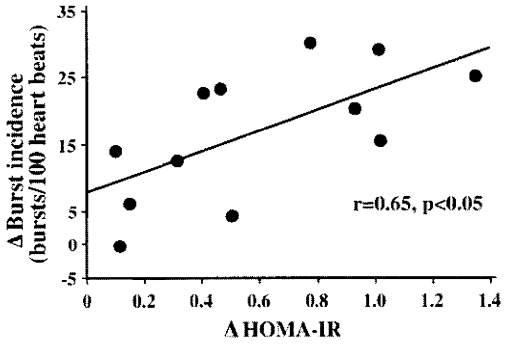

Fig. 3. The relationship between the absolute changes in the burst incidence and HOMA-IR. A significant positive relationship was found between the changes in MSNA and HOMA-IR ($r = 0.65$, $p < 0.05$).

might have resulted in the absence of fluid retention in our DM patients.

Underlying mechanisms of disturbed autonomic nervous system in DM remain uncertain. Both insulin and glucose have been assumed to contribute to increased sympathetic outflow in DM patients (Scherrer and Sartori, 1997; Straznicky et al., 2009; Fagius et al., 1996; Fagius, 2003). It is unlikely that decreased sympathetic activity would result from a direct effect of blood glucose, because FBG in pioglitazone was of similar level to that in alpha-GI. In this study, alpha-GI might have already affected sympathetic nerve activity and insulin resistance prior to study recruitment. However, our findings indicate that changing alpha-GI to pioglitazone contributes to reduction of MSNA in association with improved insulin resistance, which would help to explain partially the beneficial effect of pioglitazone in type 2 DM patients.

4.1. Study limitations

First, the study enrolled relatively few subjects. Nevertheless, our results were sufficient to detect a difference in the decreased response of MSNA to pioglitazone. Second, our study was an observational study, and did not include *placebo*-controlled study. However, because our recruited DM patients had been controlled without changing dose of alpha-GI during more than 3 months, it is unlikely that additional 3 months of treatment with alpha-GI would have significantly reduced the HOMA-IR and MSNA. Third, ongoing pharmacological treatment of our study is a limitation of our study. There is no report about an effect of antihypertensive drug and statin on MSNA in patients with type 2 DM. We did not hold these drugs on the study day of nerve recording to avoid rebound cardiovascular responses and its influence on sympathetic nerve activity. Finally, we measured multi-unit MSNA but did not evaluate cardiac sympathetic activity. However, evidence indicates a close relationship between the resting MSNA in the peroneal nerve and cardiac norepinephrine spillover in healthy normotensive individuals (Wallin et al., 1992).

4.2. Conclusions

These results suggest that improved insulin resistance with pioglitazone provides an additional favorable effect on sympathetic nerve activity, which might partly explain the contribution of pioglitazone to the improvement of cardiovascular mortality and morbidity in type 2 DM patients.

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References

Anderson, E.A., Hoffman, R.P., Balon, T.W., Sinkey, C.A., Mark, A.L., 1991. Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans. J. Clin. Invest. 87, 2246–2252.

Berne, C., Fagius, J., Pollare, T., Hjemdahl, P., 1992. The sympathetic response to euglycaemic hyperinsulinaemia. Evidence from microelectrode nerve recordings in healthy subjects. Diabetologia 35, 873–879.

Bohm, M., Flesch, M., Schnabel, P., 1997. Beta-adrenergic signal transduction in the failing and hypertrophied myocardium. J. Mol. Med. 75, 842–848.

Bonora, E., Targher, G., Alberiche, M., et al., 2000. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. Diab. Care 23, 57–63.

Chaudhri, B., Del Monte, F., Hajjar, R.J., Harding, S.E., 2002. Interaction between increased SERCA2a activity and beta-adrenoceptor stimulation in adult rabbit myocytes. Am. J. Physiol. Heart Circ. Physiol. 283, H2450–H2457.

Chiasson, J.L., Josse, R.G., Gomis, R., Hanefeld, M., Karasik, A., Laakso, M., 2003. Acarbose treatment and the risk of cardiovascular disease and hypertension in patients with impaired glucose tolerance: the STOP-NIDDM trial. JAMA 290, 486–494.

Communal, C., Singh, K., Pimentel, D.R., Colucci, W.S., 1998. Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the beta-adrenergic pathway. Circulation 98, 1329–1334.

Dormandy, J.A., Charbonnel, B., Eckland, D.J., Erdmann, E., Massi-Benedetti, M., Moules, I.K., Skene, A.M., Tan, M.H., Lefebvre, P.J., Murray, G.D., Standl, E., Wilcox, R.G., Wilhelmsen, L., Betteridge, J., Birkeland, K., Golay, A., Heine, R.J., Koranyi, L., Laakso, M., Mokan, M., Norkus, A., Pirags, V., Podar, T., Scheen, A., Scherbaum, W., Schernthaner, G., Schmitz, O., Skrha, J., Smith, U., Taton, J., 2005. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. Lancet 366, 1279–1289.

Fagius, J., 2003. Sympathetic nerve activity in metabolic control—some basic concepts. Acta Physiol. Scand. 177, 337–343.

Fagius, J., Wallin, B.G., 1993. Long-term variability and reproducibility of resting human muscle nerve sympathetic activity at rest, as reassessed after a decade. Clin. Auton. Res. 3, 201–205.

Fagius, J., Ellerfelt, K., Lithell, H., Berne, C., 1996. Increase in muscle nerve sympathetic activity after glucose intake is blunted in the elderly. Clin. Auton. Res. 6, 195–203.

Finfer, S., Chittock, D.R., Su, S.Y., Blair, D., Foster, D., Dhingra, V., Bellomo, R., Cook, D., Dodek, P., Henderson, W.R., Hebert, P.C., Heritier, S., Heyland, D.K., McArthur, C., McDonald, E., Mitchell, I., Myburgh, J.A., Norton, R., Potter, J., Robinson, B.G., Ronco, J.J., 2009. Intensive versus conventional glucose control in critically ill patients. N Engl J. Med. 360, 1283–1297.

Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M., Evans, R.M., 1995. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. Cell 83, 803–812.

Fujisawa, K., Nishikawa, T., Kukidome, D., Imoto, K., Yamashiro, T., Motoshima, H., Matsumura, T., Araki, E., 2009. TZDs reduce mitochondrial ROS production and enhance mitochondrial biogenesis. Biochem. Biophys. Res. Commun. 379, 43–48.

Gao, L., Wang, W., Li, Y.L., Schultz, H.D., Liu, D., Cornish, K.G., Zucker, I.H., 2004. Superoxide mediates sympathoexcitation in heart failure: roles of angiotensin II and NAD(P)H oxidase. Circ. Res. 95, 937–944.

Grassi, G., Seravalle, G., Dell'Oro, R., Arenare, F., Facchetti, R., Mancia, G., 2009. Reproducibility patterns of plasma norepinephrine and muscle sympathetic nerve traffic in human obesity. Nutr. Metab. Cardiovasc. Dis. 19, 469–475.

Guan, Y., Hao, C., Cha, D.R., Rao, R., Lu, W., Kohan, D.E., Magnuson, M.A., Redha, R., Zhang, Y., Breyer, M.D., 2005. Thiazolidinediones expand body fluid volume through PPARgamma stimulation of ENaC-mediated renal salt absorption. Nat. Med. 11, 861–866.

Hoffman, R.P., Sinkey, C.A., Anderson, E.A., 1998. Microneurographically determined muscle sympathetic nerve activity levels are reproducible in insulin-dependent diabetes mellitus. J. Diab. Complications 12, 307–310.

Huggett, R.J., Scott, E.M., Gilbey, S.G., Stoker, J.B., Mackintosh, A.F., Mary, D.A., 2003. Impact of type 2 diabetes mellitus on sympathetic neural mechanisms in hypertension. Circulation 108, 3097–3101.

Lago, R.M., Singh, P.P., Nesto, R.W., 2007. Congestive heart failure and cardiovascular death in patients with prediabetes and type 2 diabetes given thiazolidinediones: a meta-analysis of randomised clinical trials. Lancet 370, 1129–1136.

Matsumoto, K., Yano, M., Miyake, S., Ueki, Y., Yamaguchi, Y., Akazawa, S., Tominaga, Y., 1998. Effects of voglibose on glycemic excursions, insulin secretion, and insulin sensitivity in non-insulin-treated NIDDM patients. Diab. Care 21, 256–260.

Meneilly, G.S., Ryan, E.A., Radziuk, J., Lau, D.C., Yale, J.F., Morais, J., Chiasson, J.L., Rabasa-Lhoret, R., Maheux, P., Tessier, D., Wolever, T., Josse, R.G., Elahi, D., 2000. Effect of acarbose on insulin sensitivity in elderly patients with diabetes. Diab. Care 23, 1162–1167.

Muntzel, M.S., Anderson, E.A., Johnson, A.K., Mark, A.L., 1995. Mechanisms of insulin action on sympathetic nerve activity. Clin. Exp. Hypertens. 17, 39–50.

Murai, H., Takata, S., Maruyama, M., Nakano, M., Kobayashi, D., Otowa, K., Takamura, M., Yuasa, T., Sakagami, S., Kaneko, S., 2006. The activity of a single muscle sympathetic vasoconstrictor nerve unit is affected by physiological stress in humans. Am. J. Physiol. Heart Circ. Physiol. 290, H853–H860.

Murai, H., Takamura, M., Maruyama, M., Nakano, M., Ikeda, T., Kobayashi, D., Otowa, K., Ootsuji, H., Okajima, M., Furusho, H., Takata, S., Kaneko, S., 2009. Altered firing pattern of single-unit muscle sympathetic nerve activity during handgrip exercise in chronic heart failure. J. Physiol. 587, 2613–2622.

Nagai, Y., Sugiyama, Y., Nakatsumi, Y., Abe, T., Nomura, G., 2003. Rapid amelioration of muscle sympathetic nerve activity by pioglitazone in an obese Type 2 diabetic patient. Diabet. Med. 20, 868–869.

Otowa, K., Takamura, M., Murai, H., Maruyama, M., Nakano, M., Ikeda, T., Kobayashi, D., Ootsuji, H., Okajima, M., Furushou, H., Yuasa, T., Takata, S., Kaneko, S., 2008. Altered interaction between plasminogen activator inhibitor type 1 activity and sympathetic nerve activity with aging. Circ. J. 72, 458–462.

Pereira, M.A., Kartashov, A.I., Ebbeling, C.B., Van Horn, L., Slattery, M.L., Jacobs Jr, D.R., Ludwig, D.S., 2005. Fast-food habits, weight gain, and insulin resistance (the CARDIA study): 15-year prospective analysis. Lancet 365, 36–42.

Scherrer, U., Sartori, C., 1997. Insulin as a vascular and sympathoexcitatory hormone: implications for blood pressure regulation, insulin sensitivity, and cardiovascular morbidity. Circulation 96, 4104–4113.

Stein, P.K., Barzilay, J.I., Domitrovich, P.P., Chaves, P.M., Gottdiener, J.S., Heckbert, S.R., Kronmal, R.A., 2007. The relationship of heart rate and heart rate variability to non-diabetic fasting glucose levels and the metabolic syndrome: the Cardiovascular Health Study. Diabet. Med. 24, 855–863.

Straznicky, N.E., Lambert, G.W., Masuo, K., Dawood, T., Eikelis, N., Nestel, P.J., McGrane, M.T., Mariani, J.A., Socratous, F., Chopra, R., Esler, M.D., Schlaich, M.P., Lambert, E.A., 2009. Blunted sympathetic neural response to oral glucose in obese subjects with the insulin-resistant metabolic syndrome. Am. J. Clin. Nutr. 89, 27–36.

The Committee of Japan Diabetes Society for the Diagnostic Criteria of Diabetes Mellitus, 1999. Report of the Committee of Japan Diabetes Society on the classification and diagnostic criteria of diabetes mellitus. J. Jpn. Diab. Soc. 42 (5), 385–404.

U.K. Prospective Diabetes Study (U.K.P.D.S.) Group, 1998. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. Lancet 352, 837–853.

Vollenweider, P., Tappy, L., Randin, D., Schneiter, P., Jequier, E., Nicod, P., Scherrer, U., 1993. Differential effects of hyperinsulinemia and carbohydrate metabolism on sympathetic nerve activity and muscle blood flow in humans. J. Clin. Invest. 92, 147–154.

Wallin, B.G., Esler, M., Dorward, P., Eisenhofer, G., Ferrier, C., Westerman, R., Jennings, G., 1992. Simultaneous measurements of cardiac noradrenaline spillover and sympathetic outflow to skeletal muscle in humans. J. Physiol. 453, 45–58.

World Health Organnization, (W.H.O.), 2007. Diabetes Programme, 2006. (Available at http://www.who.int/diabe).

CD14$^+$ Monocytes Are Vulnerable and Functionally Impaired Under Endoplasmic Reticulum Stress in Patients With Type 2 Diabetes

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OBJECTIVE—Although patients with diabetes suffer from increased infections and a higher incidence of cancer due to impaired immune function, details on diabetes-induced decrease in immunity are lacking. We assessed how immune-mediating peripheral blood mononuclear cells (PBMCs) are affected in diabetes.

RESEARCH DESIGN AND METHODS—From 33 patients with type 2 diabetes and 28 healthy volunteers, we obtained PBMCs and investigated their susceptibility to apoptosis and functional alteration.

RESULTS—In a subpopulation of PBMCs, monocytes derived from patients with diabetes were more susceptible to apoptosis than monocytes from healthy volunteers. Monocytes from patients with diabetes had decreased phagocytotic activity and were less responsive to Toll-like receptor (TLR) ligands, although the expression of TLRs did not differ significantly between the two groups. Furthermore, monocytes from patients with diabetes had a distinctly different gene expression profile compared with monocytes from normal volunteers as assessed with DNA microarray analysis. Specifically, quantitative real-time detection PCR measurements showed an elevated expression of the markers of endoplasmic reticulum (ER) stress in diabetic monocytes, and electron microscopic examination of monocytes revealed morphologic alterations in the ER of cells derived from patients with diabetes. Consistently, the ER stress inducer tunicamycin increased apoptosis of otherwise healthy monocytes and attenuated the proinflammatory responses to TLR ligands.

