# Effect of the Antiplatelet Drug Dilazep Dihydrochloride on Urinary Podocytes in Patients in the Early Stage of Diabetic Nephropathy

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OBJECTIVE — To determine whether the antiplatelet drug dilazep dihydrochloride affects the number of urinary podocytes in diabetic patients with microalbuminuria.

RESEARCH DESIGN AND METHODS — Fifty patients with type 2 diabetes and microalbuminuria (30 men and 20 women, mean age 48.6 years) and 30 age-matched control subjects (18 men and 12 women, mean age 49.2 years) were included in the study. No patients showed serum creatinine levels in excess of 2.0 mg/dl. Urinary podocytes were examined by immunofluorescence microscopy with monoclonal antibodies against podocalyxin.

RESULTS — Urinary podocytes were detected in 18 of the 50 microalbuminuric diabetic patients (mean, 1.3 cells/ml). Urinary podocytes were not detected in the remaining 32 patients or in the 30 healthy control subjects. Diabetic patients positive for urinary podocytes were divided into 2 treatment groups: a dilazep dihydrochloride treatment group (300 mg/day; $n$ = 9, group A) and a placebo group ($n$ = 9, group B). Treatments were continued for 6 months. In group A, microalbuminuria decreased significantly from 146 ± 42 to 86 ± 28 μg/min ($P < 0.01$) and urinary podocytes also decreased from 1.3 ± 0.8 to 0.4 ± 0.2 cells/ml ($P < 0.01$). However, in group B, microalbuminuria and urinary podocytes changed little over the study period.

CONCLUSIONS — Podocyte injury may occur in patients with early diabetic nephropathy, and dilazep dihydrochloride may be useful for preventing glomerular injury.

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Microalbuminuria is closely linked to diabetic nephropathy, usually indicating the onset of this pathology. Moreover, microalbuminuric diabetic nephropathy is unlike overt nephropathy, which, according to general standards, is characterized by clinical proteinuria (1,2). Microalbuminuria is also an indication of early renal and vascular disorders, thus predicting advancing renal disease as well as the progression of cardiovascular disease (2). Microalbuminuria is thought to be the best overall predictor of mortality in type 2 diabetes (3). Poor glycemic control has been shown to predict microalbuminuria and could be a risk factor for the progression of diabetic nephropathy, whereas high blood pressure can be a more important predictor in late stages of the disease (4). Strict control of plasma glucose levels and blood pressure in the microalbuminuric stage in patients with diabetes is very important for preventing progression of renal complications. The antiplatelet drug dilazep dihydrochloride has many pharmacological actions, and it has been reported to be potentially useful for improving albuminuria and preventing deterioration of renal function in patients with microalbuminuric diabetic nephropathy (5).

The podocyte is a highly differentiated cell that is strategically located on the outside of the glomerular capillary wall. Podocytes, in addition to the glomerular basement membrane, play an important role in glomerular filtration (6). The most severe lesions occur when podocytes detach from the glomerular basement membrane. These cells appear subsequently in the urine. Hara and colleagues (7,8) have reported the urinary podocytes to be clinically useful for evaluating disease activity in various kinds of glomerular diseases in children. However, little is known about the podocytes in diabetic nephropathy in adults or the effects of antiplatelet drugs on the number of urinary podocytes. Here, we examined urinary podocytes in patients with early diabetic nephropathy and the effect of dilazep dihydrochloride on these cells.

## RESEARCH DESIGN AND METHODS

— Fifty patients with type 2 diabetes (30 men and 20 women, age 48.6 ± 11.6 years) and 30 healthy age-matched control subjects (18 men and 12 women, age 49.2 ± 10.8 years) were included in this study. Type 2 diabetes was diagnosed according to World Health Organization criteria. Insulin was not necessary for at least 3 years from the onset of diabetes, and body weight was stable within recent years (2.7 ± 0.1%) (9). Informed consent was obtained from each patient, and the study protocol was approved by the local committee. The inclusion criteria were as follows: *1*) no history of ketoacidosis, *2*) treatment by diet alone, *3*) in tight metabolic control ($HbA_{1c}$ <8.0% at any time in the previous year), *4*)

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Abbreviations: PBS, phosphate-buffered saline.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

