N^2-Carboxyethyl-2'-deoxyguanosine, a DNA glycation marker, in kidneys and aortas of diabetic and uremic patients

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Short title: CEdG in Kidneys and Aortas

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Background. Advanced glycation end product (AGE)-mediated modification of proteins is enhanced both in the kidneys and aortas of diabetic and uremic patients. However, AGE modification of DNA has not yet been reported in these patients.

Methods. We performed immunohistochemistry of kidneys and aortas using a monoclonal antibody against N^2-carboxyethyl-2'-deoxyguanosine (CEdG), a marker of AGE-linked DNA. A total of 20 kidneys and 20 aortas were obtained by autopsy. The kidney samples consisted of 2 groups: non-diabetic non-kidney disease (control) and diabetic nephropathy. The aorta samples consisted of 4 groups: non-diabetic non-kidney disease (control), diabetes, hemodialysis, and diabetic hemodialysis.

Results. In the kidneys CEdG was detected predominantly in the nuclei of glomerular epithelial cells, glomerular mesangial cells, glomerular endothelial cells, parietal epithelial cells, and tubular cells. The number of CEdG-positive cells in the glomeruli was significantly increased in diabetic nephropathy compared with control. In the aortic walls CEdG was detected predominantly in the nuclei of macrophages and myofibroblasts. The number of CEdG-positive cells in aorta was significantly increased in hemodialysis patients and diabetic hemodialysis patients compared with control. The highest number of CEdG-positive cells in the aorta was observed in diabetic hemodialysis patients.

Conclusion. AGE-mediated modification of DNA is enhanced in the kidney of diabetic nephropathy and the aorta of uremic atherosclerosis, and may induce a loss of genetic integrity in these diseases.

Key words

N^2-carboxyethyl-2'-deoxyguanosine, advanced glycation end products; kidney; aorta; diabetes; hemodialysis

In the Maillard reaction, free amino groups of proteins react with reducing sugars (e.g. glucose) and sugar degradation products (e.g. glyoxal, methylglyoxal, 3-deoxyglucosone) to form advanced glycation end products (AGEs). The accumulation of AGEs in tissues plays an important role in the pathogenesis of diabetic nephropathy, chronic renal failure, atherosclerosis, cataract, Alzheimer's disease, and rheumatoid arthritis [1-8]. AGEs are formed by modification of lysine and arginine residues of proteins with glucose. Protein glycation end products such as N^{ε}-(carboxymethyl)lysine (CML), pentosidine and imidazolone are accumulated in the kidneys and aortas of diabetic and uremic patients [9-17]. These findings suggest that AGE modification of protein is one of the pathogenetic factors contributing to the progression of diabetic kidney damage and uremic vascular injury.

Recently, it has been reported that AGEs are also formed by modification of free nucleosides with reducing sugars such as glucose [18-21]. Genomic damage in peripheral lymphocytes, possibly induced by DNA glycation, is enhanced in uremic patients [22,23]. However, DNA glycation products have not yet been demonstrated to be present in human tissues, and their role has yet to be understood in the pathogenesis of kidney disease and uremic vascular injury. N^2-Carboxyethyl-2'-deoxyguanosine (CEdG) was identified as a major product found in the reaction mixtures of guanosine and glucose *in vitro*, and can be used as a marker of advanced glycation of DNA [19-21]. This study aimed to investigate the role of CEdG in the pathogenesis of diabetic nephropathy and uremic vascular complications by using immunohistochemical detection.

METHODS

Subjects

A total of 20 kidneys and 20 aortas were obtained by autopsy. The kidney samples consisted of 2 groups (10 each): non-diabetic non-kidney disease (control) and diabetic nephropathy (DN). The aorta samples consisted of 4 groups (5 each): non-diabetic non-hemodialysis (control), diabetes, hemodialysis and diabetic hemodialysis. Table 1 shows the characteristics of the patients.

Antibodies

A monoclonal anti-CEdG antibody (MAb M-5.1.6) produced by M. Pischetsrieder recognizes two diastereometers of $CEdG_{A,B}$ with high affinity and specificity [21]. The IC_{50} value of the antibody for $CEdG_{A,B}$ was 2.1 ng/mL, whereas the other modified nuclueobases and AGE proteins showed negligible cross-reactivity.

A peroxidase-labeled monoclonal anti-CD68 antibody, a peroxidase-labeled monoclonal anti-α-smooth muscle actin (SMA) antibody, and a peroxidase-labeled monoclonal anti-CD31 antibody were obtained from DAKO (Glostrup, Denmark). A biotin-labeled rabbit anti-mouse antibody was obtained from Nichirei (Tokyo, Japan).