CONCLUSIONS—These data suggest that monocytes comprise a substantially impaired subpopulation of PBMCs in patients with diabetes and that ER stress is involved in these pathologic changes mechanistically. This implies that the affected monocytes should be investigated further to better understand diabetic immunity. ***Diabetes*** **59:634–643, 2010**

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Type 2 diabetes is the most frequent metabolic disease and the leading cause of human morbidity and mortality (1,2). Based on epidemiologic data, patients with diabetes are immunocompromised and have an increased incidence of infections in the respiratory tract, urinary tract, and skin (3–5). The high incidence of colorectal, breast, and pancreatic malignancies in patients with diabetes is also considered to be a consequence of diabetes-associated defects in immune function (6,7).

Although studies on immune cells and circulating cytokines have shed some light on this diabetic immunologic phenomenon, conflicting results have been reported and do not adequately explain the perturbed immune function in patients with diabetes. Controversial results concerning the phagocytotic activity of polymorphonuclear neutrophils and monocytes are in part due to differences in the patients themselves, insufficient numbers in the study populations, or inconsistencies in the collection of the cell populations under investigation (8–11). Therefore, further studies are needed to explain the decreased immune function of patients with diabetes.

We previously investigated the gene expression signatures of peripheral blood mononuclear cells (PBMCs) in patients with diabetes and observed transcriptional expression features that were distinct from those of healthy volunteers (12). Apoptosis-related genes were upregulated in the PBMCs of patients with diabetes. Based on this result, we investigated apoptotic activity and immunologic function in PBMCs from patients with type 2 diabetes.

We observed that the CD14$^+$ monocyte fraction was the most affected subpopulation of PBMCs from these patients; these cells were especially vulnerable to apoptosis compared with other cell subpopulations. We also found that CD14$^+$ monocytes demonstrated attenuated phagocytotic activity and deficient Toll-like receptor (TLR) signaling, both of which are important for innate immunity (13,14). Transcriptional analysis and electron microscopic examination of monocytes from patients with diabetes showed evidence of endoplasmic reticulum (ER) stress, which may underlie the functional defects in these cells. Collectively, the data presented herein show that CD14$^+$ monocytes are a vulnerable cell population under ER stress in these patients that could contribute to decreases in immune function in diabetes.

RESEARCH DESIGN AND METHODS

Thirty-three patients with type 2 diabetes (male/female, 15/18; age 62.0 ± 8.6 years; A1C 9.2 ± 2.0%) and 28 healthy volunteers (male/female, 15/13; age 58.2 ± 10.2 years; A1C 5.4 ± 0.7%) were enrolled consecutively for the

TABLE 1
Characteristics of the study subjects

	Diabetic patients (n = 33)	Healthy volunteers (n = 28)	P
Age (years)	62.0 ± 8.6	58.2 ± 10.2	NS
Sex (male/female)	15/18	15/13	NS
BMI (kg/m^2)	23.5 ± 4.2	23.6 ± 4.8	NS
White blood cell counts (ml)	4,800 ± 1,700	5,600 ± 1,900	NS
Lymphocytes (%)	23.5 ± 3.5	22.7 ± 2.5	NS
Monocytes (%)	5.2 ± 1.6	6.1 ± 2.3	NS
Hemoglobin (g/dl)	14.1 ± 1.3	13.6 ± 1.6	NS
Total cholesterol (mg/dl)	182 ± 24	180 ± 35	NS
Triglyceride (mg/dl)	138 ± 37	163 ± 33	NS
FPG (mg/dl)	185 ± 38	86 ± 7.4	<0.001
A1C (%)	9.2 ± 2.0	5.4 ± 0.7	<0.001
Diabetic complications (+/−)*	19/14	NA	
Insulin treatment (+/−)	10/23	NA	

Data are means ± SD. *Diabetic complications: nephropathy, neuropathy, retinopathy, macroangiopathy. FPG, fasting plasma glucose; NA, not applicable.

apoptosis assay (Table 1). The groups were not significantly different in terms of their clinical parameters, except for the fasting plasma glucose and A1C levels. The patients with diabetes (n = 16) from whom adequate numbers of monocytes were obtained were enrolled for additional experiments along with 17 other patients with diabetes (male/female, 8/9; age 60.5 ± 7.2 years; A1C 8.8 ± 1.8%) whose clinical profiles fit the diabetic profile (Table 1). Informed consent for this study was obtained from all subjects. The experimental protocol was carried out in accordance with the Declaration of Helsinki.

Isolation of subpopulations of PBMCs and flow cytometric analysis. PBMCs were freshly isolated from heparinized venous blood using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) as previously described (12). CD4$^+$ T-cell and CD14$^+$ monocyte subpopulations were isolated using a magnetic cell sorting system in accordance with the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated cells were purified by >90% as measured by flow cytometric analysis using FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). To assess the expression of TLRs on monocytes, PBMCs were incubated with phosphatidylethanolamine (PE)-labeled anti-TLR2, -TLR3, or -TLR4 (eBioscience, San Diego, CA) and fluorescein isothiocyanate (FITC)-labeled anti-CD14 antibodies (BD Biosciences) and analyzed by flow cytometry. Data were analyzed using CELLQuest Software (BD Biosciences).

Quantitative real-time detection PCR. Real-time detection (RTD)-PCR was performed as previously described (15). Briefly, total RNA obtained from cells using a MicroRNA isolation kit (Stratagene, La Jolla, CA) was reverse-transcribed using 1 μg oligo (dT) primer and Super Script II Reverse transcriptase (Invitrogen, Carlsbad, CA). The relative quantities of mRNA expression were analyzed by RTD-PCR using ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). All primer pairs and probes were obtained from the TaqMan assay reagents library. Expression levels of genes were calculated with the $2^{-\Delta\Delta Ct}$ method using either β-actin or GAPDH as internal control genes.

Apoptotic cell detection assay. Freshly isolated PBMCs were incubated with AIM-V (Invitrogen) serum-free culture media containing 5 or 30 mmol/l glucose at 37°C with 5% CO_2 for up to 24 h. The cells were incubated with FITC-labeled anti-CD4, -CD14, or -CD56 antibodies (BD Biosciences) and with PE-labeled annexin-V and 7-amino-actinomycin D (7-AAD) (BD Biosciences) in PBS containing 2% BSA (Sigma-Aldrich). Apoptotic cells were determined by flow cytometry as the fraction of cells labeled with annexin-V that were 7-AAD negative. At least 10,000 cells per sample were analyzed.

Phagocytosis assay. Phagocytotic activity was assessed using a Phagotest Kit (Orpegen Pharma, Heidelberg, Germany) and FITC-labeled opsonized *Escherichia coli* in accordance with the manufacturer's protocol. Briefly, heparinized whole blood obtained from the 33 patients with diabetes and 28 healthy volunteers was incubated with FITC-labeled *E. coli* for 10 min at 37°C. After removing the erythrocytes, the remaining cells were incubated with propidium iodide to detect viable leukocytes by flow cytometry. Monocyte populations were assessed based on cellular granularity and size as side scatter and forward scatter, respectively, and FITC-positive cells were assessed as monocytes with phagocytosed FITC-labeled *E. coli*.

TLR ligand stimuli and expression of proinflammatory cytokine genes. Peptidoglycan (PGN; 1 μg/ml) from *Streptomyces sp.* (Sigma-Aldrich), Poly (I:C) (5 μg/ml; Sigma-Aldrich), and lipopolysaccharide (LPS; 2 μg/ml) from *E. coli* (Sigma-Aldrich), which are TLR2, TLR3, and TLR4 ligands, respectively, were added to monocytes (3×10^5 cells) freshly isolated from the 33 patients and 28 healthy volunteers in AIM-V media. Before and 3 h after incubation, the expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) was analyzed by RTD-PCR.

Analysis of gene expression by DNA microarray. Total RNA was obtained from CD14$^+$ monocytes using MicroRNA isolation kit (Stratagene), and the mRNA was amplified twice using the Amino Allyl MessageAmp aRNA Kit (Ambion, Austin, TX). The reference RNA sample was isolated from CD14$^+$ monocytes from a 30-year-old healthy male volunteer and amplified in the same manner. Amplified mRNA was labeled with cyanin (Cy) 5 or Cy3 (Amersham, Buckinghamshire, U.K.). Equal amounts of the amplified mRNAs were hybridized to an oligo-DNA chip (AceGene Human Oligo Chip 30K; Hitachi Software Engineering, Yokohama, Japan) overnight and washed prior to image scanning.

The fluorescence intensity of each spot on the oligo-DNA chip was obtained using cDNA Microarray Scan Array G (PerkinElmer, Wellesley, MA). The obtained images were quantified using DNAsis array V2.6 software (Hitachi Software Engineering). For normalization, the intensity of each spot with oligo DNA was subtracted from that of spots without oligo DNA in the same block. The spot was validated when the intensity was within the intensity plus or minus a twofold range of SD within each block. By calibrating the median as the base value, the intensities of all spots were adjusted for normalization between Cy5 and Cy3. Hierarchic clustering of gene expression was calibrated using the method described above using BRB Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). The nonfiltered data were log-transformed and applied to the average linkage clustering with centered correlation. For the functional analysis of the 813 upregulated genes, we used GenMAPP (http://www.genmapp.org), a computer program designed for viewing and analyzing genome-scale data on MAPPs representing biological pathways and any other groups of genes.

Electron microscopy. Monocytes obtained from three healthy volunteers and three patients with diabetes were fixed with 2.5% glutaraldehyde and then postfixed in 1% (vol/vol) cacodylate-buffered osmium tetroxide. Samples were dehydrated in a graded series of ethanol, transferred to propylene oxide, and embedded in Epon-Araldite (Sigma-Aldrich). Ultrathin sections were obtained and observed under a Hitachi H-7500 electron microscope (Hitachi High-Technologies, Hitachinaka, Japan).

Caspase-3 assay and enzyme-linked immunosorbent assay (ELISA) of cytokines. Monocytes from a healthy volunteer were harvested and treated with tunicamycin (1 or 5 μg/ml) in AIM-V media. Every 3 h up to 12 h after tunicamycin treatment, we assessed apoptosis by flow cytometry as described above. After 12 h of incubation, the expression levels of BCL-2, C/EBP homologous protein (CHOP), and BiP (immunoglobulin heavy chain binding protein) were assessed by RTD-PCR. The DEVD-cleaving activity of active caspase-3 was measured using labeled Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) as the substrate and the Caspase-3 Colorimetric Assay Kit (Promega, Madison, WI) in accordance the manufacturer's protocol. The pNA light emission was quantified using a microtiter plate reader at a wavelength of 405 nm. In addition, we measured the production of proinflammatory cytokines by RTD-PCR 6 h after treatment of monocytes (3×10^5 cells) with tunicamycin (1 or 5 μg/ml) or the TLR ligands PGN (1 μg/ml), Poly (I:C) (5 μg/ml), and LPS (2 μg/ml). The concentrations of TNF-α, IL-1β, and IL-6 in the culture supernatants were measured using an ELISA kit (eBioscience).

Statistical analysis. Data are expressed as means ± SEM. The Mann-Whitney U test was applied to assess the significant differences between the two groups. Statistical significance was determined as $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS

Increased apoptosis of CD14$^+$ monocytes from patients with diabetes. We first assessed the frequency of apoptosis in the PBMC fractions from 33 patients with diabetes and 28 nondiabetic healthy volunteers. Apoptosis of the isolated cells was assessed after 3-h incubation in AIM-V serum-free media containing 5 mmol/l glucose (physiological concentration in blood). As shown in Fig. 1*A*, a significant difference in the frequency of apoptosis was observed in the PBMCs isolated from patients with diabetes and healthy volunteers. Adding serum to AIM-V serum-free media did not affect the difference in apoptosis (data

