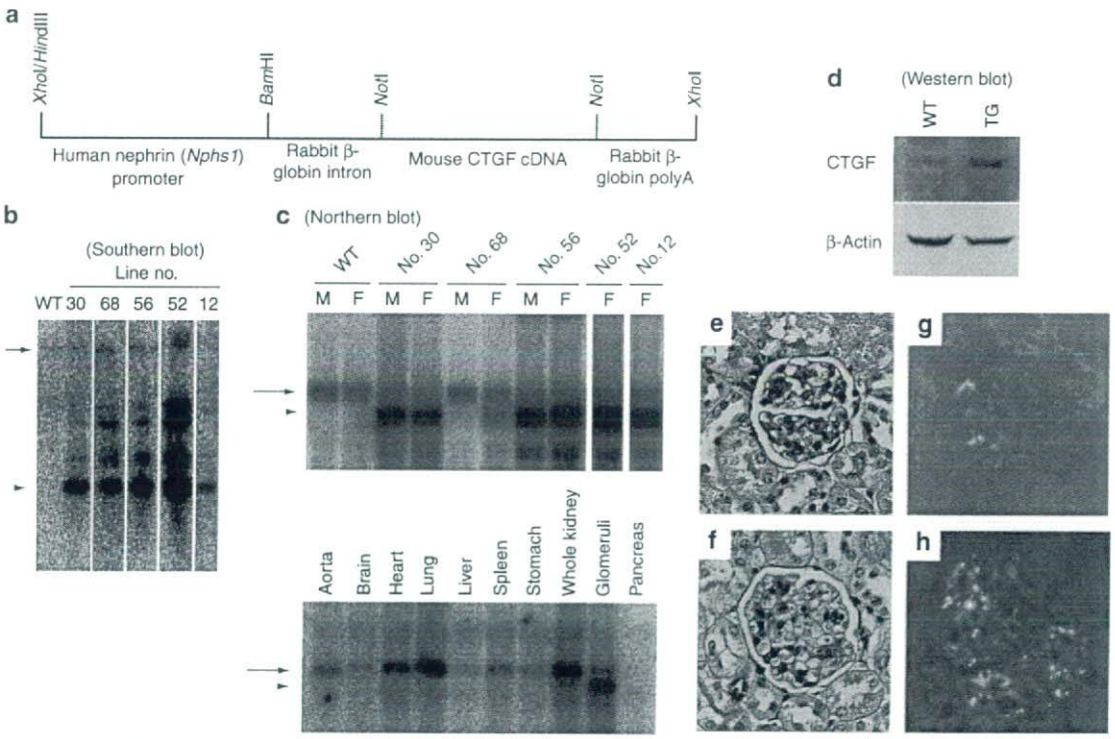


**Figure 1 | Construction of podocyte-specific CTGF transgenic mice.** (**a**) Schematic structure of the transgene. The transgene construct carried a 1.2-kb fragment of *Nphs1* promoter, β-globin intron, 1.1-kb mouse CTGF cDNA, and β-globin polyA. (**b**) Southern blot analysis of genomic DNA extracted from the mouse tail and digested with *NotI*. Five founder mouse lines were identified, and the 1.2-kb transgene (arrowhead) was present in lines 30, 68, 56, 52, and 12. An arrow indicates the genomic CTGF gene. WT, wild-type mice. (**c**) Northern blot analysis of CTGF mRNA in the glomeruli of wild-type and CTGF-transgenic mice (CTGF-Tg mice) lines 30, 68, 56, 52, and 12 (upper panel), and in the aorta, brain, heart, lung, liver, spleen, stomach, whole kidney, glomeruli, and pancreas of CTGF-Tg mice line 12 (lower panel). The size of CTGF mRNA expression from the transgene is 1.7 kb (arrowhead), which is smaller than the endogenous one (2.3 kb, arrow). (**d**) Western blot analysis of CTGF protein in the glomeruli of wild-type and CTGF-Tg mice line 12. TG, CTGF-Tg mice. (**e–h**) Histological examination and immunohistochemical study for CTGF in the kidney of non-Tg mice and CTGF-Tg mice line 12. Light microscopy of the kidney from non-Tg mice (**e**) and CTGF-Tg mice (**f**) with periodic acid–Schiff staining. Immunofluorescent examination of CTGF showed CTGF upregulation at podocytes in CTGF-Tg mice (**h**) compared with non-Tg mice (**g**).

All CTGF-Tg mice were fertile, grew normally, and showed normal gross appearance including kidney size and renal histology, as represented in line 12 (Figure 1f). Immunofluorescent analysis revealed that CTGF expression in podocytes was upregulated compared with their non-transgenic (non-Tg) littermates (Figure 1g and h). Blood pressure, blood glucose, serum creatinine, urea nitrogen, and urinary albumin excretion in CTGF-Tg mice line 12 were not different from those in control non-Tg littermates (Table 1). Another CTGF-Tg line (line 52) showed essentially similar results (data not shown).

### Characteristics of diabetic CTGF-transgenic mice

Next, to examine the role of CTGF expressed at podocytes in diabetic nephropathy, we induced diabetes in these mice by intraperitoneal injection of streptozotocin. We used CTGF-Tg mice line 12 and line 52, both with high transgene expression, in this experiment. Blood glucose, HbA1c, and serum creatinine levels as well as body weights in diabetic CTGF-Tg mice were not different from those in diabetic non-Tg mice at 12 weeks after disease induction (Table 2). Diabetic CTGF-Tg mice exhibited a tendency of renal hypertrophy compared with diabetic non-Tg mice, as shown by the increased kidney weight to body weight ratio. Systolic blood pressure and urine volume were not significantly different among diabetic groups. All diabetic groups showed increased albuminuria by 4 weeks after induction. Interestingly, diabetic CTGF-Tg mice revealed more pronounced elevation of urinary albumin excretion than diabetic non-Tg mice at 4 weeks, and became more aggravated during the experimental period (Figure 2). At 12 weeks, diabetic non-Tg mice exhibited 3.6-fold higher albumin excretion than non-diabetic control, and both lines of diabetic CTGF-Tg mice revealed significantly enhanced albuminuria by 2.8-fold compared with diabetic non-Tg mice (Figure 2). These results indicate that overexpression of CTGF in podocytes enhances proteinuria in diabetic nephropathy.