Immunohistochemistry

CEdG in tissues was detected by indirect immunostaining. Formalin-fixed and paraffin-embedded kidney and aorta were cut into 3 μm thick sections and mounted on silane-coated slides. The sections were dewaxed and dehydrated. Antigen retrieval was performed by microwave in 0.01 M citrate buffer (pH6.0) for 5 min at 600W twice. After being washed in PBS (pH7.4, 5 min 3 times), the sections were treated with 0.3% H_2O_2 in methanol for 10 min to block endogenous peroxidase activity, and then blocked with 10% rabbit serum (Nichirei, Tokyo, Japan)

for 30 min at room temperature. Followed by washing in PBS (5 min, 3 times), slices were incubated with monoclonal anti-CEdG antibody (2.5 μg/ml dissolved in 3% BSA/PBS) at 4°C overnight. The slides were incubated with the 2nd antibody (biotin-labeled rabbit-anti-mouse antibody) at room temperature for 30 min, and then with peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan) at 37°C for 30 min. After another wash with PBS, peroxidase activity was detected by reaction with 3,3'-diaminobenzidine tetrahydrochloride (DAB tablet, Merck KGaA, Germany, 30 mg/ml, containing 0.03% H_2O_2) as the chromogen. Finally, sections were counterstained by 1% methylene green, dehydrated, and mounted.

Aorta tissues were additionally stained in serial sections with a monoclonal anti-CD68 antibody and a monoclonal anti-SMA antibody to identify cells increased in tissues, either macrophages or myofibroblasts, respectively. To ensure the localization of endothelial cells, a monoclonal anti-CD31 antibody was also used.

All the sections were examined under light microscopy (Nikon E600), and their pictures were taken with a digital net camera (Nikon DN100). CEdG-positive cells were counted in ten glomeruli, and in ten 1-mm-length areas within the intimal layer of aorta.

Statistics

The data are given as mean±SE. Statistical analysis was performed using analysis of variance (ANOVA) and a P value less than 0.05 was considered significant.

RESULTS

Immunohistochemical detection of CEdG in kidneys

Figure 1 shows the chemical structure of CEdG non-enzymatically formed by the reaction of D-glucose with 2'-deoxyguanosine. CEdG was detected predominantly in the nuclei of glomerular epithelial cells, glomerular mesangial cells, glomerular endothelial cells, parietal epithelial cells, and tubular cells in the kidneys of diabetic nephropathy (Figure 2 A-D). However, the presence of CEdG-positive nuclei was not so prominent in the kidneys of non-diabetic non-kidney disease (control) (Figure 2 E-H) as compared with diabetic nephropathy. The cells with brown nuclei in the glomeruli were counted as CEdG-positive, and the cells with blue nuclei were considered to be CEdG-negative. The numbers of CEdG positive cells per glomerulus were 58.0±5.2 in control and 84.4 ±3.5 in diabetic nephropathy (mean±SE, n=10, respectively, Figure 3). The number of CEdG-positive cells per glomerulus in diabetic nephropathy was significantly increased as compared with control ($P<0.001$).

Immunohistochemical detection of CEdG in aortic walls

CEdG was also detected in the cell nuclei of aortic walls (Figure 4). Most CEdG cells were CD-68 positive or SMA-positive, indicating that the CEdG-positive cells consisted of macrophages and myofibroblasts. The CD-31-positive endothelial cells were detected in the control aorta, but hardly in the diabetic hemodialysis patients. Thus, the endothelial damage was observed in the aorta of diabetic hemodialysis patients. The numbers of CEdG-positive cells in the aortic walls were 109.0±5.1 in control, 171.4±47.3 in diabetes mellitus, 271.6±50.2 in hemodialysis patients, and 328.2±63.0 in diabetic hemodialysis patients (mean±SE, n=5, respectively, Figure 5). The number of CEdG-positive cells in the aorta was significantly increased in both hemodialysis patients and diabetic hemodialysis patients as compared with control ($P<0.01$).

DISCUSSION

AGE modification of proteins is well known to be involved in the development of diabetic glomerulosclerosis and uremic atherosclerosis with or without diabetes. AGEs cause protein cross-linking, modulate the expression of growth factors and cytokines, and consequently induce pathogenic changes in kidney tissues [6,7]. The accumulation of AGEs in vessel walls provides a common mechanism for the high restenosis rates in patients with diabetes and renal failure by upregulating extracellular matrix production [9]. Much of current research is focused on the pathogenic role of protein glycation end products, such as CML, pentosidine, and imidazolone. CML is a major AGE in renal basement membranes in diabetic nephropathy, and its accumulation involves upregulation of the receptor for AGE (RAGE) on glomerular epithelial cells [10]. Elevated serum levels of fluorescent AGE and CML as well as decreased urinary excretion of CML are found in diabetic patients with renal impairment [11]. CML is localized in the mesangial area, and pentosidine is localized in glomerular basement membrane in diabetic retinopathy, while their expression is significantly correlated with the index of diabetic glomerulosclerosis [12]. The association between pentosidine and arteriosclerosis is found in patients receiving hemodialysis [13]. Accumulation of CML in the aorta is enhanced by chronic renal failure, and may be involved in the accelerated development of atherosclerotic lesions in patients with end-stage renal disease [14]. We found that high levels of imidazolone in kidney and aortas of diabetic patients implicate its role in diabetic complications such as nephropathy and atherosclerosis [15]. Thus, there is no doubt as to the important role of protein-linked AGEs in renal and vascular lesion.