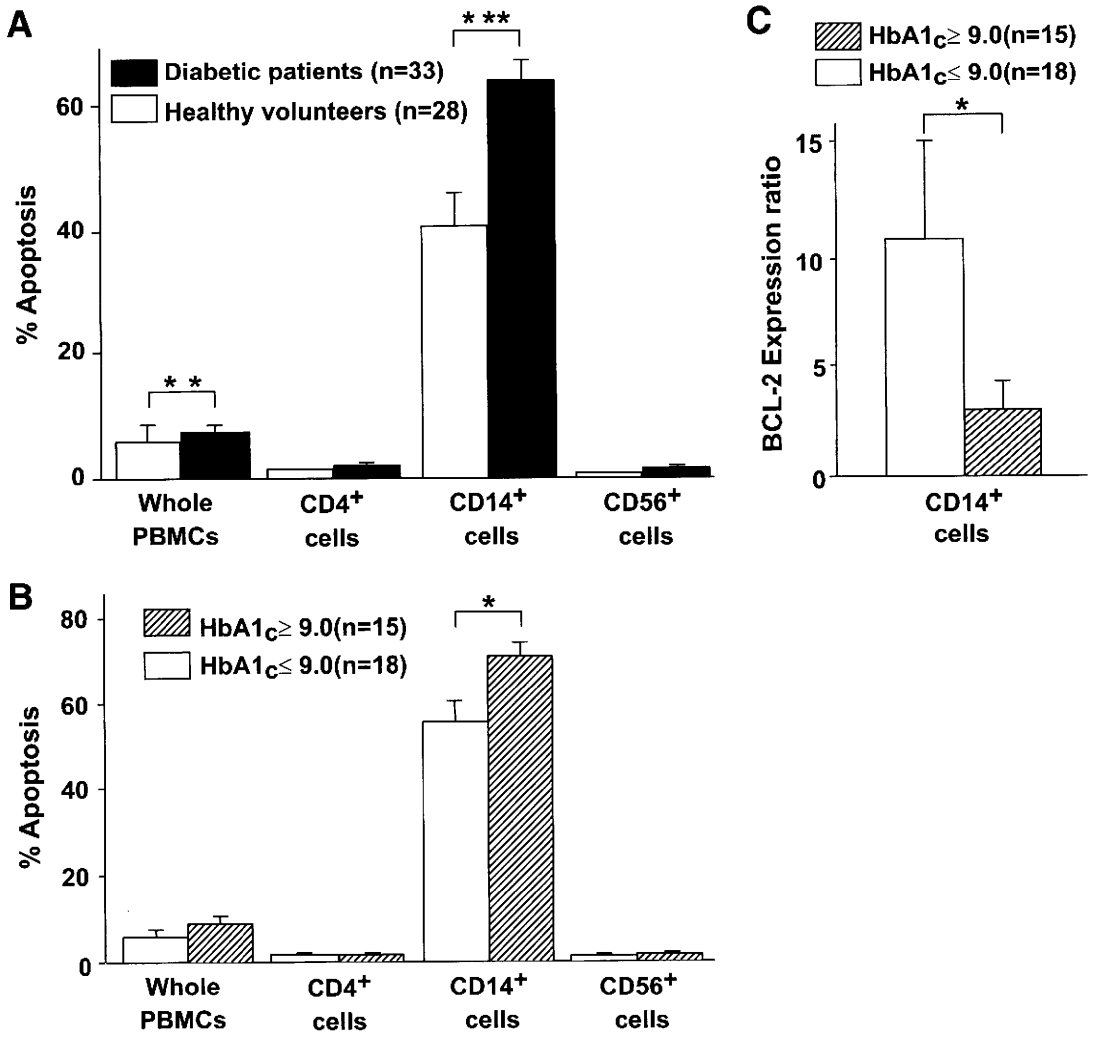

FIG. 1. Monocytes contributed to the vulnerability of the PBMCs in patients with diabetes. *A*: PBMCs were obtained from 33 patients with diabetes and 28 healthy volunteers. Isolated PBMCs were harvested in AIM-V serum-free culture media supplemented with 5 mmol/l glucose for 3 h and incubated with FITC-labeled anti-CD4, -CD14, or -CD56 antibodies, together with PE-labeled annexin-V to assess the frequency of apoptotic cells in each subpopulation of PBMCs. Apoptotic cells were identified by double-staining with PE-labeled annexin-V and 7-AAD by flow cytometry. The frequencies of apoptotic cells determined as the annexin-V–positive and 7-AAD–negative population are expressed as means ± SEM with statistical comparisons for both groups. The nonparametric Mann-Whitney *U* test was used to calculate the *P* value. **$P < 0.01$, ***$P < 0.001$. The PBMCs of patients with diabetes were more susceptible to apoptosis than those of healthy volunteers, and CD14$^+$ monocytes were contributors. *B*: Among the 33 patients with diabetes, those with poor glycemic control reflected as A1C ≥9.0% were more susceptible to apoptosis in CD14$^+$ monocytes. Data are expressed as means ± SEM with a statistical comparison of both groups. *$P < 0.05$. *C*: Monocytes were isolated from 15 patients with A1C ≥9.0% and 18 patients with A1C <9.0%. The expression of the BCL-2 gene in their monocytes before and after incubation in AIM-V serum-free media was assessed by RTD-PCR. After 3-h incubation, the expression of BCL-2 was not upregulated in the poor glycemic control group (A1C ≥9.0%), compared with the fair control group (A1C <9.0%). Data are expressed as means ± SEM with statistical comparisons of both groups. *$P < 0.05$.

not shown). The numbers of whole PBMCs and CD4$^+$, CD14$^+$, and CD56$^+$ cells were similar in both diabetic and healthy subjects (data not shown). CD14$^+$ monocytes were observed to be the major contributor to the increased apoptosis measured in the PBMCs. In contrast, apoptosis of CD4$^+$ T-cells and CD56$^+$ natural killer (NK) cells was not significantly different between the two groups (Fig. 1*A*). When the incubation period in culture media with or without serum was extended to 24 h, ~20% of the CD56$^+$ NK cells of both patients with diabetes and healthy volunteers were induced to undergo apoptosis. When incubation period was extended to 5 days, ~5% of CD4$^+$ T-cells of both patients with diabetes and healthy volunteers were induced to undergo apoptosis; there was no significant difference in cell viability of CD56$^+$ NK cells and CD4$^+$ T-cells between the two groups (data not shown). BCL-2 expression of CD4$^+$ T-cells was not different between the two groups (data not shown). Apoptosis of PBMC subpopulations incubated in culture media containing 30 mmol/l glucose was not different from cells incubated in 5 mmol/l glucose-containing media (data not shown). Moreover, the susceptibility of PBMCs from patients with diabetes to apoptosis was not related to clinical features such as vascular complications, insulin treatment, and fasting plasma glucose concentrations (data not shown).

However, among the 33 patients with diabetes, the frequency of apoptotic CD14$^+$ monocytes from those with poor glycemic control (A1C ≥9.0%) was elevated compared with patients with fair glycemic control (A1C <9.0%) (Fig. 1*B*). Furthermore, after 3-h incubation, the increased ratio of the expression of the antiapoptotic gene, BCL-2, was substantially lower in monocytes from the 15 patients with A1C ≥9.0% compared with the 18

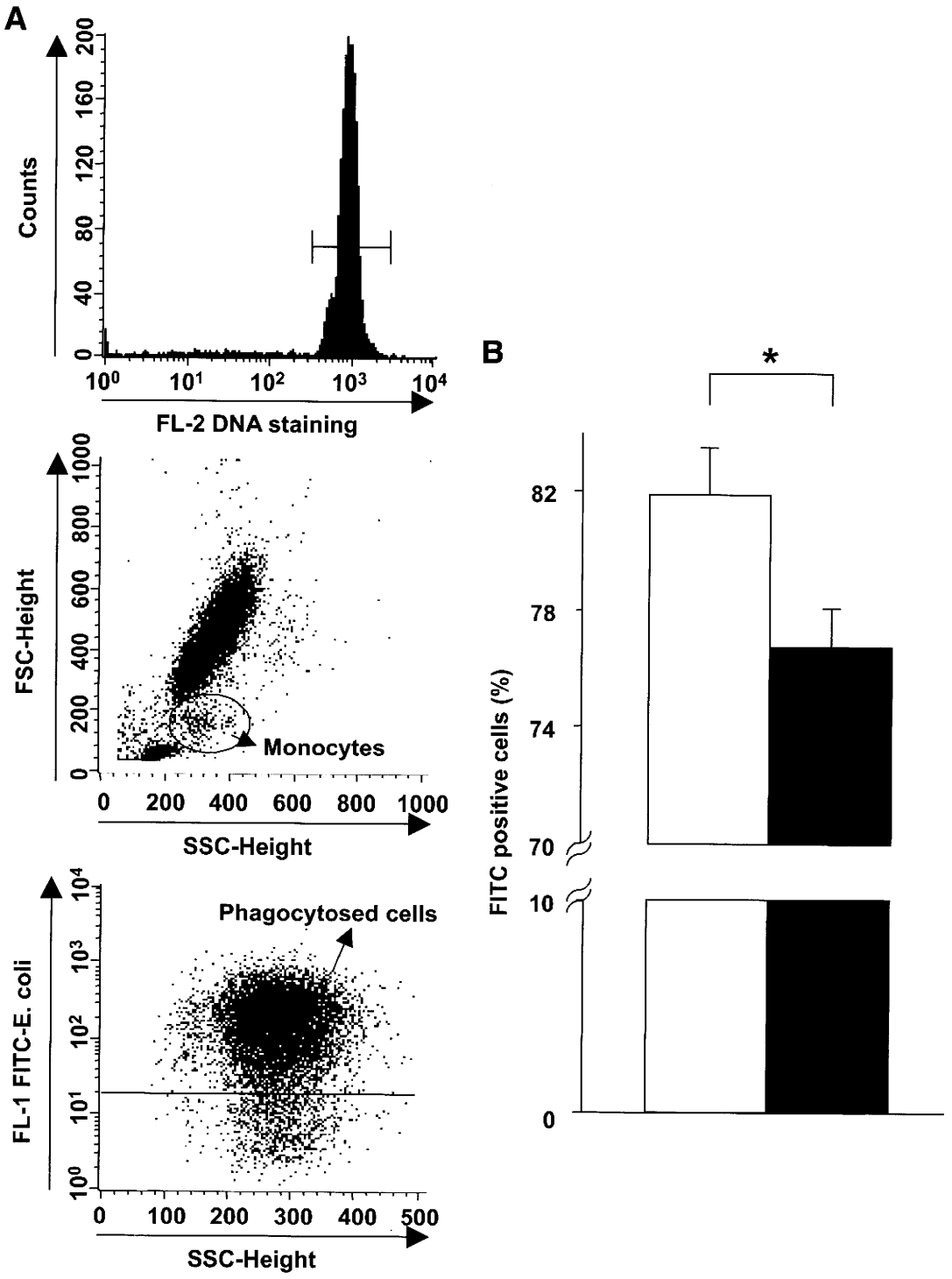

FIG. 2. Attenuated phagocytosis activity in diabetic monocytes. Whole PBMCs were incubated with FITC-labeled *E. coli* for 10 min followed by propidium iodide staining and flow cytometric analysis. *A*: Gated propidium iodide–positive populations were viable leukocyte populations (*upper panel*). The monocyte population was assessed using granularity (side scatter) and size (forward scatter) (*middle panel*). For the gated cells indicating viable monocytes, FITC-positive cells were assessed as monocytes containing phagocytosed FITC-labeled *E. coli* (*lower panel*). *B*: The frequency of monocytes containing phagocytosed *E. coli* in patients with diabetes (■, $n = 33$) was less than that in healthy volunteers (□, $n = 28$). Data are expressed as means ± SEM. $^*P < 0.05$.

patients having A1C <9.0%, as assessed by RTD-PCR (Fig. 1*C*). These data suggest that the monocytes of patients with diabetes are susceptible to apoptosis, especially under conditions of poor glycemic control.

Attenuated function of monocytes from patients with diabetes. To determine whether functional alterations exist in monocytes isolated from the 33 patients with diabetes, we cocultured the monocytes with FITC-labeled *E. coli* and counted the number of fluorescent monocytes that phagocytosed the labeled *E. coli* by flow cytometry. The ratio of monocytes that phagocytosed *E. coli* to all monocytes in patients with diabetes was higher than in the healthy volunteers (Fig. 2*A* and *B*). No significant correlation was observed between the ratio of phagocy-

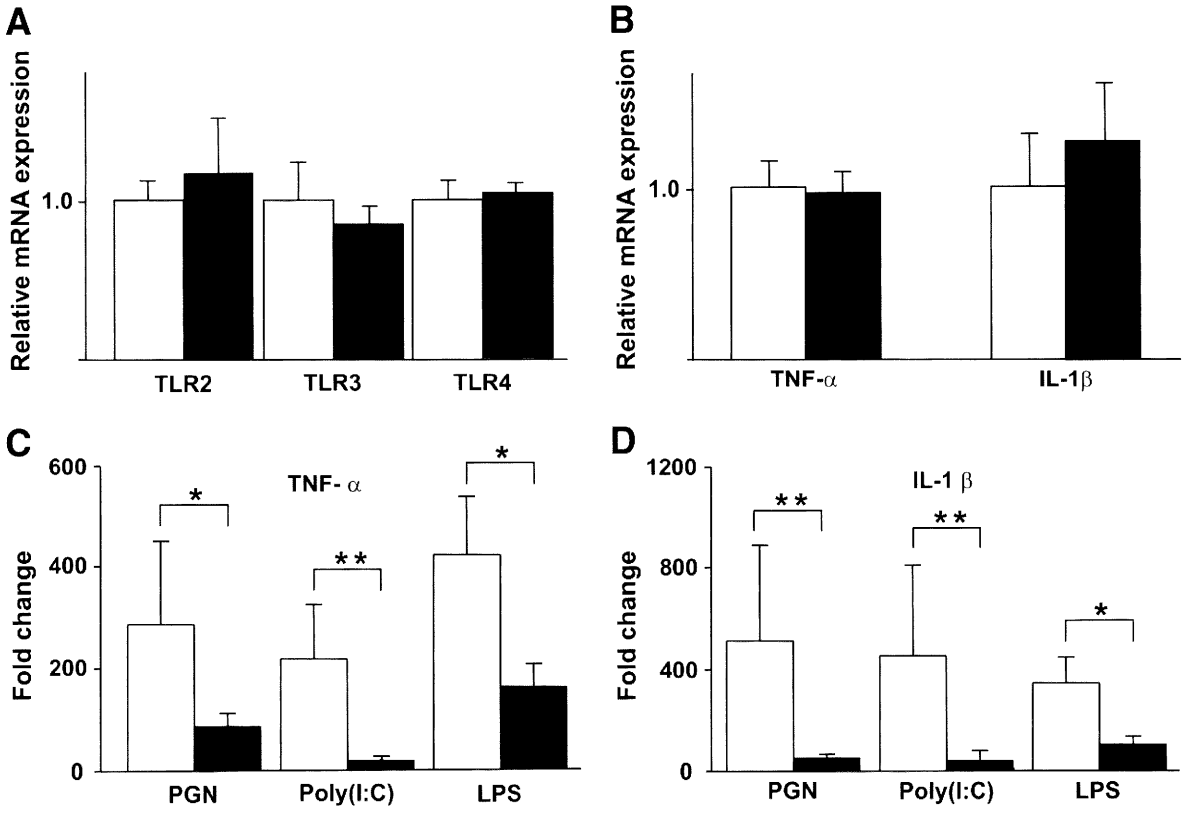

FIG. 3. Hyporesponsiveness to TLR ligand stimuli by the monocytes of patients with diabetes. *A–D*: Isolated CD14$^+$ monocytes from 33 patients with diabetes (■) and 28 healthy volunteers (□) were cultured in AIM-V serum-free media supplemented with each TLR ligand: PGN, Poly (I:C), and LPS. After 3-h incubation, RNA was isolated from the monocytes, and the expression levels of the TNF-α and IL-1β genes were analyzed by RTD-PCR. The basal (prestimuli) expression of TLR2, TLR3, and TLR4 (*A*) and TNF-α and IL-1β (*B*) did not differ significantly between the two groups. The TLR ligand–induced expression of TNF-α (*C*) and IL-1β (*D*) was downregulated in the monocytes of patients with diabetes. Data are expressed as means ± SEM. *$P < 0.05$, **$P < 0.01$.

tosed *E. coli* and A1C levels among the patients (data not shown).

