Advanced Glycation End Product-Mediated Matrix Metalloproteinase-9 and Apoptosis via Renin-Angiotensin System in Type 2 Diabetes

Toshiyuki Ishibashi¹, Michiko Kawaguchi¹, Koichi Sugimoto¹, Hironori Uekita¹, Nobuo Sakamoto¹, Keiko Yokoyama¹, Yukio Maruyama¹,², and Yasuchika Takeishi¹

Toshiyuki Ishibashi and Michiko Kawaguchi contributed equally to this work

¹Department of Cardiology and Hematology, Fukushima Medical University, Fukushima, Japan
²Hoshi General Hospital, Koriyama, Japan

Aim: Advanced glycation end products (AGE) play a key role in diabetic vascular complications, whereas matrix metalloproteinases (MMPs) and apoptosis contribute to plaque instability. This study was conducted to investigate the association of AGE-mediated MMP-9 and apoptosis with the renin-angiotensin system (RAS). We also examined the correlation between plasma HbA1c levels and plaque parameters.

Methods: We used autopsy specimens from the aortae and coronary arteries of patients with or without diabetes (n = 11, each group) for the immunohistochemistry of AGE, MMP-9, angiotensin-converting enzyme (ACE), and the receptor for AGE (RAGE). Apoptosis was determined by TUNEL staining.

Results: The proportions of AGE accumulation, MMP-9 expression and apoptosis in intimal areas of both aortic and coronary specimens of diabetics were greater than in nondiabetics. MMP-9 expression and apoptosis were correlated with AGE accumulation. RAGE expression was significantly increased in diabetic specimens compared to nondiabetes. Interestingly, the expression of ACE in diabetic specimens was increased and also correlated with AGE accumulation, RAGE expression, MMP-9 expression, and apoptosis in all specimens from diabetics and nondiabetics. Plasma levels of HbA1c were linearly correlated with AGE accumulation, MMP-9, apoptosis, and ACE expression.

Conclusion: The present study shows that AGE/RAGE-related MMP-9 expression and apoptosis were correlated with ACE expression in diabetic plaques and that RAS may be involved in AGE-dependent diabetic vascular complications.


Key words: Diabetic vascular complications, RAGE, ACE, Plaque destabilization

Introduction

Type 2 diabetes is characterized by hyperglycemia, which is reflected by plasma HbA1c levels. Since protein glycation occurs in diabetes, advanced glycation end products (AGE) are accumulated in ath-
sis play a crucial role in the formation of vulnerable plaques. It has been shown that in patients with coronary artery disease, serum levels of secreted MMPs, such as MMP-2, MMP-8 and MMP-9, are higher in patients accompanied by type 2 diabetes than in nondiabetics, indicating a role of MMPs in the pathogenesis of coronary atherosclerosis complicated with diabetes. Moreover, increased apoptosis has been shown in coronary plaques from patients with acute coronary syndromes. Thus, the possibility that plasma HbA1c levels and AGE accumulation may be associated with MMP expression and apoptosis in coronary atherosclerosis in diabetes needs to be elucidated in terms of plaque instability.

Clinical trials have shown that angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARB) reduce the incidence of cardiovascular events in hypercholesterolemic and hypertensive patients complicated with diabetes. This suggests the association of the renin-angiotensin system (RAS) with the pathogenesis of coronary atherosclerosis in diabetes.

We hypothesize that the accumulated AGE stimulates the expression of MMPs and apoptosis mediated via RAS, leading to the formation of vulnerable plaques in coronary artery disease. Thus, in the present study we examined the multiple possible correlations among AGE accumulation, MMP-9 expression, apoptosis, and ACE expression using human autopsy specimens, as well as the correlation between these variables and plasma levels of HbA1c.

**Methods**

**Patient Population and Tissue Preparation**

Autopsy specimens of aortic and coronary artery segments were obtained from 11 patients with type 2 diabetes mellitus (DM) and 11 patients without DM between 1996 and 2004. Patient characteristics are summarized in the Table 1. DM was defined by the WHO Study Group. Hypertension was defined by the Joint National Committee VI and hypercholesterolemia as cholesterol >220 mg/dL. One and 7 patients died of acute myocardial infarction (AMI) in nondiabetic and diabetic groups, respectively, against 10 and 4 non-cardiovascular diseases, respectively. Tissue specimens were obtained from the middle of the descending thoracic aorta and the left anterior descending coronary artery (LAD), 4–5 cm distal from the bifurcation of LAD and left circumflex coronary artery. This study was approved by the Ethics Committee of Fukushima Medical University.