**Table 1 | Baseline characteristics of non-Tg and CTGF-Tg mice**

| | Non-Tg mice | CTGF-Tg mice line 12 |
|---|---|---|
| Body weight (g) | 20.8 ± 1.6 | 20.0 ± 0.4 |
| Systolic blood pressure (mm Hg) | 108.5 ± 3.4 | 108.0 ± 2.5 |
| Blood glucose (mg per 100 ml) | 162 ± 11 | 151 ± 11 |
| Serum creatinine (mg per 100 ml) | 0.12 ± 0.01 | 0.13 ± 0.01 |
| Serum urea nitrogen (mg per 100 ml) | 30.7 ± 2.6 | 30.7 ± 3.5 |
| Urine volume (ml per day) | 1.0 ± 0.2 | 1.3 ± 0.2 |
| Urinary albumin excretion (μg per mg Cr) | 41.3 ± 4.2 | 42.8 ± 5.4 |

CTGF, connective tissue growth factor; Tg, transgenic.
Values are expressed as the mean ± s.e. for non-Tg mice ($n$=13) and CTGF-Tg mice line 12 ($n$=9).

### Glomerular extracellular matrix accumulation and podocyte injury in diabetic CTGF-Tg mice

Microscopic examination showed that diabetic mice at 12 weeks after induction of diabetes exhibited marked mesangial expansion with glomerular hypertrophy (Figure 3c and d). Morphometric analysis revealed a significant increase in the mesangial area of diabetic CTGF-Tg mice line 12 compared with diabetic non-Tg mice (Figure 3f).

In electron microscopic analysis, podocytes of both lines of diabetic CTGF-Tg mice revealed diffuse vacuolation (Figure 4b and c), which was rarely observed in diabetic non-Tg mice (Figure 4a) or non-diabetic CTGF-Tg mice. The thickness of the GBM was similar among diabetic groups (Figure 4d–f). Thus, CTGF overexpressed at podocytes causes enhanced mesangial matrix expansion and podocyte structural changes under diabetic conditions.

### Effect of CTGF overexpression on gene and protein expression in glomeruli

Since we found enhanced ECM accumulation in diabetic CTGF-Tg mice compared with diabetic non-Tg mice, we investigated glomerular expression of total CTGF, endogenous CTGF, and TGF-β1, which are key inducers of ECM, as well as fibronectin, α1(IV) collagen (COL4A1), and α3(IV) collagen (COL4A3), which are representative ECM components. Total CTGF indicates the sum of transgene-derived

**Table 2 | Characteristics of diabetic mice at 12 weeks after induction of diabetes**

| | Control | | | Diabetes | | |
|---|---|---|---|---|---|---|
| | Non-Tg | Tg line 12 | Tg line 52 | Non-Tg | Tg line 12 | Tg line 52 |
| Blood glucose (mg per 100 ml) | 144 ± 14 | 123 ± 20 | 106 ± 16 | 732 ± 134* | 718 ± 48* | 804 ± 156* |
| HbA1c (%) | 3.3 ± 0.2 | 3.1 ± 0.2 | 2.9 ± 0.2 | 11.4 ± 0.7* | 10.0 ± 0.5* | 9.3 ± 1.8* |
| Serum creatinine (mg per 100 ml) | 0.16 ± 0.02 | 0.14 ± 0.01 | 0.17 ± 0.02 | 0.21 ± 0.03# | 0.25 ± 0.03# | 0.24 ± 0.06# |
| Serum urea nitrogen (mg per 100 ml) | 25.7 ± 1.8 | 24.3 ± 2.3 | 29.6 ± 3.0 | 49.7 ± 2.5* | 44.0 ± 3.6* | 50.6 ± ± 10.4* |
| Body weight (g) | 24.6 ± 1.8 | 25.7 ± 0.6 | 23.1 ± 1.0 | 23.1 ± 0.5 | 22.1 ± 2.2 | 22.2 ± 0.4 |
| Kidney weight (g) | 0.14 ± 0.02 | 0.13 ± 0.01 | 0.12 ± 0.02 | 0.22 ± 0.02* | 0.22 ± 0.01* | 0.24 ± 0.05* |
| Kidney weight/body weight ratio (g per 100 g BW) | 0.56 ± 0.02 | 0.52 ± 0.02 | 0.47 ± 0.02 | 0.96 ± 0.04* | 0.99 ± 0.09* | 1.04 ± 0.04* |
| Systolic blood pressure (mm Hg) | 105.4 ± 1.1 | 107.2 ± 0.3 | 110.3 ± 0.3 | 108.2 ± 0.3 | 108.6 ± 1.0 | 110.8 ± 1.6 |
| Urine volume (ml per day) | 1.1 ± 0.3 | 1.2 ± 0.4 | 0.5 ± 0.5 | 32.1 ± 0.9* | 26.6 ± 7.4* | 25.8 ± 5.3* |

BW, body weight; Hb, hemoglobin; Tg, transgenic.
Values are expressed as the mean ± s.e. for control non-Tg mice ($n$=4), control CTGF-Tg mice line 12 ($n$=5), control CTGF-Tg mice line 52 ($n$=5), diabetic non-Tg mice ($n$=10), diabetic CTGF-Tg mice line 12 ($n$=8), and diabetic CTGF-Tg mice line 52 ($n$=7).
*$P$<0.01 vs control non-Tg mice.
#$P$<0.05 vs control non-Tg mice.

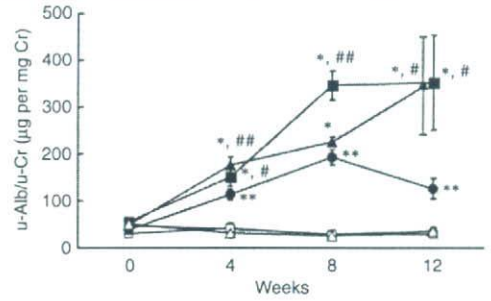


**Figure 2 | Urinary albumin excretion after induction of diabetes in CTGF-Tg mice and non-Tg mice.** The open circles, squares, and triangles represent vehicle-treated non-Tg mice, CTGF-Tg mice line 12, and CTGF-Tg line 52, respectively. The closed circles, squares, and triangles represent diabetic non-Tg mice, CTGF-Tg mice line 12, and CTGF-Tg line 52, respectively. Urinary albumin excretion showed a marked increase in diabetic CTGF-Tg mice throughout the course. Mean ± s.e. for control non-Tg mice ($n = 4$), control CTGF-Tg mice line 12 ($n = 5$), control CTGF-Tg mice line 52 ($n = 5$), diabetic non-Tg mice ($n = 10$), diabetic CTGF-Tg mice line 12 ($n = 8$), and diabetic CTGF-Tg mice line 52 ($n = 7$). $^{*}P < 0.01$, versus non-diabetic CTGF-Tg mice in the same genotype at each time point; $^{**}P < 0.01$, versus non-diabetic non-Tg mice at each time point; and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus diabetic non-Tg mice at each time point.