Next, we assessed the responsiveness of monocytes to external pathogenic stimuli in vitro. Monocytes typically express pattern-recognition molecules such as the TLRs that are important for innate immunity against various pathogens (13,14). The expression levels of TLR2, TLR3, and TLR4 were not significantly different between monocytes from patients with diabetes and those from healthy volunteers, as assessed by RTD-PCR (Fig. 3*A*) and flow cytometry (data not shown). We also found that transcriptional expression of TLR signal molecules (MyD88, IRAK1, and TRAF6 for TLR2 and TLR4 signaling and TRIF for TLR3 signaling) was not altered in diabetic monocytes compared with nondiabetic monocytes (data not shown). Next, we exposed the monocytes from the patients with diabetes and healthy volunteers to the TLR ligands, PGN (a TLR2 ligand), Poly (I:C) (a TLR3 ligand), and LPS (a TLR4 ligand) and measured the expression of the proinflammatory cytokine genes, TNF-α and IL-1β. After incubation, the expression of the cytokines was not significantly different between the groups (Fig. 3*B*), but the responsiveness to PGN, Poly (I:C), and LPS was significantly attenuated in monocytes from patients with diabetes compared with those from healthy volunteers as assessed by RTD-PCR (Fig. 3*C* and *D*). These results demonstrate that the monocytes of patients with diabetes are functionally impaired, which implies that they could contribute to immune deficiency in diabetes.

ER stress is a molecular feature of impaired monocytes. To elucidate the molecular features of the diabetic monocytes that were distinctly susceptible to apoptosis, DNA microarray analysis was performed on CD14$^+$ cells isolated from five randomly selected patients with diabetes and five healthy volunteers. These subjects demonstrated clinical features near the median of all study subjects. Unsupervised hierarchic clustering analysis was performed to assess the gene expression profiles of monocytes obtained from patients with diabetes and healthy volunteers; 17,184 filtered genes were evaluated after excluding genes that were not expressed or those with low expression levels that prevented their analysis in 50% of the cases. As shown in Fig. 4*A*, two completely discernible clusters formed between the patients with diabetes and the healthy volunteers.

We identified 813 genes that were upregulated in the monocytes from patients with diabetes compared with those of healthy volunteers ($P < 0.05$, Student t test). Analysis of the biological processes concerning these genes was performed using GenMAPP. The identified genes were shown to be involved in posttranslational protein modification systems occurring in the Golgi apparatus or were involved in ER stress (Table 2 and supplementary Table 1, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0659/DC1). The elevated expression of genes related to ER stress, such as CHOP and BiP, was confirmed using RTD-PCR; the expression of these genes was significantly higher in the monocytes from the 33

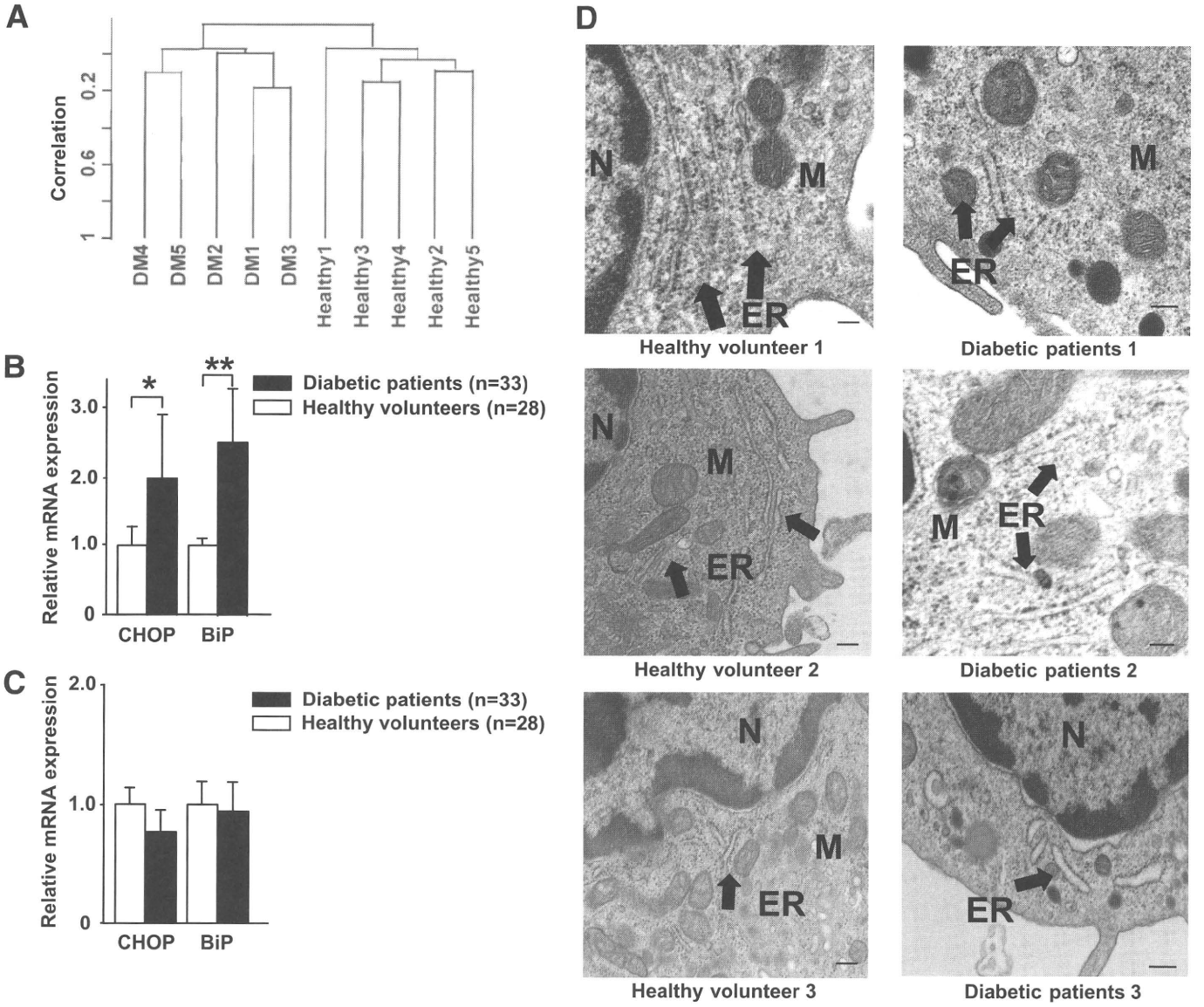

FIG. 4. Monocytes of patients with diabetes were under ER stress. *A*: The gene expression profiles of representative vulnerable CD14$^+$ monocytes obtained from five patients with diabetes and five healthy volunteers were analyzed using a DNA microarray. Unsupervised hierarchic clustering using 17,184 filtered genes produced two clusters that separated the patients with diabetes from the healthy volunteers without exception. *B* and *C*: The gene expression levels of the ER stress markers, such as CHOP and BiP, on CD14$^+$ monocytes and CD4$^+$ T-cells obtained from 33 patients with diabetes and 28 healthy volunteers were analyzed using RTD-PCR. *B*: The expression levels of CHOP and BiP in monocytes of patients with diabetes were significantly upregulated, compared with the monocytes of healthy volunteers. Data are expressed as means ± SEM. *$P < 0.05$, **$P < 0.01$. *C*: The expression levels of CHOP and BiP in T-cells of patients with diabetes were similar to those of healthy volunteers. Data are expressed as means ± SEM. *D*: Monocytes were obtained from three healthy volunteers and three patients with diabetes (healthy volunteer 1: 64-year-old man, A1C 5.7%; healthy volunteer 2: 66-year-old man, A1C 4.9%; healthy volunteer 3: 68-year-old woman, A1C 5.6%; diabetic patient 1: 56-year-old man, A1C 9.1%; diabetic patient 2: 64-year-old woman, A1C 8.2%; diabetic patient 3: 71-year-old man, A1C 10.2%) and examined using electron microscopy. In the three patients with diabetes, the concentric, continuous, and regular layer structures of the ER were corrupted, with fewer ribosomes on the ER membrane compared with the ER of the healthy volunteer. ER, endoplasmic reticulum; M, mitochondrion; N, nucleus. Scale bars indicate 100 nm.

patients with diabetes than in those from the 28 healthy volunteers (Fig. 4*B*). In contrast, no significant difference in the expression of these genes was observed in CD4$^+$ T-cells from patients with diabetes and healthy volunteers (Fig. 4*C*).

Electron microscopy further confirmed ER stress in the monocytes derived from patients with diabetes. As shown in Fig. 4*D*, morphologic alterations of the ER such as corruption of concentric, continuous, and regular layer structure and a decreased number of ribosomes on the ER membrane were evident from the electron photomicrographic images.

ER stress–induced apoptosis and attenuation of TLR signaling in human monocytes. The results described above indicated that the monocytes from patients with diabetes have compromised immunologic function and that ER stress is a distinct feature in these cells. To determine whether ER stress could be a mechanism underlying the observed increase in apoptosis and decreased responsiveness to TLR ligands, CD14$^+$ cells isolated from a healthy volunteer were treated with the ER stress inducer, tunicamycin (1 μg/ml), in AIM-V media. As shown in Fig. 5*A* and *B*, an increased number of apoptotic cells was observed among monocytes treated with tunica-

TABLE 2
Biological processes for upregulated genes in monocytes of diabetic patients

	Z score	Permute *P* value
MAPP name		
Golgi apparatus	3.383	0.000
Ribosomal proteins	3.691	0.002
Unfold protein binding	2.471	0.026
Intracellular protein transport	2.310	0.029
Enzyme-linked receptor protein signaling pathway	2.175	0.042
Nuclear receptor	2.316	0.043
Gametogenesis	−1.998	0.049

mycin compared with untreated monocytes after >6 h of incubation. Treatment of monocytes with a higher concentration of tunicamycin (5 μg/ml) induced more apoptosis (Fig. 5*A* and *B*), and when monocytes were treated with tunicamycin for 12 h, the activity of the proapoptotic protease, caspase-3, significantly increased (Fig. 5*C*). Treatment with tunicamycin coordinately decreased the expression of BCL-2 (Fig. 5*D*) and increased the expression of the ER stress markers, CHOP and BiP (Fig. 5*E*). These results suggest that ER stress promotes apoptosis of human monocytes.

Next, we investigated how tunicamycin-induced ER stress affected the responsiveness of human monocytes to TLR ligands. Treatment of monocytes with tunicamycin for 6 h did not affect the transcriptional and translational expression of TLR2 and TLR4 (data not shown). As shown in Fig. 6*A–C*, however, the expression of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 was downregulated after stimulation with TLR2 and TLR4 ligands. Furthermore, the production of TNF-α, IL-1β, and IL-6 in media was measured by ELISA and found to decrease after treatment of human monocytes with tunicamycin and after stimulation with TLR2 or TLR4 ligands (Fig. 6*D–F*). However, tunicamycin-induced ER stress did not affect expression after treatment of monocytes with the TLR3 ligand, Poly (I:C) (data not shown).