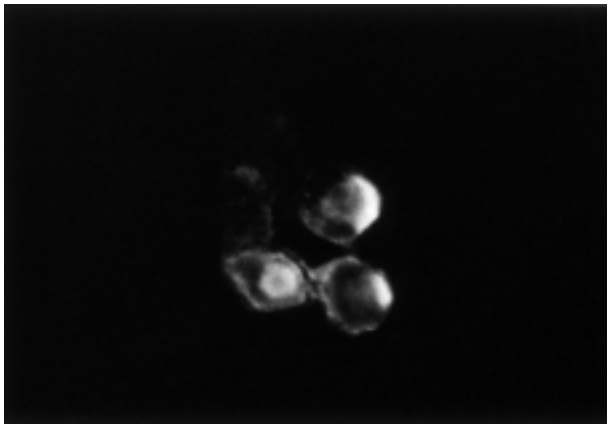

**Figure 1—***Urinary podocytes visualized by immunofluorescence (×400).*

microalbuminuria, and 5) diabetic duration between 10 and 15 years (mean 12 ± 3 years). No patient showed serum creatinine levels in excess of 2.0 mg/dl at the time of the study. None were given antihypertensive drugs or other antiplatelet agents such as dipyridamole, ticlopidine, or trapidil. No patient had a malignancy or history of heart disease, cerebrovascular disease, liver disease, or collagen disease. Clinical and laboratory data for each patient were obtained at the first examination (baseline) at 3 and 6 months after the first examination. After an overnight fast, blood was drawn from an antecubital vein for measurement of plasma glucose, glycated hemoglobin, serum creatinine concentration, and blood urea nitrogen. Plasma glucose concentrations were determined by the glucose oxidase method, and plasma glycated hemoglobin concentrations were measured by spectrophotometric assay (Bio-Rad, Richmond, CA; normal range 3.5–6.5%). On the basis of the median urinary albumin excretion in at least 3 consecutive 4-h urine collections (taken in the morning), microalbuminuria was defined as a median urinary albumin excretion of 20–200 μg/min (10). All 50 diabetic patients were at a microalbuminuric stage in which urinary albumin levels ranged from 50 to 198 μg/min at the time of dilazep dihydrochloride administration. Patients were randomly assigned to 2 groups. Dilazep dihydrochloride (Comelian; Kowa, Nagoya, Japan) was administered orally at a dose of 300 mg/day for 6 months (group A) to 9 diabetic patients that showed urinary podocytes, as described previously (5). Another 9 diabetic patients showing urinary podocytes were treated with a placebo for 6 months (group B). First-voided morning urine specimens were obtained from all subjects before treatment and at 3 and 6 months after treatment. Urinary podocytes were stained by immunofluorescence, as reported previously (7,8). The urine specimens were processed within 30 min after voiding. Freshly voided urine (10 ml) was centrifuged for 5 min at 700*g*. The supernatant was aspirated, and the sediment was washed with 0.01 mol/l phosphate-buffered saline (PBS), pH 7.2. The sediment was then resuspended in 10 ml PBS, cytocentrifuged onto poly-L-lysin–coated microscope slides for 5 min at 700*g* in an Autosmear (Sakuraseiki, Tokyo, Japan), then air-dried for at least 30 min. Slides were then fixed in acetone for 5 min at 4°C. The urine sediments collected on the slides were partitioned into 6 areas of 1.0 × 1.0 $cm^2$ each by a PAP pen (Dako, Japan). After washing with PBS, each area was incubated for 60 min with 20 μl anti-human podocalyxin monoclonal antibody PHM-5 (Australian Monoclonal Development, Artamon, NSW, Australia) at a 1:200 dilution (7). This antibody reacts with podocalyxin (165- to 170-kDa band) (7,8). After washing, the slides were incubated with fluorescein isothiocyanate–labeled $F(ab^1)_2$ fragments of affinity-purified anti-mouse IgG (Cappel/ICN Biomedicals). Slides were washed and examined by immunofluorescence microscopy. Nuclei of the cells were counterstained with ethidium bromide before mounting. Quantitative analysis was performed by 3 different pathologists in a blinded fashion to control for subjective interpretation of results. Urinary podocyte number was shown as cells per milliliter urine. The freshly voided urine was collected for 5 consecutive days, and the urinary podocytes were counted every day. We reported previously that podocalyxin was present in the urine sediments obtained from patients with glomerular diseases in children in the following 3 patterns: cast, fine granules, and entire cells (7,8). In the present study, we measured entire cells, not cell fragments, in the urine.

Data were expressed as means ± SD. Results were analyzed using 2-way analysis of variance, and specific comparisons between groups were carried out by 2-tailed