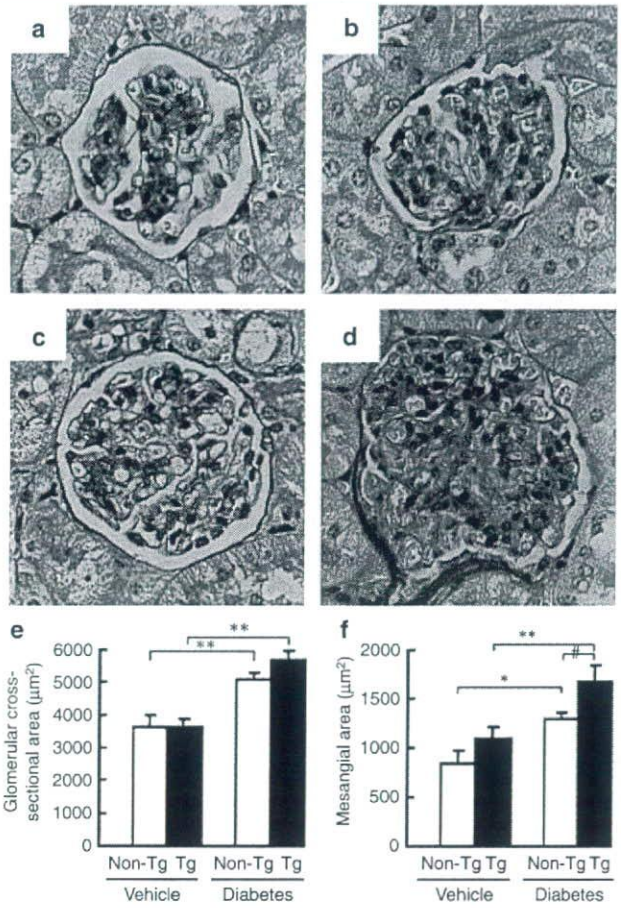


**Figure 3 | Histological examination of streptozotocin-induced diabetic nephropathy in CTGF-Tg mice.** Representative views on light microscopy at 12 weeks after induction of diabetes of the kidneys from (**a**) non-diabetic non-Tg mice, (**b**) non-diabetic CTGF-Tg mice line 12, (**c**) diabetic non-Tg mice, (**d**) and diabetic CTGF-Tg mice line 12 are shown. The kidney sections were stained with periodic acid-Schiff. (**e**) Glomerular cross-sectional area (**f**) and mesangial area at 12 weeks after induction of diabetes. Values were expressed as the mean ± s.e. for control non-Tg mice ($n = 4$), control CTGF-Tg mice line 12 ($n = 5$), diabetic non-Tg mice ($n = 10$), and diabetic CTGF-Tg mice line 12 ($n = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{\#}P < 0.05$.

and endogenous CTGF. Total CTGF expression in glomeruli from non-diabetic CTGF-Tg mice was upregulated by 2.5-fold compared with control non-Tg mice (Figure 5a). In diabetic CTGF-Tg mice, total CTGF mRNA expression was significantly upregulated by 4.0-fold compared with diabetic non-Tg mice (Figure 5a). Endogenous CTGF expression in diabetic CTGF-Tg mice was 3.0 times higher than that in diabetic non-Tg mice, indicating that CTGF upregulation in diabetic CTGF-Tg mice was due to the increase of endogenous CTGF. Fibronectin mRNA expression in glomeruli was increased in diabetic mice, but the levels were not significantly different between diabetic CTGF-Tg and diabetic non-Tg mice (Figure 5a). TGF-β1 and COL4A1 mRNA expressions in glomeruli also increased or tended to increase in diabetic conditions, but there was no significant difference among diabetic groups. There was no significant alteration in COL4A3 mRNA expression between CTGF-Tg and non-Tg mice in diabetic or non-diabetic conditions (Figure 5a). Next, we investigated glomerular expression of ECM degradation enzymes. Matrix metalloproteinase (MMP)-2 mRNA expression in glomeruli was significantly increased in diabetic condition (Figure 5a). In diabetic CTGF-Tg mice, MMP-2 mRNA expression was reduced compared with diabetic non-Tg mice. Expressions of tissue inhibitor of metalloproteinase (TIMP)-1 and -2, inhibitors of MMPs, were also upregulated in diabetic conditions, but there was no significant difference among diabetic groups. We next examined glomerular MMP-2 activity in diabetic CTGF-Tg mice, using gelatin zymography. The gelatinase activity of pro- and active- MMP-2 was significantly elevated in diabetic non-Tg mice (Figure 5b). In diabetic CTGF-Tg mice, the gelatinase activity was significantly decreased compared with diabetic non-Tg mice (Figure 5b). These findings suggest that CTGF causes ECM accumulation in mesangium by suppression of degradation rather than by enhancement of ECM expression itself.

The number of podocytes was evaluated by immunohistochemical study for the Wilms' tumor gene (WT1). The number of WT1-positive cells was significantly (by 29%) low in a glomerular cross-section of diabetic CTGF-Tg mice line 12 (Figure 6d) than in diabetic non-Tg mice (Figure 6c). CTGF-Tg line 52 showed virtually similar results (not shown).

Immunofluorescent study revealed that in diabetic non-Tg mice CTGF expression was upregulated in podocytes and mesangial cells (Figure 7c). Diabetic CTGF-Tg mice showed further increased CTGF protein expression in podocytes and mesangial cells compared with diabetic non-Tg mice

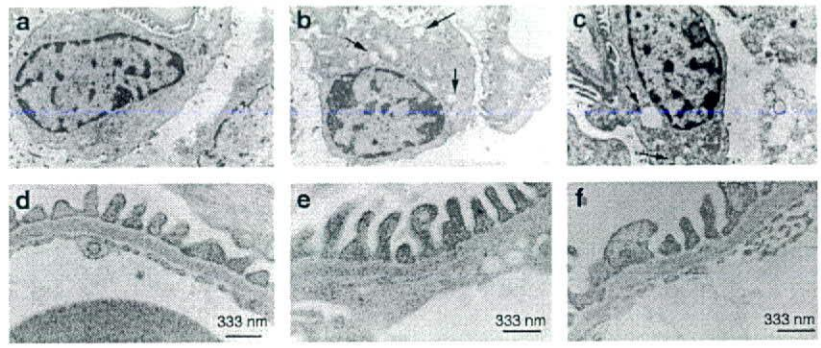


**Figure 4 | Electron microscopic analysis of a glomerulus in diabetic nephropathy in CTGF-Tg mice.** Diabetic non-Tg mice showed intact podocytes (**a**), no vacuolar formation, and almost normal foot process structure with mild thickening of the GBM (**d**). Diabetic CTGF-Tg (**b**, line 12; **c**, line 52) contained several vacuoles in podocytes (arrows; **b** and **c**). However, foot process structure was well maintained with mild GBM thickening (**e**, line 12; **f**, line 52).