DISCUSSION

In the present study, we observed that PBMCs from patients with diabetes were more susceptible to apoptosis compared with PBMCs from healthy volunteers and that

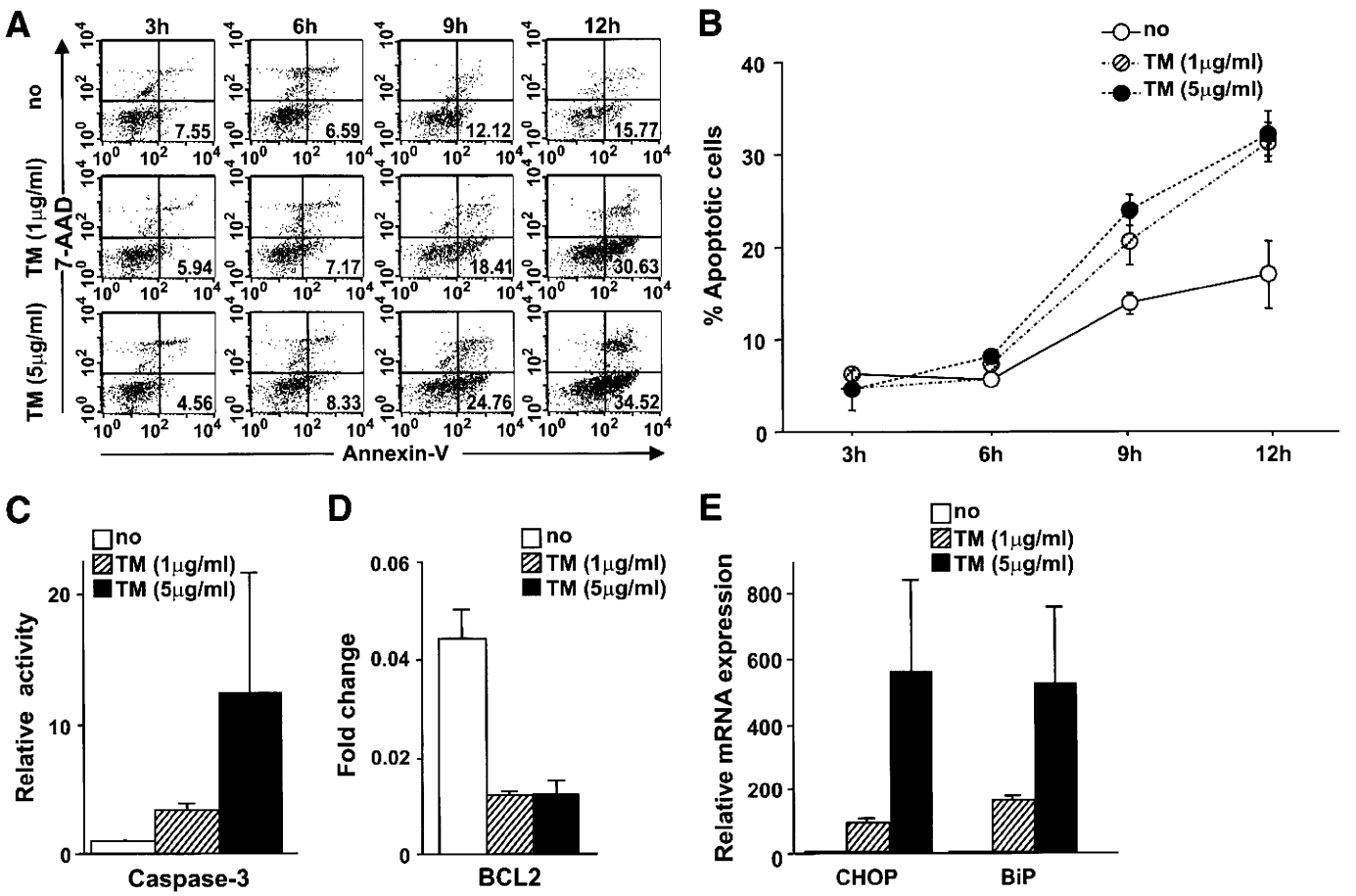

FIG. 5. ER stress enhanced the susceptibility of human monocytes to apoptosis. *A* and *B*: Human CD14$^+$ monocytes obtained from a healthy volunteer were incubated in AIM-V culture media supplemented with tunicamycin (TM) (1 or 5 μg/ml). The frequency of apoptotic cells was analyzed by flow cytometry every 3 h for 12 h. More apoptotic cells were observed among monocytes treated with tunicamycin for >6 h of incubation, compared with untreated monocytes. *A*: Representative scattergram of annexin-V and 7-AAD for monocytes treated with tunicamycin. The numbers in each quadrant indicate the percentage of apoptotic cells. *B*: Apoptotic cells were assessed in triplicate for each condition. Data are expressed as means ± SEM. *C*: Caspase-3 activity in monocytes treated with tunicamycin increased significantly at 12 h of incubation. *D*: The BCL-2 expression in monocytes incubated with tunicamycin for 12 h was downregulated. *E*: The expression levels of the ER stress markers CHOP and BiP in monocytes incubated with tunicamycin for 12 h were significantly upregulated. Data are expressed as means ± SEM of three independent experiments. □, No treatment; ▨, treatment with tunicamycin (1 μg/ml); ■, treatment with tunicamycin (5 μg/ml).

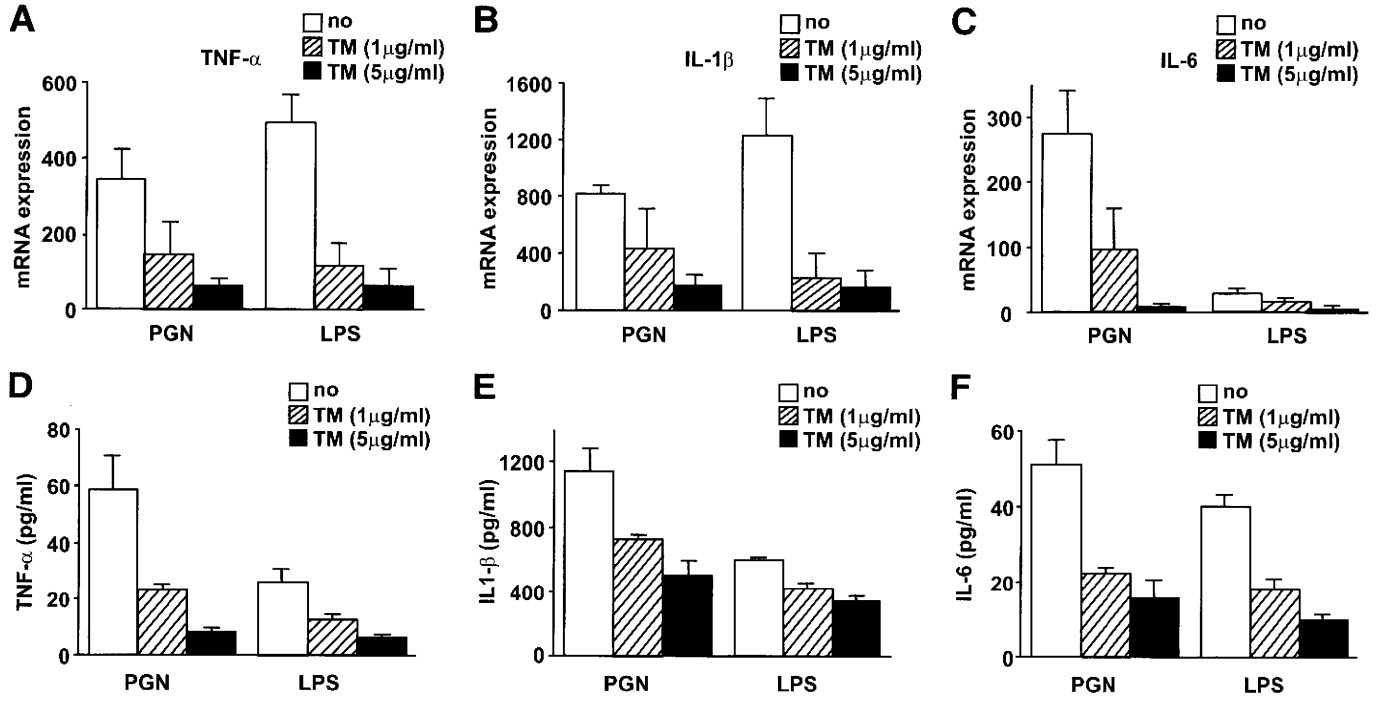

FIG. 6. Expression of proinflammatory cytokines in response to TLR ligand stimuli decreased in human monocytes treated with tunicamycin (TM). Isolated human CD14$^+$ monocytes were incubated in AIM-V culture media with tunicamycin (1 or 5 μg/ml) and stimulated using TLR ligands, PGN, and LPS for 6 h. *A–C*: RTD-PCR analysis showed that the expression of TNF-α (*A*), IL-1β (*B*), and IL-6 (*C*) was downregulated in human CD14$^+$ monocytes treated with tunicamycin, especially at the higher concentration (5 μg/ml). *D–F*: ELISA showed that the production of TNF-α (*D*), IL-1β (*E*), and IL-6 (*F*) in culture media decreased in human monocytes treated with tunicamycin, especially at the higher concentration (5 μg/ml). Data are expressed as means ± SEM of four independent experiments. □, No treatment; ▨, treatment with tunicamycin (1 μg/ml); ■, treatment with tunicamycin (5 μg/ml).

CD14$^+$ monocytes comprised the primary PBMC subpopulation undergoing apoptosis. We also found that CD14$^+$ monocytes from patients with diabetes were hyporesponsive to TLR ligands and that they had attenuated phagocytotic activity. Transcriptional analysis and electron microscopy revealed the presence of ER stress in the affected diabetic monocytes. Consistently, monocytes isolated from nondiabetic patients showed a similar increase in apoptosis and a weakened response to TLR ligands, when they were treated with tunicamycin, indicating that ER stress may be a pivotal mechanism underlying the decreased immunologic function observed in patients with diabetes.

As innate immune-defense mediators, monocytes are capable of ingesting exogenous pathogens to protect the host from infectious diseases. Previous studies have shown that phagocytosis in diabetic neutrophils and monocytes is attenuated (10,11). Similarly, in our study population, monocytes from patients with diabetes were less capable of phagocytosing *E. coli* pathogens compared with monocytes derived from healthy volunteers. This novel finding might explain, at least in part, the decrease in immune function characteristic of patients with diabetes (16). Nevertheless, the detailed mechanisms underlying diabetes-induced decreases in phagocytotic activity remain unclear, because simple high-glucose concentration neither affected the phagocytotic activity and TLR expression nor induced ER stress in nondiabetic monocytes in vitro (data not shown).

The TLRs are pattern-recognition receptors that are important for recognizing pathogens, inducing proinflammatory responses, and preventing the host from acquiring infectious diseases (17–20). The expression of TLR2, TLR3, and TLR4 in CD14$^+$ monocytes was similar between patients with diabetes and healthy volunteers. The administration of a high dose of insulin downregulates TLR expression (21). Transformed monocyte-lineage blastoma cells showed increased TLR expression under hyperglycemic conditions in vitro (22). Type 2 diabetes is characterized as a state of inadequately controlled glycemia associated with hyperinsulinemia due to peripheral insulin resistance (1). Taken together, the TLR expression may be affected by hyperglycemia and hyperinsulinemia in a complex manner. In contrast to the previous finding that monocytes from patients with diabetes were hypersensitive to the TLR ligand, LPS (23,24), we observed that the TNF-α and IL-1β expression from monocytes derived from patients with type 2 diabetes diminished after exposure to PGN, Poly I:C, and LPS—ligands of the TLR2, TLR3, and TLR4 receptors, respectively. These data suggest that diabetes perturbs signaling downstream of the TLRs. In this study, we collected CD14$^+$ monocytes from PBMCs via enrichment using magnetic beads; this protocol was used to remove T-cells, NK cells, B-cells, dendritic cells, and basophils from the PBMC mixture. This is in contrast to the methodology used to isolate these cells in many other studies, in which monocytes were obtained as adherent cells in the culture dish or by a rosetting technique (25,26). CD14$^+$ cells have been shown to be composed of multiple subtypes of activated states; the classical monocyte-isolation methods used in the other studies might unknowingly remove the fraction of monocytes that are susceptible to apoptosis (27). More than half of the CD14$^+$ diabetic monocytes isolated in this study were dead after 12-h incubation, even in media containing physiological concentration of glucose (data not shown).

Our current data showing attenuation of TLR responsiveness to ligands in diabetic monocytes suggest that initial immune responses that are normally triggered by viruses, bacteria, and parasites could be impaired in diabetes, which is consistent with epidemiologic data showing a high incidence of infection in patients with diabetes (3–5).

Gene expression and electron microscopic analysis of monocytes derived from patients with diabetes showed active signatures of ER stress; this is important because ER is an organelle essential for the proper folding and glycosylation of proteins after protein synthesis (28). When cells are under ER stress, protein kinase R–like ER kinase, inositol-requiring enzyme 1, and activating transcription factor 6 are activated and function in the adaptation to stress, proper folding of proteins, and removal of harmful unfolded proteins, respectively (29,30). However, prolonged ER stress leads to apoptotic cell death, which is mediated by CHOP (31). CHOP is a crucial and specific molecule for ER stress–induced apoptosis and alters the transcription of the *BCL-2* gene family members (32). The current study showed that diabetic monocytes had increased levels of ER stress–related apoptotic molecules. Moreover, nondiabetic monocytes treated with tunicamycin, an ER stress inducer, underwent apoptosis in a manner similar to monocytes derived from patients with diabetes. From these data, we conclude that ER stress contributes to the susceptibility of diabetic monocytes to apoptosis.

We also observed that tunicamycin-induced ER stress diminished TLR2 and TLR4 signaling without altering expression of TLRs. Tunicamycin induces ER stress by disturbing N-linked glycosylation (33), and previous reports suggest that perturbations in this glycosylation attenuate TLR2 and TLR4 signaling in vitro (34,35). Hence, these data collectively indicate that ER stress may underlie decreases in TLR2 and TLR4 signaling and affect immune function in patients with diabetes.

TLR3 signaling is different from the other TLR signaling pathway; for example, it is independent of MyD88. TLR2 and TLR4 are expressed on the cell surface, whereas TLR3 is expressed in intracellular compartments such as endosomes (13), and its ligands require internalization before signaling occurs. This suggests that disturbances in TLR3 signaling in diabetic monocytes may be due to reasons other than ER stress. Further investigations are needed to elucidate the detailed mechanisms of attenuated TLR signaling in monocytes from patients with diabetes.

ER stress has been shown to be a mainstay of the diabetic condition. Its pathologic importance in diabetes is especially important in pancreatic β-cells, in which glucose toxicity results in ER stress and insufficient insulin secretion (36–38). The current study suggests that monocytes are yet another population of cells vulnerable to hyperglycemia-induced ER stress and dysfunction. Nevertheless, the mechanisms that render pancreatic β-cells and monocytes vulnerable to ER stress in patients with diabetes remain uncertain.

Diabetes is considered a chronic inflammatory disease. Activated macrophages that produce proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 are thought to contribute to insulin resistance in muscle and adipose tissues (39,40). Furthermore, the atherosclerotic complications in patients with diabetes have a basis in inflammation; local inflammatory foci in atherosclerotic lesions are commonly composed of foam cells derived from activated macrophages (41,42). Further studies are needed to determine whether different subpopulations of monocyte-derived cells, for example, systemically circulating and locally residing inflammatory cells, are susceptible to hyperglycemia-induced ER stress and dysfunction.

In conclusion, our findings show that $CD14^+$ monocytes are susceptible to ER stress–induced alterations in inflammatory signaling and apoptosis, which may play a role in the decreased immune function observed in patients with diabetes. Further investigations are needed to discern the mechanisms of diabetes-induced ER stress and perturbations in inflammatory signaling in $CD14^+$ monocytes.