Table 1—*Urinary podocyte number in 18 type 2 diabetic patients*

| Patient | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Mean |
|---|---|---|---|---|---|---|
| 1 | 0.8 | 0.7 | 0.8 | 0.8 | 0.7 | 0.76 |
| 2 | 1.7 | 1.5 | 1.7 | 1.7 | 1.6 | 1.64 |
| 3 | 1.3 | 1.2 | 1.3 | 1.4 | 1.2 | 1.28 |
| 4 | 1.0 | 1.4 | 1.2 | 1.3 | 1.3 | 1.24 |
| 5 | 1.6 | 1.7 | 1.6 | 1.8 | 1.7 | 1.68 |
| 6 | 0.5 | 0.6 | 0.7 | 0.7 | 0.6 | 0.62 |
| 7 | 1.2 | 1.6 | 1.4 | 1.3 | 1.4 | 1.38 |
| 8 | 1.8 | 1.7 | 1.9 | 1.9 | 1.8 | 1.82 |
| 9 | 1.6 | 1.7 | 1.6 | 1.7 | 1.7 | 1.66 |
| 10 | 0.9 | 1.0 | 1.1 | 1.0 | 1.1 | 1.02 |
| 11 | 1.4 | 1.6 | 1.9 | 1.7 | 1.6 | 1.64 |
| 12 | 1.5 | 1.7 | 1.4 | 1.5 | 1.6 | 1.54 |
| 13 | 0.9 | 1.2 | 1.1 | 1.0 | 1.4 | 1.12 |
| 14 | 1.9 | 2.2 | 2.0 | 1.9 | 2.0 | 2.00 |
| 15 | 0.4 | 0.4 | 0.4 | 0.4 | 0.5 | 0.42 |
| 16 | 0.9 | 1.1 | 1.0 | 1.1 | 1.1 | 1.04 |
| 17 | 1.4 | 1.3 | 1.4 | 1.5 | 1.1 | 1.34 |
| 18 | 1.3 | 1.2 | 1.3 | 1.3 | 0.9 | 1.20 |

Data are given in cells per milliliter.

Table 2—*Clinical and laboratory data before and 3 and 6 months after dilazep dihydrochloride administration*

| | Before treatment | After treatment | |
|---|---|---|---|
| | | 3 Months | 6 Months |
| Serum creatinine (mg/dl) | 0.88 ± 0.10 | 0.90 ± 0.12 | 0.92 ± 0.11 |
| Creatinine clearance (ml/min) | 98 ± 12 | 92 ± 14 | 94 ± 12 |
| Blood urea nitrogen (mg/dl) | 17 ± 4 | 16 ± 3 | 17 ± 4 |
| Fasting blood sugar (mg/dl) | 152 ± 12 | 146 ± 14 | 144 ± 16 |
| $HbA_{1c}$ (%) | 7.8 ± 0.6 | 7.5 ± 0.4 | 7.6 ± 0.4 |
| Systolic blood pressure (mmHg) | 116 ± 14 | 120 ± 16 | 118 ± 10 |
| Diastolic blood pressure (mmHg) | 78 ± 10 | 80 ± 6 | 74 ± 10 |

Data are means ± SD.

Student's *t* test. Statistical significance was determined at a *P* value of $<0.05$.

RESULTS — Urinary podocytes were detected in 18 of the 50 patients with microalbuminuric diabetes (range 0.4–2.2 cells/ml, mean 1.3) (Fig. 1). Table 1 shows the urinary podocyte number in each patient ($n$ = 18). No urinary podocytes were detected in the remaining 32 diabetic patients or the 30 control subjects. No significant difference in microalbuminuria was detected between the urinary podocyte-positive patients ($n$ = 18) and the urinary podocyte-negative patients ($n$ = 32). Patients 1–9 were treated by dilazep dihydrochloride (group A), and patients 10–18 were treated by placebo (group B). There were no significant differences in the levels of serum creatinine, blood urea nitrogen, fasting blood glucose, glycated hemoglobin, or blood pressure over the study period (6 months) between group A and group B. Table 2 shows the data of group A. In addition, there were no significant differences in urinary albumin excretion or urinary podocyte number between group A and group B before treatments. Microalbuminuria decreased significantly, from 146 ± 42 μg/min before dilazep dihydrochloride treatment to 120 ± 32 μg/min at 3 months ($P < 0.05$) and 86 ± 28 μg/min at 6 months ($P < 0.01$) after treatment in group A (Fig. 2). The number of urinary podocytes also decreased significantly after treatment, from 1.3 ± 0.8 to 0.8 ± 0.4 cells/ml at 3 months ($P < 0.05$) and 0.4 ± 0.2 cells/ml at 6 months ($P < 0.01$) (Fig. 2). However, microalbuminuria and urinary podocytes showed little change in group B throughout the study period.

CONCLUSIONS — We found evidence that podocyte injury occurs in the early stages of diabetic nephropathy and that dilazep dihydrochloride treatment may be effective for podocyte injury in these patients. Diabetic nephropathy is characterized by hypertrophy of both glomerular and tubular structures, thickening of the basement membranes, and progressive accumulation of the extracellular matrix in the mesangium as well as in the interstitium (11,12). Despite a reported demonstration that high glucose stimulates extracellular matrix synthesis in cultured visceral glomerular epithelial cells (13), the growth profile of these cells in diabetic patients has not been investigated. It is widely postulated that glomerular epithelial cells rarely replicate in vivo, possibly because of continuous overexpression of cell cycle-arresting proteins (14,15). Podocytes respond to injury or an alteration in homeostasis by undergoing hypertrophy or apoptosis (15). Podocyte hypertrophy and broadening of the foot processes associated with a loss in total number per glomerulus have been described recently in patients with type 2 diabetes (16). Hypertrophy may lead eventually to degeneration of glomerular visceral epithelial cells, a process that underlies the development of proteinuria and glomerulosclerosis in diabetes (17). Recently, Fioretto et al. (18) reported that close to one-third of their microalbuminuric subjects had a normal glomerular structure when examined by light microscopy. However, other investigators have reported that microalbuminuria usually indicates detectable renal structural damage to the glomerulus (19).