(Figure 7c and d). Immunostaining of podocin became downregulated in diabetic CTGF-Tg mice as compared with diabetic non-Tg mice (Figure 7g and h). Double immunostaining for CTGF and podocin showed that CTGF protein in non-diabetic CTGF-Tg mice was located mainly in podocytes (yellow) (Figure 7j) and that CTGF was increased in the mesangial area in diabetic CTGF-Tg mice (green) (Figure 7l). These findings suggest that CTGF overexpression at podocytes resulted in loss of and functional impairment of podocytes and augmented induction of CTGF mainly at the mesangial area in streptozotocin-induced diabetic nephropathy.

## DISCUSSION

In this study, to clarify the role of CTGF in diabetic nephropathy, we have generated podocyte-specific CTGF-Tg mice under the control of the human nephrin promoter.[26] Glomerular expressions of CTGF mRNA and protein of CTGF-Tg mice were increased 2.5 and 5 times, respectively, compared with non-Tg mice. Immunofluorescent analysis revealed that CTGF expression was upregulated at podocytes in CTGF-Tg mice. Both lines of CTGF-Tg mice exhibited normal blood pressure, serum urea nitrogen, and urinary albumin excretion levels as well as normal renal histology, suggesting that increased CTGF expression at podocytes alone is not sufficient to evoke renal abnormality.

After induction of diabetes, CTGF-Tg mice exhibited enhanced CTGF mRNA expression by 4.0-fold as compared with diabetic non-Tg mice. In contrast, non-diabetic CTGF-Tg mice showed only 2.5-fold elevated CTGF mRNA compared with control, non-Tg mice. The origin of CTGF upregulation in diabetic CTGF-Tg mice was mainly endogenous CTGF in glomeruli determined by real-time reverse transcription-PCR (RT-PCR). Immunofluorescent staining for CTGF also revealed that diabetic CTGF-Tg mice exhibited enhanced CTGF expression mainly in the mesangial area. Such upregulation of CTGF expression associated with diabetes suggests a 'vicious cycle' whereby transgene-derived CTGF induces endogenous CTGF gene expression. It has already been shown that CTGF administration stimulates CTGF expression in cultured mesangial cells,[19] supporting this hypothesis.

Diabetic CTGF-Tg mice showed significantly increased mesangial expansion. Several lines of evidence indicate that humoral factors produced by podocytes have effects on mesangial and endothelial cells. Quaggin *et al.*[27] have reported that heterozygous disruption of vascular endothelial growth factor allele in podocytes results in endothelial cell damages and mesangial expansion. Another report showed that podocyte-specific injury in adult mice leads to mesangial expansion and glomerulosclerosis.[28] Taken together, it is well conceivable that CTGF produced by podocytes should exert an effect on mesangial cells to stimulate CTGF expression and ECM accumulation in diabetic conditions. CTGF has been shown to inhibit ECM degradation in mesangial cells and fibroblasts.[12,19,25] This study showed that glomerular COL4A3 or fibronectin mRNA expression was not changed among diabetic groups. Of note, glomerular MMP-2 mRNA expression in diabetic CTGF-Tg mice was significantly decreased compared with diabetic non-Tg mice. Glomerular MMP-2 activity in diabetic CTGF-Tg mice was also reduced compared with diabetic non-Tg mice. MMP-2 mRNA expression has been shown to be located in mesangial cells, as well as in visceral epithelial, glomerular epithelial, and endothelial cells in the kidney from patients of type I diabetes with diabetic nephropathy.[29] Our results, that the reduced activity of MMP-2 in glomeruli is associated with mesangial expansion in diabetic CTGF-Tg mice, may be consistent with this finding. Although the mechanism for the enhanced mesangial expansion in CTGF-Tg mice should await further clarification, our study suggests that the enhanced mesangial expansion in diabetic CTGF-Tg mice may be due to inhibition of ECM degradation through decreased expression and activity of MMP-2.

Increased CTGF expression has already been demonstrated in various cell types of human and rodent diabetic kidneys,[19–24,30,31] providing one of potential mechanisms for development and progression of diabetic nephropathy. High