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No potential conflicts of interest relevant to this article were reported.

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REFERENCES

1. Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. Lancet 2005;365:1333–1346
2. Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. Nature 2001;414:782–787
3. Joshi N, Caputo GM, Weitekamp MR, Karchmer AW. Infections in patients with diabetes mellitus. N Engl J Med 1999;341:1906–1912
4. Shah BR, Hux JE. Quantifying the risk of infectious diseases for people with diabetes. Diabetes Care 2003;26:510–513
5. Finney SJ, Zekveld C, Elia A, Evans TW. Glucose control and mortality in critically ill patients. JAMA 2003;290:2041–2047
6. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity 2004;21:137–148
7. Karin M, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. Cell 2006;124:823–835
8. Delamaire M, Maugendre D, Moreno M, Le Goff MC, Allannic H, Genetet B. Impaired leucocyte functions in diabetic patients. Diabet Med 1997;14:29–34
9. Geerlings SE, Hoepelman AI. Immune dysfunction in patients with diabetes mellitus (DM). FEMS Immunol Med Microbiol 1999;26:259–265
10. Katz S, Klein B, Elian I, Fishman P, Djaldetti M. Phagocytotic activity of monocytes from diabetic patients. Diabetes Care 1983;6:479–482
11. Geisler C, Almdal T, Bennedsen J, Rhodes JM, Klendorf K. Monocyte functions in diabetes mellitus. Acta Pathol Microbiol Immunol Scand C 1982;90:33–37
12. Takamura T, Honda M, Sakai Y, Ando H, Shimizu A, Ota T, Sakurai M, Misu H, Kurita S, Matsuzawa-Nagata N, Uchikata M, Nakamura S, Matoba R, Tanino M, Matsubara K, Kaneko S. Gene expression profiles in peripheral blood mononuclear cells reflect the pathophysiology of type 2 diabetes. Biochem Biophys Res Commun 2007;361:379–384
13. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783–801
14. Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. Microbes Infect 2004;6:1382–1387
15. Tateno M, Honda M, Kawamura T, Honda H, Kaneko S. Expression profiling of peripheral-blood mononuclear cells from patients with chronic hepatitis C undergoing interferon therapy. J Infect Dis 2007;195:255–267
16. Stuart LM, Ezekowitz RA. Phagocytosis and comparative innate immunity: learning on the fly. Nat Rev Immunol 2008;8:131–141
17. Thoma-Uszynski S, Stenger S, Takeuchi O, Ochoa MT, Engele M, Sieling PA, Barnes PF, Rollinghoff M, Bolcskei PL, Wagner M, Akira S, Norgard MV, Belisle JT, Godowski PJ, Bloom BR, Modlin RL. Induction of direct antimicrobial activity through mammalian toll-like receptors. Science 2001;291:1544–1547
18. Barton GM, Medzhitov R. Toll-like receptor signaling pathways. Science 2003;300:1524–1525
19. Sabroe I, Parker LC, Dower SK, Whyte MK. The role of TLR activation in inflammation. J Pathol 2008;214:126–135
20. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004;10:987–995
21. Ghanim H, Mohanty P, Deopurkar R, Sia CL, Korzeniewski K, Abuaysheh S, Chaudhuri A, Dandona P. Acute modulation of Toll-like receptors by insulin. Diabetes Care 2008;31:1827–1831

22. Dasu MR, Devaraj S, Zhao L, Hwang DH, Jialal I. High glucose induces toll-like receptor expression in human monocytes: mechanism of activation. Diabetes 2008;57:3090–3098
23. Desfaits AC, Serri O, Renier G. Normalization of plasma lipid peroxides, monocyte adhesion, and tumor necrosis factor-alpha production in NIDDM patients after gliclazide treatment. Diabetes Care 1998;21:487–493
24. Ohno Y, Aoki N, Nishimura A. In vitro production of interleukin-1, interleukin-6, and tumor necrosis factor-alpha in insulin-dependent diabetes mellitus. J Clin Endocrinol Metab 1993;77:1072–1077
25. Renier G, Mamputu JC, Serri O. Benefits of gliclazide in the atherosclerotic process: decrease in monocyte adhesion to endothelial cells. Metabolism 2003;52:13–18
26. Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol 2008;26:421–452
27. Wahl LM, Wahl SM, Smythies LE, Smith PD. Isolation of human monocyte populations. Curr Protoc Immunol 2006;Chapter 7:Unit 7.6A
28. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 2007;8:519–529
29. Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. J Clin Invest 2005;115:2656–2664
30. Bukau B, Weissman J, Horwich A. Molecular chaperones and protein quality control. Cell 2006;125:443–451
31. Wang XZ, Ron D. Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. Science 1996;272:1347–1349
32. McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 2001;21:1249–1259
33. Kataoka H, Yasuda M, Iyori M, Kiura K, Narita M, Nakata T, Shibata K. Roles of N-linked glycans in the recognition of microbial lipopeptides and lipoproteins by TLR2. Cell Microbiol 2006;8:1199–1209
34. Ohnishi T, Muroi M, Tanamoto K. N-linked glycosylations at Asn(26) and Asn(114) of human MD-2 are required for toll-like receptor 4-mediated activation of NF-kappaB by lipopolysaccharide. J Immunol 2001;167:3354–3359
35. Weber AN, Morse MA, Gay NJ. Four N-linked glycosylation sites in human toll-like receptor 2 cooperate to direct efficient biosynthesis and secretion. J Biol Chem 2004;279:34589–34594
36. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Görgün C, Glimcher LH, Hotamisligil GS. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 2004;306:457–461
37. Oyadomari S, Takeda K, Takiguchi M, Gotoh T, Matsumoto M, Wada I, Akira S, Araki E, Mori M. Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. Proc Natl Acad Sci U S A 2001;98:10845–10850
38. Schenk S, Saberi M, Olefsky JM. Insulin sensitivity: modulation by nutrients and inflammation. J Clin Invest 2008;118:2992–3002
39. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006;444:840–846
40. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest 2005;115:1111–1119
41. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414:813–820
42. Liang CP, Han S, Senokuchi T, Tall AR. The macrophage at the crossroads of insulin resistance and atherosclerosis. Circ Res 2007;100:1546–1555

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特集：プロテオミクス

腎臓病学におけるプロテオミクス

山本　格

はじめに

日本の透析患者数はこれまで年間約1万人ずつ増え続け，その総数は2008年12月末現在で約28万人にのぼっている。増加の主な原因は慢性腎臓病(CKD)患者，特に糖尿病性腎症患者が増加し，それが進行して透析療法を余儀なくされているためである[1]。糖尿病性腎症のほかには慢性(糸球体)腎炎や腎硬化症が透析療法を必要とする病態に進展する主なCKDである。これらのCKDのうち糖尿病性腎症と腎硬化症はそれぞれ糖尿病と高血圧症による腎臓病で，生活習慣病と密接に関連している。そのため，腎臓障害を早期に発見し，生活習慣を改善することでその発症や進行を阻止することが世界的に目指されている。また，降圧薬として開発されたACEI(angiotensin converting enzyme inhibitor)やARB(angiotensin II receptor blocker)が糸球体内圧を低下させることで糸球体の負荷を軽減し，糸球体障害を抑制し，さらにはその降圧作用とは別に，それらの“腎臓保護作用”による治療効果が期待され，CKDの治療薬として第一選択の薬剤となっている。

このように，糖尿病性腎症や腎硬化症は生活習慣の改善や降圧薬によりその進行を抑制できると期待される。一方，慢性(糸球体)腎炎はその病因が不明のため有力な根治的療法はなく，生活習慣の改善や高血圧の抑制だけではその進行を十分には抑制できていない。また，糖尿病性腎症や腎硬化症でも，糖尿病や高血圧がどのような分子機構で糸球体を傷害するのかは明らかではない。そのため，CKDの病因や病態の分子機構の解明はその治療法の開発に非常に重要である。本稿ではそれらの解明に期待されているプロテオミクスについて概説する[2]。

Proteomics in nephrology
新潟大学大学院医歯学総合研究科 腎研究施設構造病理学部門

慢性腎臓病の病因と進行機序の研究

CKDの根治的あるいは有力な治療薬がまだ開発されていないのは，主に次の3点が問題であったためと思われる。

第一に，これまで腎臓病の研究は先人の偉大な研究にとらわれすぎていたのかもしれないという点である。すなわち，多くのヒトの病気の研究がそうであったように，動物に疾患モデルを求め，それを解析し，ヒトに当てはめようとする研究が多くなされてきた。しかし近年，病因や病態が明らかになったヒトの病気の多くは，ヒトを対象に研究し，それらが解明されたものが多い。

第二に，糸球体という部位で起きている病変は，糸球体の構造や機能から離れて理解するのは危険と考えられる点である。例えば，同じ分子が糸球体とそれ以外の組織に作用しても，異なった応答が起こる可能性がある。すなわち，糸球体以外の部位で発見された分子や傷害機序を糸球体に当てはめ検討しても，それらは必ずしも糸球体と他の部位とが同じとは限らない。また，糸球体構造の複雑さを単純化するために培養糸球体細胞を用いた研究もなされてきたが，培養細胞は生体内と全く異なった環境で生存し分裂を続けているので，それらを用いた研究成果も直ちに生体内の現象を反映しているとは言えない。

第三は，近年，末期慢性腎不全に進行する可能性のある疾患をCKDとして総括してしまったために，全く異なった病因や病態で発症した病気の病因や初期の傷害機構の研究ではなく，末期の腎不全に進行する共通的機序の研究に注目が集まってしまった可能性がある点である。

このようなことから，ヒトの疾患糸球体の初期の組織に存在する分子の全容を解析し，そのなかから原因や中心的な病態形成の分子機構を解明することや，さらにはタンパク尿より早期にCKDを発見できる尿中バイオマーカーの発見などが必要と考えられる。近年急速に発展しているタンパク質を網羅的に把握，解析するプロテオミクスは，そ

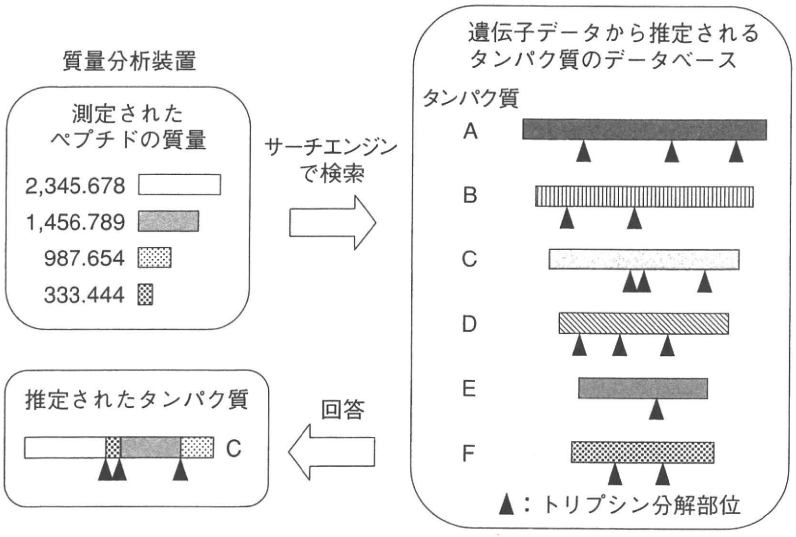

図 1 フィンガープリント法
あるタンパク質をトリプシンで分解して得られるペプチドの質量を測定することで，そのようなペプチドを生ずるタンパク質を推定する。

の解明と発見のための強力な手法となると考えられる。

プロテオミクスとは

プロテオミクス(proteomics)とは，ある生物学的な系において存在しているタンパク質の総体(プロテオーム，proteome)を総合的に研究する科学である。生物学的な系とは，生物全体，臓器，組織，細胞，細胞内器官，タンパク質の機能的複合体などを指し，プロテオミクスは，生物学的な系全体のタンパク質を把握し，タンパク質同士の相互作用などを推定することで，その系を総合的に理解することを目指している。また，生物学的な系のプロテオームを生理的状態と病的状態で比較することにより，病的状態の分子機構を理解することが可能になると期待されている。

現在，プロテオミクスのほかに，遺伝子全体を対象とするゲノミクス，発現遺伝子全体を対象とするトランスクリプトミクス，代謝産物全体を対象とするメタボロミクスなどがオミックスと総称され，その研究が盛んに行われている。いずれも個々の分子から出発して，その分子と相互作用をする分子を探索していく従来の研究手法と異なり，対象分子を含む生物学的系全体の分子を捉え，それらの分子の相互作用などからその系を理解しようとするものである。その利点は，ある生物学的な系全体を実態(分子)として把握できる点であり，従来の研究のように研究者の“センス”に頼った仮説を実証する研究とは異なる新しい研究手法でもある。

プロテオミクスは広範な科学分野を含んでおり，さまざまな手法が用いられている。はじめに，臓器，組織，細胞，あるいは細胞内器官など対象となる生物学的系を分離，精製し，そこにあるタンパク質を網羅的(プロテオーム)に同定することから始める。それには，生物学的系からタンパク質を精製するが，その後の解析はさまざまである。ここでは，通常行われている主な 2 種類の解析法を簡単に紹介する。

一つはある生物学的系のタンパク質群を二次元ゲル電気泳動法などで分離し，分離したタンパク質を染色し，タンパク質を含むゲル(スポット)を切り出し，その中のタンパク質をリジンあるいはアルギニンの C 末端を加水分解するトリプシンなどで消化し，複数のペプチドに分解する。それらのペプチド質量を MALDI-TOF(Matrix-Assist Laser Dissociation Ionization-Time of Flight)型などの質量分析計で正確に測定する。次に，その生物の遺伝子データベースから推定される全タンパク質をトリプシンで分解したとき，質量分析計で実測された複数のペプチドと同じ質量のペプチドを生じるタンパク質をコンピュータで検索し，そのタンパク質を推定するのである(フィンガープリント法，**図 1**)。