More recently, Meyer et al. (20) reported that among the glomerular morphological characteristics used to diagnose nephropathy, urinary podocyte number was the best predictor in diabetic patients. Moreover, morphological measurements other than urinary podocyte number displayed little relation to subsequent urinary albumin excretion. The loss of podocytes from the glomerular basement membrane results in severe damage to the glomerular filtration processes. It is possible to count the number of lost podocytes in urine, since podocytes are located in the urinary space of the

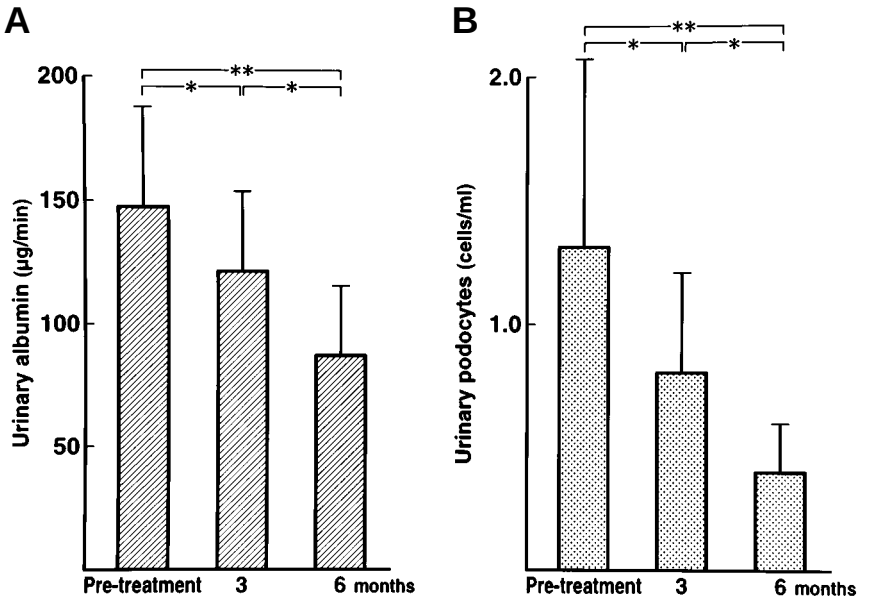


**Figure 2**—*Urinary albumin excretion (*A*) and the number of urinary podocytes (*B*) before and 3 and 6 months after dilazep dihydrochloride treatment of patients with diabetic nephropathy at the microalbuminuric stage.* *P $< 0.05$, **P $< 0.01$.

glomerular capillary walls. Therefore, podocytes appear in the urine if they detach from the glomerular basement membrane. Hara and colleagues (7,8) have shown that detection of urinary podocytes is useful for estimating the severity of active glomerular injury and also as a predictor of disease progression. A quantitative measurement of podocytes in the urine would be very helpful, i.e., number per grams of creatinine or number per hour or 24 h. In the preliminary studies, we examined urinary podocytes at certain points (8:30 A.M., 10:30 A.M., 5:30 P.M., and 9:30 P.M.) in 8 type 2 diabetic patients with urinary podocytes. We recognized that the urinary podocyte number was almost the same at any time point. We are now studying what is the best quantitative measurement of urinary podocytes.

Dilazep dihydrochloride exerts its antiplatelet effects by increasing adenosine levels in the fluid of the extracellular space (21). Dilazep dihydrochloride shows the following pharmacological effects: *1*) suppression of platelet adhesion and coagulation, *2*) suppression of the platelet phospholipase activity, and *3*) maintenance of glomerular filtration rate due to increased renal blood flow (5). Dilazep dihydrochloride is also known to have a vasodilating effect in coronary, cerebral, and renal vessels and is often used in patients with ischemic heart disease, cerebral ischemia, or renal dysfunction (22). Here we report that dilazep dihydrochloride treatment reduced microalbuminuria and the number of urinary podocytes, probably by suppressing platelet hypersensitivity, suppressing decreases in anionic charge, and/or increasing renal blood flow (23–25). However, the precise mechanisms are still unclear.

In summary, our data suggest that podocyte injury may occur early in patients with diabetic nephropathy and that dilazep dihydrochloride may be useful in preventing renal deterioration in these patients.

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