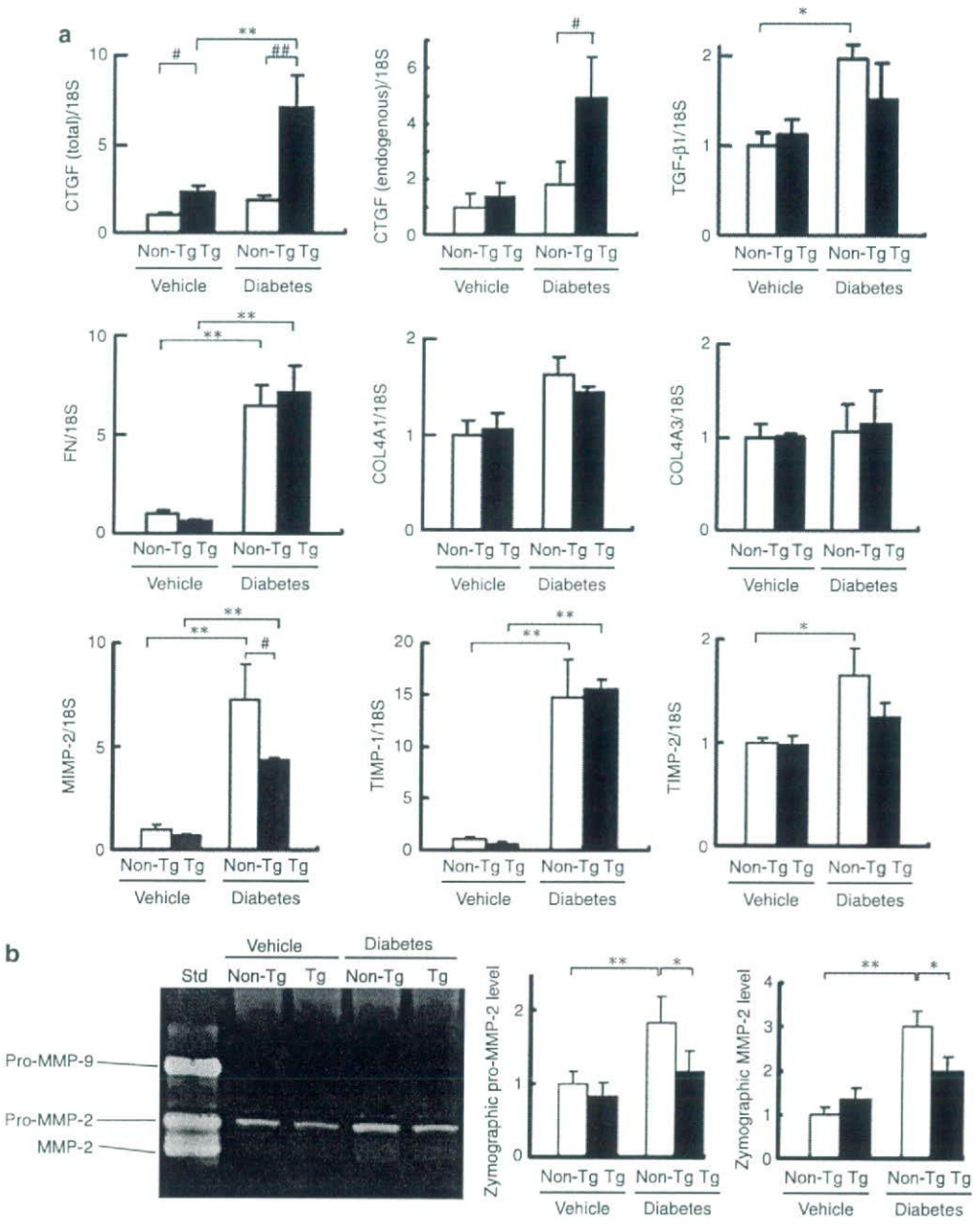


**Figure 5 | Glomerular mRNA expressions and MMP-2 zymographic activity.** (**a**) Real-time RT-PCR analyses of CTGF (total), CTGF (endogenous), TGF-β1, fibronectin (FN), α1(IV) collagen (COL4A1), α3(IV) collagen (COL4A3), matrix metalloproteinase 2 (MMP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1), and TIMP-2 mRNA expression in glomeruli of the kidneys at 12 weeks after induction of diabetes. CTGF (total) recognizes both transgene-derived and endogenous CTGF. Ribosomal RNA 18S expression is used as control. Mean ± s.e. for control non-Tg mice ($n = 4$), control CTGF-Tg mice line 12 ($n = 5$), diabetic non-Tg mice ($n = 10$), and diabetic CTGF-Tg mice line 12 ($n = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$. (**b**) Representative gelatin zymography performed 12 weeks after induction of diabetes. A mixture of human MMP-2 and MMP-9 served as a standard (std). Densitometric analysis of pro- and active forms of MMP-2 (72 and 62 kDa) activity ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$.

glucose stimulation induces CTGF mRNA in human and rat mesangial cells.[19,21,30] Advanced glycation end products also induce CTGF mRNA and protein in mesangial cells, through a TGF-β-independent pathway.[31,32] Furthermore, plasma and urinary CTGF levels are significantly increased in type I diabetic patients with nephropathy.[33] Very recently, Guha *et al.*[34] have reported that downregulation of CTGF using antisense oligonucleotide attenuates urinary albuminuria and ECM accumulation in diabetic mice. The present study reveals that overexpression of CTGF in podocytes enhances urinary albumin excretion in the diabetic milieu, indicating that augmented podocyte expression of

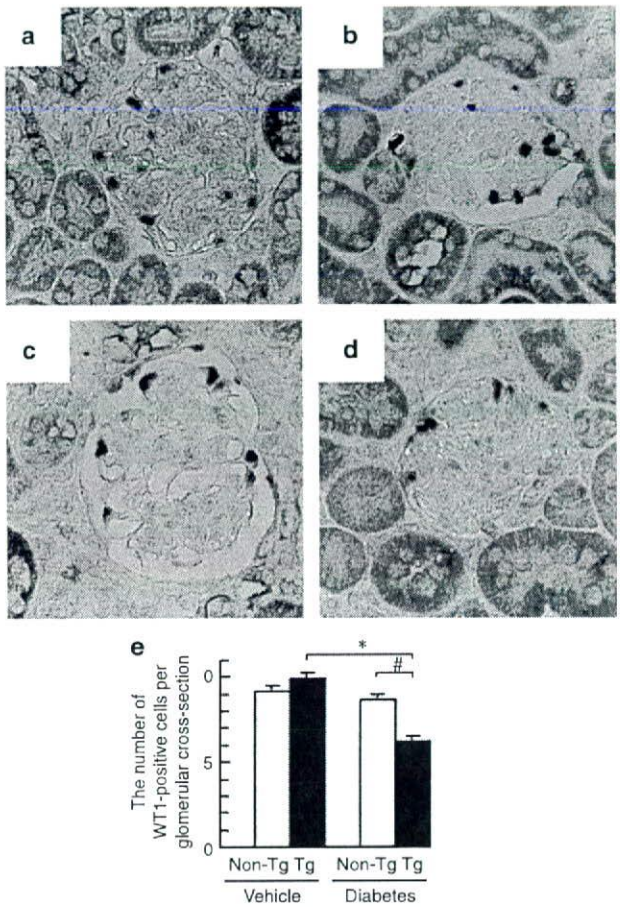


**Figure 6 | Immunohistochemical study of WT1 and number of podocytes.** (**a**) Non-diabetic non-Tg mice, (**b**) non-diabetic CTGF-Tg mice line 12, (**c**) diabetic non-Tg mice, (**d**) and diabetic CTGF-Tg mice line 12 at 12 weeks after induction of diabetes. (**e**) Number of WT1-positive cells per glomerular cross-section was counted. Diabetic CTGF-Tg mice had significantly fewer WT1-positive cells per glomerular cross-section as compared with diabetic non-Tg mice. Values were expressed as the mean ± s.e. for control non-Tg mice ($n = 4$), control CTGF-Tg mice line 12 ($n = 5$), diabetic non-Tg mice ($n = 10$), and diabetic CTGF-Tg mice line 12 ($n = 8$). $^{*}P < 0.01$, $^{\#}P < 0.01$.

CTGF accelerates advancement of diabetic nephropathy (Figure 8).