もう一方は，ショットガン法とも呼ばれ，生物学的系の全タンパク質を分離せずにトリプシンでペプチドに分解し，そのペプチドを液体クロマトグラフィなどで分離し，分離されたペプチドの質量を ESI-MS/MS (Electron-Spray Ionization Mass Spectrometer)などの質量分析装置で測定する(MS)。さらに，質量分析計のなかで個々のペプチドのアミノ酸の結合をランダムに分解し，その分解産物の質量を正確に測定すること(MS/MS)により，そのペプチドのアミノ酸配列が推定できる(**図 2**)。その結果，ペプチドの質量やその

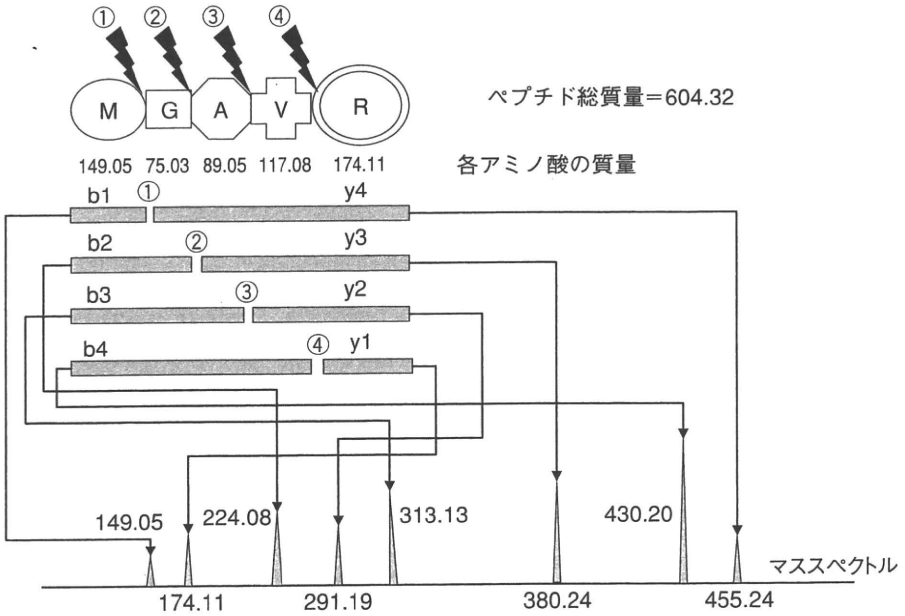

図 2　ショットガン法

トリプシンで分解されたペプチド(質量＝604.32)が質量分析計の中でアミノ酸の結合がランダムに壊され(①～④)，分解されたペプチド(b1～b4，y1～y4)を測定することでアミノ酸配列(MGAVR)が決定される。

アミノ酸配列情報からタンパク質が特定されるのである。

このような方法でプロテオミクスでは数日で数百～数千のタンパク質を同定することも可能で，1 つのタンパク質を精製して，そのアミノ酸配列を決定して 1 つのタンパク質を同定する従来の方法に比べて格段にスループットが高い。

1 つのタンパク質由来のペプチドの数をカウントするなどして，そのタンパク質の凡その量を推定することも可能である。そのため，複数の生物学的な系の間でプロテオームを比較することにより，ある系で増減するタンパク質を探索できる。増減するタンパク質はその系で特徴的な生物反応に関係するタンパク質と推定することができる。また，疾患患者と健常者の尿のプロテオームを比較することで，疾患の早期診断や予後判定の新しいバイオマーカーが見つかるのではないかと期待されている。

バイオインフォマティクス

このようにタンパク質が同定され，ある系のタンパク質のカタログができるが，それだけでその系の生物学的意味を理解するのは容易ではない。そのために，タンパク質の立体構造や翻訳後修飾の解析，これまでの研究成果から推定される他のタンパク質との相互作用(パスウエイ)，ある生物学的系に特徴的なタンパク質群を選択するソフトウエアなどコンピューターを用いた解析手法が発展している。このような分野をバイオインフォマティクスと言い，その発展によって，初めてある系のタンパク質のカタログから意味のある情報を取り出せる。パスウエイの代表的データベースに KEGG (Kyoto Encyclopedia of Genes and Genomes，http://www.kegg.jp/ja/)[3]がある。

プロテオミクスの重要性と困難性

一般にゲノムは同一生物種内では変化しないが，そこから作られるトランスクリプトーム(転写産物)，プロテオーム(翻訳産物)は細胞の種類や状態によって大きく変化しうる。特にタンパク質は機能を担っている分子であり，複雑なタンパク質の集合であるプロテオームを知りその系の全容を推定することは，その系を理解するために重要である。

これまで，タンパク質が存在するかどうかは主に抗体により検出されてきた。しかし，現在推定されるヒト遺伝子約 2 万個のうち，その翻訳産物であるタンパク質が捉えられている遺伝子は約 1 万個で，残りの約 1 万個の遺伝子についてはそれに由来するタンパク質の実態はいまだに検出されていないと言われている。その原因の一つは，そのタンパク質に対する抗体が用意されていないことによる。しかし，質量分析計による同定では抗体がなくてもタンパク質を同定できるため，同定の網羅性はより高くなる。

一方，タンパク質を網羅的に解析することは，遺伝子よ

りもはるかに困難である。その理由の一つは，遺伝子は実験室で増幅して解析することができるが，タンパク質はそれができない。また，遺伝子は4種類の塩基配列の決定で同定できるが，タンパク質を構成するアミノ酸は20種類あるため，核酸の配列決定よりも難しい。さらに，1つの遺伝子から数種類のバリアントができうるが，そこから翻訳されるタンパク質は，分解，結合，翻訳後修飾(リン酸化，糖鎖修飾，アセチル化など)を受け，そのバリエーションは数十倍に増え，対象が多く複雑である。

そのほか，質量分析計によるタンパク質の同定はあくまで推定であるので，抗体などによる検証が必要とされていたが，近年の質量分析計の精度の向上により，同定のための抗体が不必要な時代が来るかもしれない[4]。

ヒトプロテオーム機構とヒト腎臓・尿プロテオームプロジェクト

ヒトプロテオームの解析を標準化，規格化して行うために2001年にヒトプロテオーム機構(Human Proteome Organization：HUPO, http://www.hupo.org/)が組織され，その傘下にいくつかのプロジェクト(イニシアチブ)が行われている(**表**)。われわれも2001年頃からCKDの組織や尿をプロテオーム解析し，その病因，病態，バイオマーカーの発見，検証を目指して国際連携研究を行うヒト腎臓・尿プロテオームプロジェクト(Human Kidney and Urine Proteome Project：HKUPP)を開始した。

このプロジェクトは2008年にはHUPOイニシアチブとして認知され[5]，現在はHUPOイニシアチブのHuman Antibody Initiative(Protein Atlas, http://www.proteinatlas.org/)やHuman Plasma Proteomeと連携して，ヒト腎臓・尿プロテオームのデータベースの構築やプロテオーム間の比較解析の共同研究が行われている[6]。

腎臓病のバイオマーカーの探索を目指した尿のプロテオミクスも盛んに行われている。HKUPPでは尿プロテオーム解析のための尿の採取，保存に関する検討を行い，その標準方法をガイドラインとして提唱している。日本でも尿中バイオマーカーを探索する研究が盛んとなり，2008年に日本腎臓学会に尿中バイオマーカーパネル化に関する小委員会が設けられ，2009年から全国多施設で同じ規格で尿を採取，保存し，さらに研究者に提供する尿バンクがスタートした(http://www.urinebank.org/)。これまで尿中バイオマーカーの探索，検証研究はほとんど研究室単位で行ってきたものが多く，大規模検証研究による臨床的有用性の実証研究は十分には行われていなかった。尿バンクは多検体の尿を提供することで，それを可能にするため，日本からの腎臓病患者の早期診断や予後予測に有用なバイオマーカーの提唱が期待される。また，同一の尿検体でいくつかのバイオマーカー候補を測定することにより，それぞれの特性を解析し，その結果からいくつかのバイオマーカーを組み合わせ(パネル化)て測定することで，早期発見や予後予測に有用な新しいシステムの構築を産学連携で目指すことも目標にしている。この尿バンクには多くの施設の協力が必要で，多くの先生方のご協力をお願いしたい。

表 HUPOイニシアチブ

1. Human Liver Proteome Project (HLPP), http://www.hlpp.org/Index/index.php/
2. Human Brain Proteome Project (HBPP), http://www.hbpp.org/
3. Proteomic Standard Initiative (PSI), http://www.psidev.info/
4. Human Antibody Initiative (HAI), http://www.proteinatlas.org/
5. Plasma Proteome Project (PPP), http://www.bioinformatics.med.umich.edu/hupo/ppp/
6. Human Disease Glycomics/Proteome Initiative (HGPI), http://www.hgpi.jp/menuD.html/
7. Mouse Models of Human Disease (MMHD)
8. Disease Biomarkers Initiative (DBI)
9. HUPO Cardiovascular Initiative (HCVI), http://www.hupocvi.org/
10. Proteome Biology of Stem Cells Initiative
11. Human Kidney and Urine Proteome Project (HKUPP), http://www.hkupp.org/

文 献

1. Imai E, Horio M, Iseki K, et al. Prevalence of chronic kidney disease (CKD) in Japanese population predicted by MDRD equation modified by a Japanese coefficient. Clin Exp Nephrol 2007；11：156-163.
2. 高橋信弘，礒辺俊明．細胞機能解析に向けたプロテオミクス基盤技術．細胞工学 2006；25：590-595.
3. Kanehisa MI, Goto S. KEGG. Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 2000；28：27-30.
4. Mann M. Can proteomics retire the western blot? J Proteome Res 2008；7：3065.
5. Cottingham K. HKUPP becomes a full-fledged initiative of its own. J Proteome Res 2008；7：484.
6. Yamamoto T, Langham RG, Ronco P, et al. Towards standard protocols and guidelines for urine proteomics. Proteomics 2008；8：2156-2159.

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Association of Asymmetric Dimethylarginine with Severity of Kidney Injury and Decline in Kidney Function in IgA Nephropathy

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Key Words
Asymmetric dimethylarginine · Chronic kidney disease · Endothelium · Glomerular sclerosis · Interstitial fibrosis

Abstract
Background: Asymmetric dimethylarginine (ADMA) plays important roles in the pathogenesis of chronic kidney disease (CKD). We have recently found that ADMA is involved in glomerular sclerosis and tubulointerstitial fibrosis in an animal model of CKD. However, relationship between plasma ADMA levels and severity of renal damage in CKD patients remains unknown. ***Methods:*** Relatively young 109 biopsy-proven IgA nephropathy (IgAN) patients (age: 32.7 ± 13.2; estimated glomerular filtration rate, eGFR: 86.5 ± 28.8 ml/min/1.73 m^2) were enrolled. We retrospectively investigated whether plasma levels of ADMA were associated with severity of the renal tissue damage and could be a predictor of the disease progression in our subjects. ***Results:*** ADMA levels were higher in IgAN patients than age-, sex- and mean eGFR-matched healthy volunteers (0.53 ± 0.14 vs. 0.43 ± 0.08 μM, $p < 0.01$). ADMA levels were associated with the severity of glomerular and tubulointerstitial injury. Multiple stepwise regression analysis revealed that ADMA, but not proteinuria was an independent determinant for the disease progression assessed by annual reduction rates of eGFR. In univariate analyses, ADMA levels were correlated with proteinuria, total cholesterol, triglycerides, and uric acid. Proteinuria was a sole independent correlate of ADMA in multiple stepwise regression analysis. ***Conclusion:*** The present study demonstrated that ADMA was correlated with the severity of the renal tissue damage and could be a predictor of disease progression in IgAN patients.

Introduction

Nitric oxide (NO) is synthesized by stereospecific oxidation of the terminal guanidine nitrogen of L-arginine by the action of the NO synthase (NOS) [1, 2]. The synthesis of NO is blocked by inhibition of the NOS active site with guanidino-substituted analogues of L-arginine such as asymmetric dimethylarginine (ADMA) [1, 2]. We, along with others, have shown that plasma concentrations of ADMA are elevated in patients at high risk for cardiovascular disease (CVD) such as patients with hypertension [3], diabetes [4], dyslipidemia [5], and end-stage renal disease [6], thus being one of the biomarkers for predicting future cardiovascular events in these patients [4, 6–9]. In addition, ADMA levels have recently been found to increase even in stage 1 chronic kidney dis-

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ease (CKD) patients [10, 11]. These observations suggest that ADMA is one of the key molecules that could link CKD to increased risk for CVD [2, 12, 13].

Since NOS inhibitors are reported to elicit systemic and glomerular hypertension and cause glomerulosclerosis, tubulointerstitial injury and proteinuria in several animal models [14, 15], it is conceivable that ADMA may play a role in the progression of CKD by inhibiting endogenous NO generation. Indeed, two epidemiological studies have shown that plasma ADMA levels are significantly associated with disease progression in CKD patients, which is assessed by doubling of serum creatinine (Cr), halving estimated glomerular filtration rate (eGFR) and/or renal replacement therapy [16, 17]. Further, we have recently found that overexpression of dimethylarginine dimethylaminohydrolase (DDAH), a rate-limiting enzyme that degrades ADMA, ameliorates peritubular and glomerular capillary loss and subsequently blocks tubulointerstitial fibrosis, glomerular sclerosis and proteinuria in 5/6 subtotal-nephrectomized rats, an experimental model of CKD by lowering ADMA levels [18, 19]. These findings suggest that plasma ADMA levels could be associated with disease severity and also become a marker for disease progression in CKD patients. However, as far as we know, there is no report that has evaluated a relationship between plasma ADMA levels and the severity of renal tissue damage in CKD patients with IgA nephropathy (IgAN). Therefore, in this study, we retrospectively investigated whether plasma levels of ADMA could be associated with the degree of renal tissue damage and predict a faster rate of renal function loss in patients with IgAN. We further studied here which anthropometric and metabolic variables were independent correlates of plasma ADMA levels in IgAN patients.