**Histological Examination**

Histological examination was performed as described previously. Autopsy specimens were fixed with 10% formalin, embedded in paraffin, and sectioned serially at 4-μm thickness. All first sections were stained with hematoxylin and cosin, and Azan-Mallory’s staining was performed on every second section to evaluate the extracellular matrix (ECM). Subsequent serial sections were immunohistochemically stained by the indirect immunoperoxidase method. In the present study, we used antibodies against AGE (6D12; Transgenic Inc., Kumamoto, Japan), MMP-9 (Chemicon, Temecula, CA), RAGE (Millipore, Billerica, MA), ACE (Santa Cruz Biotechnology, Santa Cruz, CA), smooth muscle α-actin (CBL 171; Cymbus Biotechnology, Hants, England) and CD68, macrophage antigen (DAKO-CD68, Kp-1; DAKO, Glostrup, Denmark), as well as nonimmune mouse IgG serum as a negative control. Sections were incubated with each antibody at 4°C overnight and subjected to a 3-step staining procedure using the streptavidin-biotin complex method. Peroxidase activity was visualized with 3,3-diaminobenzidine for 5–10 minutes at room temperature, and the sections were finally counterstained with hematoxylin.

To identify the origin of MMP-9-positive cells, immunohistochemical double staining was performed. Sections were reacted with mouse monoclonal antibody to human MMP-9, the biotinylated secondary antibody and streptavidin-alkaline phosphatase, followed by the substrate reaction for visualization. The same sections were subsequently processed to detect smooth muscle cells (SMCs) or macrophages, followed by the biotinylated secondary antibody and streptavidin-peroxidase reaction with the same conditions used for detecting MMP-9.

Quantitative analysis of immunohistochemistry was performed using personal computer-based quantitative 24-bit color image analysis (NIH Image analysis) in a blind fashion by two researchers. The atherosclerotic lesions were graded with Stary’s classification which ranges from I (initial lesion) to grade VI (plaque rupture, fissure and hemorrhage).

**Determination of Apoptotic Cells**

To determine the presence of apoptotic cells with DNA breaks in nuclei in situ, we performed terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) on tissue sections using the Dead EndTM Colorimetric TUNEL System (Promega Co., Madison, WI) as described previously. Positive control sections were treated with DNase I (10 ng/mL; Boehringer Mannheim city, coun-
We examined 200 cells in each aortic and coronary specimen for apoptosis.

Statistical Analysis

The \( \chi^2 \) test was performed to compare demographic data and the severity of atherosclerosis. Unpaired Student's \( t \) test was performed to compare ECM extent, AGE accumulation, MMP-9, TUNEL-positive cells, ACE expression and RAGE expression in autopsy specimens between nondiabetic and diabetic groups, as well as the levels of HbA1c. Linear regression analysis was performed to correlate each parameter. A value of \( p < 0.05 \) was considered significant. Data are expressed as the means \( \pm S.D. \).

Results

The demographic characteristics of the autopsy cases are shown in the Table 1. There was no significant difference in age, sex and incidence of hypertension and hyperlipidemia between nondiabetic and diabetic groups. The diabetic group had significantly higher percentages of acute myocardial infarction (AMI) as the cause of death compared with the nondiabetic group (\( p < 0.01 \)). There were no significant differences in the grading of aortic and coronary specimens according to Stary's classification between the two groups, except for type \( \text{V} \) in coronary specimens in which the nondiabetic group had higher percentages than the diabetic group (\( p < 0.01 \)).

Proportions of ECM, AGE and MMP-9

The extent of ECM of both aortic and coronary intimal lesions in the diabetic group was significantly smaller than in the nondiabetic group (aorta: non-DM, 72.4 \( \pm \) 9.3%; DM, 37.0 \( \pm \) 13.7%; coronary: non-DM,
Fig. 1. Results of quantitative analysis for areas of extracellular matrix (ECM, A), advanced glycation end products (AGE, B) and matrix metalloproteinase-9 (MMP-9, C) in both aortic and coronary intimal lesions from autopsy specimens from nondiabetic and diabetic cases ($n = 11$, each). DM: diabetes mellitus. *$p < 0.01$, †$p < 0.0001$. D, Representative photomicrographs showing ECM, AGE and MMP-9 in autopsy specimens from coronary arteries from non-DM and DM groups. E and F, Photomicrographs showing immunohistochemical double staining with monoclonal antibodies against MMP-9 and smooth muscle $\alpha$ actin (E) or macrophages (F). Blue shows MMP-9 expression (E and F) and brown, smooth muscle $\alpha$ actin (E) and macrophage antigen (F). Arrows indicate MMP-9-positive SMCs and macrophages in a coronary autopsy specimen from a DM case.