Diabetic CTGF-Tg mice showed podocyte loss as compared with diabetic non-Tg mice by the immunohistochemical study of WT1. Kidney biopsies from type II diabetic patients have shown that the number of podocytes is reduced at the microalbuminuria stage and is gradually decreased with the progression of diabetic nephropathy,[8] through, at least in part, the detachment from the GBM.[7] Overexpression of CTGF at podocytes in the current study may have facilitated the podocyte detachment under the diabetic milieu. Our study has also demonstrated that, by electron microscopy, both lines of diabetic CTGF-Tg mice reveal diffuse vacuolar formation in podocytes, which was rarely observed in diabetic non-Tg mice. Vacuolar degeneration of podocytes has already been shown in diabetic ZDF-*fa/fa* rats.[35] In addition, renal biopsy specimens from patients with focal segmental glomerulosclerosis (FSGS) or membranous nephropathy show the vacuolar degeneration in podocytes,[36,37] and FSGS patients with podocyte vacuolation tend to develop chronic renal failure.[36] Taken together, diabetic CTGF-Tg mice showed accelerated podocyte injury with the overexpression of CTGF under diabetic condition.

Podocin, coded by the *Nphs2* gene, is identified as a causative gene responsible for a familial form of early-onset, steroid-resistant nephrotic syndrome.[38] Podocin-deficient mice show proteinuria, mesangial sclerosis, and podocyte foot process fusion,[39] indicating that podocin is essential for maintaining normal glomerular structure and function. In this study, podocin protein expression was reduced in diabetic CTGF-Tg mice, which may reflect podocyte loss.

In summary, this study reveals that overexpression of CTGF at podocytes in mice causes enhanced albumin excretion and mesangial expansion, podocyte loss and vacuolar degeneration, and downregulation of podocin protein under the diabetic milieu. These findings are consistent with an important role for CTGF in the development of diabetic nephropathy *in vivo* and suggest that CTGF can be a promising therapeutic target against diabetic nephropathy.

## MATERIALS AND METHODS

### Generation of podocyte-specific CTGF-Tg mice

The 1.2-kb promoter of the human nephrin gene (*Nphs1*) capable of podocyte-specific expression[26] was PCR amplified from the human genome using the following primers: 5′-ctgaggcagatggatcacctgagg-3′ and 5′-tcacaggtccccctactgtgaccc-3′. The product was subcloned into pSTBlue-1 AccepTor vector (Merck Biosciences, Darmstadt, Germany) and the nucleotide sequence was confirmed. A 1.2-kb *Hin*dIII/*Bam*HI fragment of the pSTBlue-1-human nephrin promoter was inserted into the transgene vector that contained a rabbit β-globin intron and the polyadenylation site.[40] *Hin*dIII and *Eco*RI restriction sites of the vector were replaced with *Xho*I and *Not*I sites, respectively. Full-length mouse CTGF cDNA was PCR amplified from C57BL/6J mouse kidney cDNA using the following primers: 5′-tcctaccgcgtcccgatcat-3′ and 5′-gctttacgccatgtctccgt-3′. The 1.1-kb PCR product was excised, subcloned into pGEM-T-Easy vector (Promega, Madison, MI, USA), and ligated into the *Not*I site of the transgene vector. The *Xho*I fragment of the fusion gene was microinjected into the pronucleus of C57BL/6J mouse eggs.[41]

To identify founder mouse lines that carried the nephrin promoter-CTGF transgene, Southern blot analysis was performed using tail tissue DNA.[41] CTGF mRNA expression was evaluated by northern blot analysis.[13,18] Briefly, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) from glomeruli that were isolated by the graded sieving method.[42,43] Hybridization was performed with $^{32}$P-labeled probes for mouse CTGF cDNA (nucleotides 119–1187).

### Phenotypic analysis

All animal experiments were approved by the Animal Experimentation Committee of Kyoto University Graduate School of Medicine. CTGF-Tg mice (lines 12 and 52) and their non-Tg littermates were followed up to 12 weeks of age and the samples were collected.

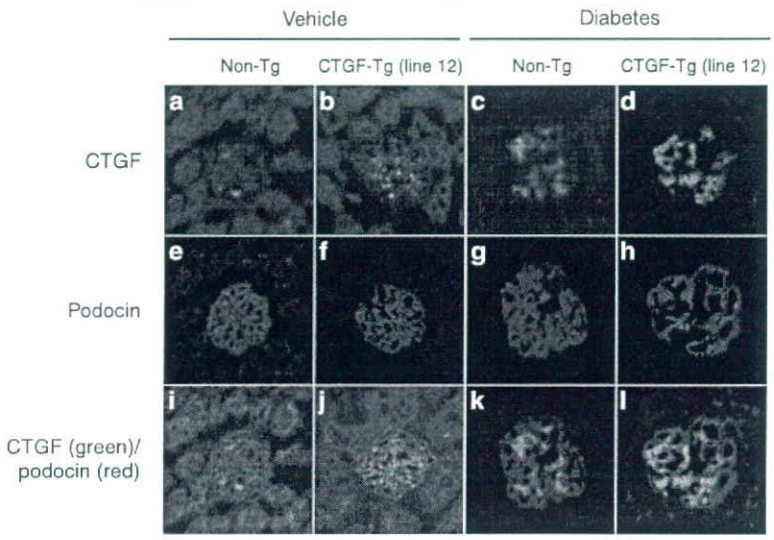


**Figure 7 | Double immunofluorescent staining for CTGF (green) and podocin (red).** (**a**–**d**) CTGF, (**e**–**h**) podocin, and (**i**–**l**) merged images in the kidney from (**a**, **e**, and **i**) non-diabetic non-Tg mice, (**b**, **f**, and **j**) non-diabetic CTGF-Tg mice line 12, (**c**, **g**, and **k**) diabetic non-Tg mice, and (**d**, **h**, and **l**) diabetic CTGF-Tg mice line 12 at 12 weeks after induction of diabetes. CTGF protein expression was increased in the mesangial area and podocytes of diabetic CTGF-Tg mice (**d** and **l**) as compared with diabetic non-Tg mice (**c** and **k**). Podocin staining was well maintained in diabetic non-Tg mice (**g**). In contrast, podocin expression was partially reduced in diabetic CTGF-Tg mice line 12 (**h**).

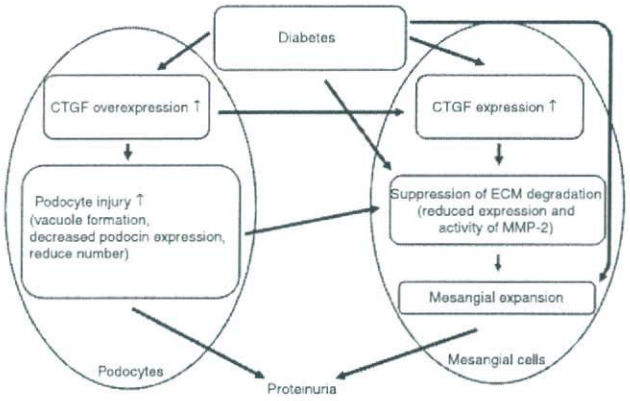


**Figure 8 | Proposed mechanism of diabetic nephropathy.** Overexpression of CTGF at podocytes accelerates podocyte injury in diabetic nephropathy and induces CTGF at mesangial cells, which inhibits ECM degradation and leads to mesangial expansion.