Patients and Methods

Subjects

One hundred and nine biopsy-proven IgAN patients (age: 32.7 ± 13.2 years old, 44 males and 65 females, 24-hour Cr clearance (24 h-Ccr): 95.2 ± 31.6 ml/min, eGFR: 86.5 ± 28.8 ml/min/1.73 m^2) and age-, sex- and mean eGFR-matched 28 healthy volunteers [age: 30.3 ± 5.3 (range 22–40) years, females/males: 14/14, eGFR: 86.7 ± 11.8 (range 65.3–110.5) ml/min/1.73 m^2] were enrolled in this study. The number of patients who received renin angiotensin system (RAS) inhibitors and statins at baseline were 18 and 2, respectively. None of the subjects received other antihypertensive drugs. We excluded any patients with diabetes, CVD, chronic liver diseases, neoplastic disorders, and any acute infections. Patients whose age was younger than 14 years old or older than 65 years old, whose serum Cr level was >2.0 mg/dl were also excluded. The study protocol was approved by the ethical committee of Kurume University, and informed consent was obtained from all study participants. The study complied with the principles of the Declaration of Helsinki.

Data Collection

Medical history and use of tobacco were ascertained by a questionnaire. Smoking was classified as current habitual use or not. Height and weight were measured, and BMI (kg/m^2) was calculated as an index of the presence or absence of obesity. Blood pressure (BP) was measured in the sitting position using an upright standard sphygmomanometer. Vigorous physical activity and smoking were avoided for at least 30 min before BP measurement. Fasting blood was drawn from the antecubital vein for determinations of total cholesterol, high-density lipoprotein cholesterol, triglycerides, uric acid, serum Cr, blood urea nitrogen, and ADMA. Plasma levels of ADMA were measured by high-performance liquid chromatography as described previously [18, 19]. Other blood chemistries were determined enzymatically at a commercially available laboratory (The Kyodo Igaku Laboratory, Fukuoka, Japan). eGFR was calculated according to the following formula: $eGFR = 194 \times \text{serum } Cr^{-1.094} \times age^{-0.287}$ (×0.739; if female). Twenty-four-hour urine collections were made at the 2nd day of admission to our hospital. Total protein excretion levels were determined with a pyrogallol red method (Wako, Osaka, Japan). eGFR was measured annually during follow-up periods.

Severity of Renal Tissue Damage

Biopsy sections were stained with periodic acid-Schiff and methenamine silver. Glomerular and tubular interstitial abnormalities were scored by 2 renal pathologists who were blinded to clinical information, according to the report of Katafuchi et al. [20] with some modification. In brief, glomerular score was calculated as the sum of indices of the following four glomerular lesions: (a) glomerular global sclerosis; (b) glomerular segmental sclerosis; (c) tuft adhesion, and (d) crescent. The index of global sclerosis was determined according to percentages of glomeruli showing the lesion of total number of glomeruli in a biopsy sample as follows: 0, none; 1, less than 10% of glomeruli; 2, 10–30% of glomeruli; 3, >30% of glomeruli. The indices of glomerular segmental sclerosis lesion and tuft adhesion were determined according to percentages of glomeruli showing each lesion as follows: 0, none; 1, <25% of glomeruli; 2, 25–50% of glomeruli; 3, >50% of glomeruli. The index of crescent was determined according to percentages of glomeruli showing the lesion as follows: 0, none; 1, <10% of glomeruli; 2, 10–30% of glomeruli; 3, >30% of glomeruli. Therefore, glomerular score ranged from 0 to 12. Tubulointerstitial score was determined according to the involved area (tubular atrophy, interstitial inflammatory cell infiltration and fibrosis) in renal cortical tissue in a biopsy sample as follows: 0, none; 1 = mild, involving <25%; 2 = medium, 25–50%; 3 = severe, >50%.

Statistics

Data were expressed as mean ± standard deviation. Correlations among ADMA, glomerular score, tubulointerstitial score, and clinical variables were determined by a linear regression analysis. To determine independent correlates of plasma ADMA levels, multiple stepwise regression analysis was performed. Statistical significance was defined as $p < 0.05$.

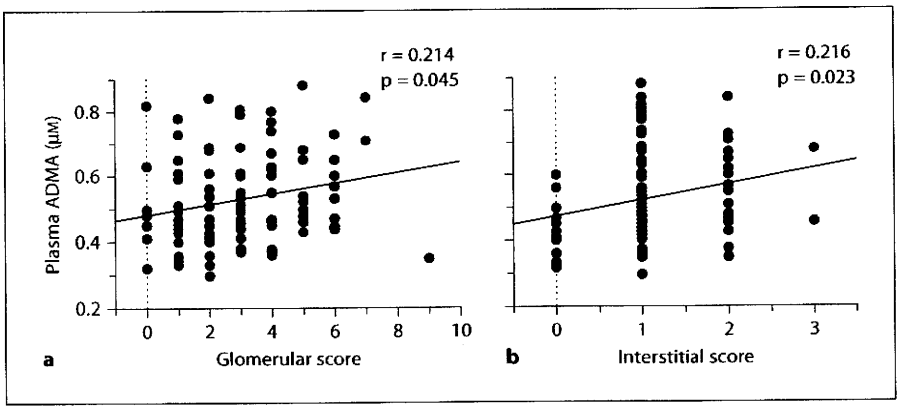

Fig. 1. Relationship between plasma ADMA levels and glomerular score (**a**) and tubulointerstitial score (**b**).

Results

Clinical Parameters

Patient characteristics at baseline are shown in table 1. Mean serum Cr, 24 h-Ccr, and eGFR of our patients were 0.80 mg/dl, 95.2 ml/min, and 86.5 ml/min/1.73 m^2, respectively. Plasma ADMA levels were significantly higher in IgAN patients than age-, sex- and mean eGFR-matched healthy volunteers (control: 0.43 ± 0.08 vs. IgAN: 0.53 ± 0.14 μM, $p < 0.01$).

Correlations between Plasma ADMA Levels and Renal Tissue Damage

We investigated whether plasma levels of ADMA were associated with the severity of renal tissue damage in IgAN patients. As shown in figure 1, plasma ADMA levels were significantly correlated with both glomerular and tubulointerstitial scores, which could reflect glomerular and tubulointerstitial injury in IgAN patients, respectively.

Correlations between 24 h-Ccr and Renal Tissue Damage

In our subjects, mean glomerular and interstitial scores were 2.92 ± 1.8 and 1.1 ± 0.6, respectively. These scores were significantly associated with 24 h-Ccr (glomerular score: $\beta = 0.30$, $p = 0.003$; tubulointerstitial score: $\beta = 0.25$, $p = 0.003$). 24 h-Ccr values in patients with glomerular score >2.92 and interstitial score >1.1 were 88.8 ± 32.5 and 79.7 ± 32.5 ml/min, respectively.

Correlations between Plasma ADMA Levels and Annual Reduction Rates of eGFR

We next examined whether plasma levels of ADMA could predict a faster rate of renal function loss in patients with IgAN. We were able to follow-up eGFR values of 40 patients for 4 years. As shown in figure 2, plasma ADMA levels were inversely associated with the 4-year changes in eGFR values ($\beta = -0.327$, $p < 0.05$). In other words, annual reduction rates of eGFR were significantly correlated with plasma levels of ADMA at admission. Over the 4 years, the mean eGFR values modestly decreased by 0.27 ± 4.61 ml/min/1.73 m^2 in our IgAN patients. When the patients were divided into two groups according to their ADMA levels, eGFR of patients with high ADMA levels (more than median, 0.5 μM) was decreased by 0.95 ± 2.05 ml/min/1.73 m^2 annually, while eGFR of patients

Table 1. Clinical characteristics of patients; mean ± SD (range)

Patients	109
Male/female	44/65
Age, years	32.7 ± 13.2 (14–63)
Body weight, kg	57.9 ± 11.8 (39.6–104.2)
Height, cm	162.3 ± 8.5 (138.5–182.6)
Body mass index	21.6 ± 3.8 (16.3–33.3)
Systolic BP, mm Hg	124 ± 18.5 (90–191)
Diastolic BP, mm Hg	75.7 ± 13.2 (40–110)
Total cholesterol, mg/dl	193.8 ± 44.3 (90–334)
HDL cholesterol, mg/dl	63.9 ± 17.4 (28.9–105.1)
Triglycerides, mg/dl	114.5 ± 70.4 (32.0–369.0)
Uric acid, mg/dl	5.4 ± 1.6 (2.9–9.5)
Serum creatinine, mg/dl	0.80 ± 0.28 (0.39–1.8)
24 h-Ccr, ml/min	95.2 ± 31.6 (34.0–190)
eGFR, ml/min/1.73 m^2	86.5 ± 28.8 (28.8–161.6)
Proteinuria, g/day	0.92 ± 1.00 (0–5.4)
Serum ADMA, μM	0.53 ± 0.14 (0.30–0.88)
Medication	
RAS inhibitors	18
Statins	2
Current smoker	24

HDL = High-density lipoprotein.

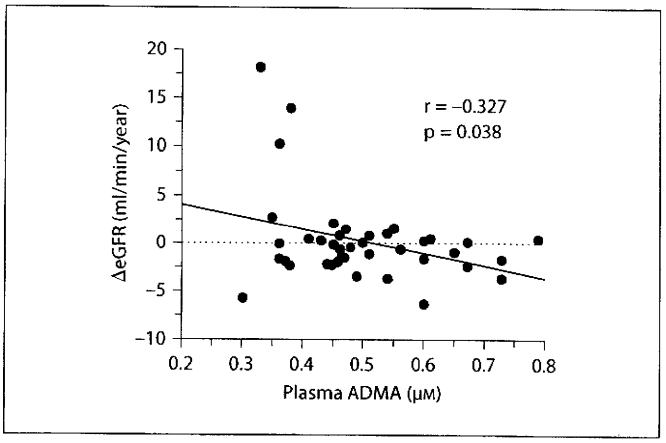

Fig. 2. Relationship between plasma ADMA levels and annual reduction rates of eGFR (ΔeGFR).

Table 2. Univariate regression analysis for correlates of ADMA

Variables	β	SE	p value
Age	0.155	0.001	0.10
BMI	0.111	0.004	0.25
Systolic BP	0.075	0.001	0.43
Diastolic BP	0.017	0.001	0.85
24 h-Ccr	0.09	0.004	0.38
Proteinuria	0.303	0.013	0.001
Total cholesterol	0.225	0.000	0.019
HDL cholesterol	−0.059	0.001	0.55
Triglycerides	0.247	0.000	0.011
Uric acid	0.246	0.008	0.009

β = Standardized regression coefficients; SE = standard error.

with low ADMA levels (≤0.5 μM) increased by 1.18 ± 5.7 ml/min/1.73 m^2 ($p < 0.05$). Multiple stepwise regression analysis revealed that ADMA, but not proteinuria was an independent determinant of annual reduction rates of eGFR in our subjects (F value: 4.5).

Univariate and Multivariate Stepwise Regression Analyses for Correlates of Plasma ADMA Levels

In univariate analyses, plasma ADMA levels were significantly correlated with proteinuria (β = 0.303, p = 0.001), total cholesterol (β = 0.225, p = 0.019), triglycerides (β = 0.247, p = 0.011), and uric acid (β = 0.246, p = 0.01; table 2). Because these parameters could be closely correlated with each other, to determine independent correlates of plasma ADMA levels, multiple stepwise regression analysis was performed. This analysis showed that proteinuria (F value: 12.75, β = 0.24) was a sole independent correlate of ADMA levels in our patients. Smoking status did not affect ADMA levels in our subjects (smokers: 0.53 ± 0.15, nonsmokers: 0.53 ± 0.13 μM).

Discussion

There is accumulating evidence that ADMA induces renal damage in experimental models [18, 21] and that its plasma level is associated with renal risk [16, 17]. The salient findings of the present study are that ADMA could be correlated with renal tissue damage and also be a predictor of annual reduction rates of eGFR in a fairly young cohort of patients with IgAN.

Although we did not clarify here the underlying mechanism by which ADMA could contribute to tissue damage and progression of IgAN, it is unlikely that ADMA promotes renal dysfunction via elevation of BP, because BP was not associated with plasma ADMA levels (table 2) or the 4-year changes in eGFR values in our subjects. ADMA may have deleterious effects in IgAN patients probably by causing chronic ischemia in the kidney via inhibition of endogenous NO generation because (1) NO protects against the development of glomerular sclerosis and tubulointerstitial fibrosis in CKD animal models by inhibiting endothelial cell apoptosis, preserving endothelial function and maintaining microvascular homeostasis in the kidney [2, 14, 15], and (2) ADMA, an endogenous inhibitor of NOS, has been shown to elicit glomerular sclerosis and tubulointerstitial injury in an animal model of CKD by causing glomerular and peritubular capillary loss [18, 19].

There is accumulating evidence that proteinuria is not merely a biomarker of the progression of CKD, but also a mediator of this devastating disorder [22–24]. Indeed, albumin, one of the major components found in proteinuria, is reported to cause proinflammatory and profibrotic changes in cultured proximal tubular cells [22–24]. In addition, proteinuria has been shown to be correlated with the severity of tubulointerstitial damage in CKD patients [22, 23]. Further, Sharma et al. [25] recently reported that ADMA could injure glomerular filtration barrier and thereby enhance albumin permeability of isolated glomeruli. Taken together, although proteinuria was not associated with annual reduction rates of eGFR in our subjects, ADMA may also be involved in progression of renal dysfunction in CKD patients partly by eliciting proteinuria. Whatever the mechanisms, the present study suggests that the inhibition of ADMA may be a novel