68.6 ± 9.9%; DM, 34.6 ± 10.5%; $p < 0.01$, each), as shown in Fig. 1A. Fig. 1B shows the increased accumulation of AGE in aortic and coronary specimens from the DM (aorta, 47.7 ± 6.7%; coronary, 39.6 ± 8.1%) compared with in the nondiabetic group (aorta, 11.5 ± 6.2%; coronary, 10.5 ± 5.7%) ($p < 0.01$, each). Fig. 1C shows significant increases in MMP-9 expression in both aortic and coronary specimens from the DM (aorta, 3.8 ± 2.3%; coronary, 2.6 ± 1.0%) compared with the nondiabetic specimens (aorta, 0.4 ± 0.3%; coronary, 0.2 ± 0.1%), ($p < 0.0001$, each).
nondiabetic and diabetic groups. Fig. 1E, 1F present the results of double staining to detect the origin of MMP-9 expression showing MMP-9-positive SMCs and macrophages, respectively.

**Various Correlations among ECM, AGE and MMP-9**

We then evaluated the correlations among the extent of ECM, AGE accumulation, and MMP-9 expression. Fig. 2A shows a significant overall inverse correlation between ECM and AGE accumulation in all aortic and coronary specimens from nondiabetic and diabetic groups (aorta: $r^2=0.707, p<0.0001$; coronary: $r^2=0.697, p<0.0001$). A similar result was observed in the correlation between ECM extent and MMP-9 expression in all specimens (aorta: $r^2=0.481, p<0.0001$; coronary: $r^2=0.492, p<0.0001$) (Fig. 2B). In contrast, regression analysis showed a significant positive correlation between AGE accumulation and MMP-9 expression in all aortic and coronary specimens from non-DM and DM, as shown in Fig. 2C (aorta: $r^2=0.625, p<0.0001$; coronary: $r^2=0.624, p<0.0001$).

**TUNEL Staining**

Next, we determined apoptosis in nondiabetic and diabetic specimens. Fig. 3A shows significant increases in TUNEL-positive cells in both aortic and coronary specimens in diabetes compared to nondiabetes (aorta: non-DM 5.5±6.1%; DM 34.8±24.5%, $p<0.0001$; coronary: non-DM 7.1±7.0%; DM 25.2±14.7%, $p<0.01$). Morphologically, the origin of predominant TUNEL-positive cells appeared to be SMCs rather than macrophages. Regression analysis demonstrated a significant positive correlation between TUNEL-positive cells and AGE accumulation in all aortic and coronary specimens (aorta: $r^2=0.526, p<0.0001$; coronary: $r^2=0.462, p<0.001$) (Fig. 3B). Similar results were obtained for the correlation between TUNEL-positive cells and MMP-9 expression (aorta: $r^2=0.423, p=0.002$; coronary: $r^2=0.318, p<0.01$), as shown in Fig. 3C.

**ACE Expression**

We then immunohistochemically assessed the involvement of RAS in AGE-triggered MMP-9 expression and apoptosis. Fig. 4A demonstrates the significant increased ACE expression of aortic and coronary specimens from diabetic compared with nondiabetic specimens (aorta: non-DM 1.7±1.8%; DM 4.1±3.6%, $p<0.05$; coronary: non-DM 0.9±0.7%; DM 2.8±0.9%, $p<0.05$). Fig. 4B shows a significant positive correlation between the ACE-positive area and AGE accumulation in all nondiabetes and diabetes specimens (aorta: $r^2=0.181, p<0.05$; coronary: $r^2=0.443, p<0.001$). The extent of ACE-positive areas was correlated with the expression of MMP-9 in all specimens (aorta: $r^2=0.228, p<0.05$; coronary: $r^2=0.561, p<0.0001$) (Fig. 4C). In addition, the proportions of TUNEL-positive areas were correlated with the extent of ACE-positive areas (aorta: $r^2=0.312, p<0.01$; coronary: $r^2=0.404, p=0.002$), as
RAGE Expression

We also examined the proportions of RAGE in aortic and coronary plaques. Fig. 5A shows significant increases in RAGE expression in diabetic specimens compared to nondiabetic specimens both in aortic and coronary specimens (aorta: non-DM, 4.8 ± 1.3%; DM, 10.1 ± 1.1%; coronary: non-DM, 5.4 ± 0.9%; DM, 9.1 ± 0.7%; *p < 0.01, each). RAGE expression significantly correlated with AGE accumulation (aorta: \( r^2 = 0.698, p < 0.0001 \); coronary: \( r^2 = 0.674, p < 0.0001 \)), MMP-9 (aorta: \( r^2 = 0.634, p < 0.0001 \); coronary: \( r^2 = 0.632, p < 0.0001 \)), apoptosis (aorta: \( r^2 = 0.531, p = 0.0001 \); coronary: \( r^2 = 0.497, p = 0.0002 \)), and ACE expression (aorta: \( r^2 = 0.198, p = 0.038 \); coronary: \( r^2 = 0.451, p = 0.0006 \)) in all aortic and coronary specimens (Fig. 5B–5E).