Blood glucose and HbA1c values were determined from whole venous blood taken from mouse tails in *ad libitum*-fed conditions using an automatic glucometer (Glutest Ace, Sanwa Kagaku, Nagoya, Japan)[42] and DCA2000 analyzer (Bayer Medical, Tokyo, Japan), respectively. For urine measurements, each animal was housed separately in a metabolic cage (Shinano Manufacturing, Tokyo, Japan). Urinary and serum creatinine levels were measured by the enzymatic method (SRL, Tokyo, Japan).[42] Urinary albumin excretion was assayed with a murine albumin ELISA kit (Exocell, Philadelphia, PA, USA).[43]

### Induction of diabetes and experimental protocols

Twelve-week-old CTGF-Tg mice line 12 (non-diabetic, $n = 5$; diabetic, $n = 8$), CTGF-Tg mice line 52 (non-diabetic, $n = 5$; diabetic, $n = 7$), and their non-Tg littermates (non-diabetic, $n = 4$; diabetic, $n = 10$) were fed on standard chow and given water *ad libitum*. Diabetes was induced by three consecutive intraperitoneal injections of streptozotocin (Sigma-Aldrich, Saint Louis, MO, USA; 100 mg per kg body weight in citrate buffer, pH 4.0);[21,42] control mice received citrate buffer only. Mice with blood glucose levels higher than 300 mg per 100 ml were used as diabetic. Blood glucose levels and body weights were measured every 4 weeks over a 12-week period. Mice were killed at 24 weeks of age under ether anesthesia and samples were collected for histological and biochemical analyses.

### Renal histology and electron microscopy

Kidney sections were fixed with 4% buffered paraformaldehyde and embedded in paraffin. One-μm-thick sections were stained with periodic acid–Schiff and examined by light microscopy.[43] The cross-sectional area and the mesangial area of 20 glomeruli from the outer cortex were measured quantitatively using a computer-aided manipulator (KS400, Carl Zeiss Vision, Munich, Germany).[43] The procedure was performed by two investigators blind to the origin of the slides, and the mean values were calculated. For electron microscopy, small blocks of kidneys were fixed in 2.5% buffered glutaraldehyde, post-fixed in 2% osmium tetroxide, dehydrated in graded ethanol, and embedded in epoxy resin.[44] Ultrathin sections (0.1-μm thick) were stained with uranyl acetate/lead citrate and examined in an electron microscope (H-7100, Hitachi, Tokyo, Japan).

### Immunohistochemistry

For double immunofluorescence analyses of CTGF and podocin, 4-μm cryostat sections fixed in cold acetone were treated with 0.1% Triton X (Nacalai Tesque, Kyoto, Japan) for 10 min, followed by incubation with human anti-CTGF antibody (a kind gift from Dr N Oliver, FibroGen Inc., South San Francisco, CA, USA)[20] and anti-podocin antibody (Sigma-Aldrich) for 1 h. After incubation with FITC-labeled donkey anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA) and Texas Red-labeled anti-rabbit IgG

(Jackson ImmunoResearch), the slides were developed by a fluorescence microscope (IX-81; Olympus, Tokyo, Japan). WT1 immunostaining was performed as described.[45] More than 20 consecutive glomerular sections in each mouse were examined, and the mean number of WT1-positive cells per glomerular cross-section was calculated.

### Glomerular RNA extraction and real-time RT-PCR analysis

Quantitative real-time RT-PCR was performed using one-step RT-PCR master mix reagents on an ABI Prism 7700 Sequence Detector (Applied Biosystems, Tokyo, Japan).[46] Primers were chosen with the analysis of the Primer Express version 1.5 (Applied Biosystems). Total CTGF, endogenous CTGF, TGF-β1, COL4A1, COL4A3, fibronectin, MMP-2, TIMP-1 and TIMP-2 mRNA expressions were evaluated with the following primers and probes: total CTGF forward primer, 5′-ttcccgagaagggtcaagct-3′; total CTGF reverse primer, 5′-tccttg ggctcgtcacaca-3′; total CTGF probe, 5′-FAM-cctgggaaatgctgcaaggagtgg-TAMRA-3′;[47] endogenous CTGF forward, 5′-ggtcaaatccctgttggtgaa-3′; endogenous CTGF reverse, 5′-aaagaagcagcaagcacttcct-3′; endogenous CTGF probe, 5′-FAM-ttaagaaatggctggctcagggtaaggtcc-TAMRA-3′ (accession number M70642); TGF-β1 forward, 5′-gacgtcactggagttgtacgg-3′; TGF-β1 reverse, 5′-gctgaatcgaaagccctgt-3′; TGF-β1 probe, 5′-FAM-agtgg ctgaaccaaggagacggaa-TAMRA-3′;[46] COL4A1 forward, 5′-ggcaggtcaagttct agcgtaga-3′; COL4A1 reverse, 5′-gtgagtttgaggaaagctggtgtt-3′; COL4A1 probe, 5′-FAM-ataaagcgcacttggtttcgtctctgga-TAMRA-3′;[48] COL4A3 forward, 5′-tgtggatgcacggtgtgtt-3′; COL4A3 reverse, 5′-gttctcttcacggtgtgc ttga-3′; COL4A3 probe, 5′-FAM-ccttccatctcctccctgacaccttagact-TAM RA-3′ (accession number AF169387); fibronectin forward, 5′-atcatttca tgccaaccagtt-3′; fibronectin reverse, 5′-tcgcactggtagaagttcca-3′; fibronectin probe, 5′-FAM-ccgacgaagagcccttacagttcca-TAMRA-3′;[13] MMP-2 forward, 5′-cctggtttcacccttttctgct-3′; MMP-2 reverse, 5′-cgagcgaagggca tacaaa-3′; MMP-2 probe, 5′-FAM-cccagatacctgcaccaccttaactgttgc-TAMRA-3′ (accession number AK148184); TIMP-1 forward, 5′-atggaaa gcctctgtggatatg-3′; TIMP-1 reverse, 5′-ggcccgtgatgagaaactc-3′; TIMP-1 probe, 5′-FAM-cacaagtcccagaaccgcagtga-TAMRA-3′ (accession number BC008107); TIMP-2 forward, 5′-aggcgttttgcaatgcag-3′; TIMP-2 reverse, 5′-ccggaatccacctccttct-3′; and TIMP-2 probe, 5′-FAM-cgtagtgatcagagcc aaagcagtgagc-TAMRA-3′ (accession number M82858). Expression of each mRNA was normalized for 18S ribosomal RNA (Taqman ribosomal RNA control reagents; Applied Biosystems).

### Western blot analysis

Western blot analysis was performed as described[18] with some modifications. Isolated glomerular extracts were electrophoresed and transferred onto Immobilon filter (Millipore, Bedford, MA, USA). Filters were incubated with human monoclonal anti-CTGF antibody (FibroGen Inc.)[20] for 1 h, and immunoblots were then developed using horseradish peroxidase-linked donkey anti-human immunoglobulin (Amersham, Arlington Heights, IL, USA) and a chemiluminescence kit (Amersham). β-Actin (antibody from Sigma-Aldrich) was used as an internal control.

### Zymography

MMP activity in 30 μg of glomerular extract was measured by gelatin zymography as previously described.[49] Briefly, isolated glomeruli were lysed on ice in zymography buffer containing 50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij35, 10 μg $ml^{-1}$ leupeptin, and 1 mM PMSF. The lysates were centrifuged at 15 000 r.p.m., and the supernatants and gelatinase zymography standards (Millipore, Temecula, CA, USA) were separated by 10% polyacrylamide gels containing 0.1% gelatin (Invitrogen). After gel electrophoresis, gels were incubated overnight at 37°C in zymogram developing buffer (Invitrogen) and stained with Coomassie stain.

### Statistical analysis

Data are expressed as the mean ± s.e. Statistical analysis was performed using one-way ANOVA. A $P$-value $< 0.05$ was considered statistically significant.

### DISCLOSURE

None.

### ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

# Variations in the *FTO* gene are associated with severe obesity in the Japanese

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**Abstract** Variations in the fat-mass and obesity-associated gene (*FTO*) are associated with the obesity phenotype in many Caucasian populations. This association with the obesity phenotype is not clear in the Japanese. To investigate the relationship between the *FTO* gene and obesity in the Japanese, we genotyped single nucleotide polymorphisms (SNPs) in the *FTO* genes from severely obese subjects [$n = 927$, body mass index (BMI) $\geq 30$ kg/m$^2$] and normal-weight control subjects ($n = 1{,}527$, BMI $< 25$ kg/m$^2$). A case-control association analysis revealed that 15 SNPs, including rs9939609 and rs1121980, in a linkage disequilibrium (LD) block of approximately 50 kb demonstrated significant associations with obesity; rs1558902 was most significantly associated with obesity. *P* value in additive mode was 0.0000041, and odds ratio (OR) adjusted for age and gender was 1.41 [95% confidential interval (CI) = 1.22–1.62]. Obesity-associated phenotypes, which include the level of plasma glucose, hemoglobin A1c, total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol, and blood pressure were not associated with the

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rs1558902 genotype. Thus, the SNPs in the *FTO* gene were found to be associated with obesity, i.e., severe obesity, in the Japanese.

**Keywords** Fat-mass and obesity-associated gene · Obesity · Japanese population · Association · SNP

## Introduction

Obesity is the most common nutritional disorder in developed countries, and it is a major risk factor for hypertension, cardiovascular disease, and type 2 diabetes (Kopelman 2000; Wilson et al. 2003). Genetic and environmental factors contribute to obesity development (Maes et al. 1997; Barsh et al. 2000; Rankinen et al. 2006). Recent progress in single nucleotide polymorphism (SNP) genotyping techniques has enabled genome-wide association studies on common diseases (Herbert et al. 2006; Frayling et al. 2007; Scuteri et al. 2007; The Wellcome Trust Case Control Consortium 2007; Hinney et al. 2007). Using a large-scale case-control association study, we found that secretogranin III (SCG3) (Tanabe et al. 2007) and myotubularin-related protein 9 (MTMR9) (Yanagiya et al. 2007) are involved in susceptibility to the obesity phenotype. Genome-wide association studies have shown that the fat-mass and obesity-associated gene (*FTO*) is also associated with the obesity phenotype (Frayling et al. 2007; Scuteri et al. 2007; Hinney et al. 2007). This association was also found in many Caucasian and Hispanic American populations (Frayling et al. 2007; Scuteri et al. 2007; Dina et al. 2007; Field et al. 2007; Andreasen et al. 2008; Wåhlén et al. 2008; Peeters et al. 2008), whereas it was not found in the Chinese Han population (Li et al. 2008). Among Japanese, body mass index (BMI) was higher in subjects who had the A allele of rs9939609, similar to that observed in Caucasians; however, this finding was not significant (Horikoshi et al. 2007). Another group reported that rs9939609 was associated with BMI in the Japanese (Omori et al. 2008). Thus, the association of SNPs in the *FTO* gene with obesity in the Japanese remains controversial.

To investigate the relationship between the *FTO* gene and obesity in the Japanese, we performed a case-control association study using patients with severe adult obesity (BMI $\geq$ 30 kg/m$^2$) and normal-weight controls (BMI $<$ 25 kg/m$^2$); we found that SNPs in intron 1 of the *FTO* gene were associated with severe adult obesity.

## Materials and methods

### Study subjects

The sample size for severely obese Japanese subjects (BMI $\geq$ 30 kg/m$^2$) was 927 (male:female ratio 419:508, age 48.7 $\pm$ 14.2 years, BMI 34.2 $\pm$ 5.4 kg/m$^2$), whereas that for Japanese normal weight controls (BMI $<$ 25 kg/m$^2$) was 1,527 (male:female ratio 685:842, age 48.1 $\pm$ 16.5 years, BMI 21.7 $\pm$ 2.1 kg/m$^2$). The severely obese subjects were recruited from among outpatients of medical institutes. Patients with secondary obesity and obesity-related hereditary disorders were not included, and neither were patients with medication-induced obesity. The normal-weight controls were recruited from among subjects who had undergone a medical examination for screening of common diseases. Clinical features of the subjects are illustrated in Table 1. Additionally, 1,604 subjects were recruited (male:female ratio 803:801, age 48.7 $\pm$ 16.9 years, BMI 22.66 $\pm$ 3.16 kg/m$^2$) from the Japanese general population. Each subject provided written informed consent, and the protocol was approved by the ethics committee of each institution and that of RIKEN.

### DNA preparation and SNP genotyping

Genomic DNA was prepared from the blood sample of each subject by using the Genomix (Talent Srl, Trieste, Italy). We searched for dbSNPs with minor allele frequencies (MAF) $>$ 0.10 in the *FTO* gene of Japanese people. We selected 90 SNPs and were able to construct Invader probes (Third Wave Technologies, Madison, WI)

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