Correlations between Plasma HbA1c Levels and Plaque Parameters

Finally, we examined the correlations between each plaque parameter of autopsy specimens and plasma HbA1c levels in the cases. Fig. 6A shows the results of the higher plasma levels of HbA1c in diabetic compared with nondiabetic cases (\( p < 0.0001 \)). Plasma levels of HbA1c were correlated with AGE accumulation, RAGE expression, the expression of MMP-9, the percentage of TUNEL-positive cells, and ACE expression in both aortic and coronary specimens (Fig. 6B, 6C).

Discussion

In the present study, immunohistochemistry using autopsy specimens from aortic and coronary arteries showed that increased AGE accumulation was accompanied by increased MMP-9 expression and apoptosis in diabetes compared with nondiabetes. Interestingly, ACE expression was also increased in diabetic specimens and correlated with AGE accumulation, MMP-9 expression, and apoptosis. The present study suggests that accumulated AGE may play a role in plaque instability via RAS signaling in type 2 diabetes.

Cipollone et al. reported a linear correlation between MMP-9 expression in carotid atherosclerotic lesions and plasma HbA1c levels\(^{22}\). In human aortic and coronary tissues from diabetes and nondiabetes, we demonstrated in the present study for the first time the close correlations of plasma HbA1c levels with MMP-9 expression, apoptotic cells, and ACE expression in autopsy specimens, as well as plaque AGE accumulation, suggesting that glycemic control might be associated with vascular responses through AGE accumulation; however, risk factors overlap in patients with type 2 diabetes. AGE accumulation and RAGE...
expression in the vascular wall appear to be regulated not only by glycemic control but also by oxidative stress. The latter is associated with other risk factors and inflammation. UKPDS23 and JDCS have shown that the greatest risk factor is the LDL cholesterol level, not hyperglycemia, in diabetic vascular compli-

Fig. 4. A, Extent of angiotensin-converting enzyme (ACE)-positive areas in aortic and coronary autopsy specimens from non-DM and DM (n = 11, each group) was determined as described in Methods. A significant increase in the extent of ACE-positive areas was observed in both aortic and coronary specimens from DM compared with non-DM. *p < 0.05. B, C and D, Correlation of extent of ACE-positive areas versus AGE accumulation (B), MMP-9 expression (C) or TUNEL-positive cells (D) in all aortic and coronary specimens from non-DM (open circle) and DM (closed circle).
Fig. 5. Proportions of RAGE expression in aortic and coronary autopsy specimens from non-DM and DM cases (n = 11, each group, *p < 0.01, A). Regression analysis showed significant correlation between RAGE and AGE, MMP-9, apoptosis, or ACE (B–E).
Thus, the results of the present study do not seem to reflect only glycemic control. Other risk factors and oxidative stress induced by these factors should be taken into account, and this issue needs to be investigated further.

It has been shown that local increased activation
of RAS is associated with the progression of coronary atherosclerosis and the pathogenesis of plaque instability. We demonstrated the increased expression of ACE in coronary and aortic specimens in diabetes compared with nondiabetes. Data also show that the extent of ACE-positive areas was correlated with the accumulation of AGE, suggesting a link between RAS and AGE accumulation in the vasculature. It has been shown that exposure to high glucose up-regulates ACE mRNA and angiotensin II generation in rat mesangial cells, whereas ACE inhibition has been shown to reduce the accumulation of AGE in experimental nephropathy. Together, our results suggest that accumulated AGE may further activate RAS in aortic and coronary atherosclerosis.

A study by Cipollone et al. has shown that diabetic plaques retrieved by carotid endarterectomy had increased the expression of MMP-2 and MMP-9 in humans, suggesting a crucial role of increased gelatinases in plaque destabilization in diabetes. It was reported that streptozotocin-induced diabetes enhances the activity of MMP-9 via oxidant stress. The present study demonstrated that vascular AGE accumulation positively correlated with MMP-9 expression, and negatively with the extent of ECM, and that MMP-9 was correlated with ACE expression, suggesting an important role of RAS in AGE-triggered MMP-9 regulation.

Burke et al. reported that RAGE expression is significantly increased in diabetes compared with nondiabetes and is associated with apoptotic cells in coronary plaques. We clearly showed that diabetic specimens from aortic and coronary arteries had higher percentages of TUNEL-positive cells compared with nondiabetic specimens, and that the frequency of apoptotic cells was associated with AGE accumulation and ACE expression. In vivo blockade of RAS has been shown to suppress diabetes-induced apoptosis in tubular cells and myocytes. Additionally, Fukushima et al. reported that plaque-increased TGF-β1 was involved in apoptosis in AMI with type 2 diabetes mellitus.

In conclusion, increased MMP-9 expression and apoptosis were associated with the AGE/RAGE axis in diabetic plaques, which may play a key role in the gene expression of diabetic vascular complications. The present study suggests that blockade of RAS may be beneficial for plaque destabilization in diabetes.

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