AGEs are also formed by nucleoside glycation [18-20]. N^2-Carboxyethyl-guanosine (CEG) is identified as an advanced glycation end product of guanosine [18]. A competitive ELISA to detect CEG was established by using polyclonal anti-CEG antiserum [20]. CEdG is identified as an advanced glycation end product of 2'-deoxyguanosine [19]. Recently, a new monoclonal antibody, which binds with high affinity and specificity to CEdG, has been used to measure AGE-modified

nucleosides in normal human urine [21]. Nucleotide glycation leads to DNA lesions including depurination, single-strand breaks and mutations such as insertions, deletions and transposition [24-30]. As a consequence, DNA glycation could contribute to the well-established loss of genomic integrity that occurs during aging, and may induce aging-related complications [31].

The present study first demonstrated an increased accumulation of CEdG in the kidneys of diabetic patients and the aortas of hemodialysis patients. The number of CEdG-positive cells in glomeruli was significantly increased in diabetic nephropathy. The number of CEdG-positive cells in aorta walls was significantly increased in hemodialysis patients with or without diabetes. These results suggest that CEdG, a DNA-linked AGE, may lead to a loss of genetic integrity in the kidney of diabetic nephropathy and the aorta of uremic atherosclerosis.

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Table 1. Characteristics of patients

Group	Sex (female /male)	Age Mean ± SD (min-max, y)	Duration on HD Mean ± SD (min-max, y)	DM history Mean ± SD (min-max, y)	Serum creatinine Mean ± SD (min-max, mg/dl)
Kidney					
Control	2/8	56.9 ± 16.4 (22-75)	-	-	0.5 ± 0.2 (0.1-0.8)
DN	2/8	66.6 ± 8.8 (53-79)	-	25.4 ± 9.9 (10-37)	3.6 ± 1.7 (2.1-6.1)
Aorta					
Control	2/3	69.6 ± 5.1 (62-74)	-	-	0.8 ± 0.6 (0.2-1.6)
DM	2/3	71.8 ± 7.9 (64-85)	-	13.2 ± 9.0 (3-25)	1.2 ± 0.6 (0.5-2.2)
HD	3/2	69.8 ± 3.8 (66-76)	15.8 ± 8.1 (4-24)	-	7.0 ± 3.0 (3.8-10.3)
HD+DM	0/5	64.6 ± 17.9 (50-90)	3.0 ± 2.0 (0.08-5)	19.2 ± 5.7 (10-25)	6.5 ± 2.9 (3.6-10.2)

CRF: chronic renal failure; DN: diabetic nephropathy, DM: diabetes mellitus, HD: hemodialysis.

Figure legends

Figure 1. Non-enzymatic formation of N^2-carboxyethyl-2'-deoxyguanosine (CEdG) by reaction of D-glucose with 2'-deoxyguanosine.

Figure 2. Immunostaining of CEdG in the kidneys

A-D) Diabetic nephropathy (A,B: glomerulus in a 66-year-old man with serum creatinine of 5.5 mg/dL, C,D: tubules in a 53-year-old man with serum creatinine of 2.1 mg/dL) (A,C: x 100, B,D: x 250)

E-H) Control (E,F: glomerulus in a 68-year-old non-kidney disease man with serum creatinine of 0.5 mg/dL, G,H: tubules in a 43-year-old non-kidney disease man with serum creatinine of 0.3 mg/dL) (E,G: x 100, F,H: x 250)

Figure 3. Number of CEdG-positive cells in the glomeruli of patients with non-diabetic non-kidney disease (control) and diabetic nephropathy (DN)

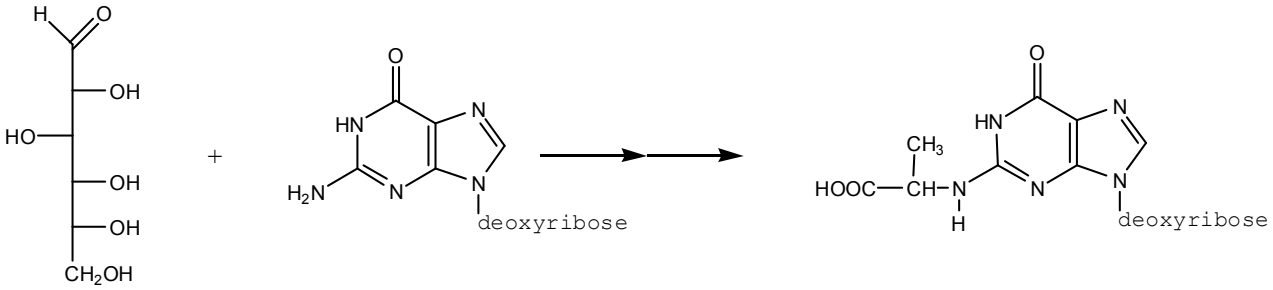
Data are expressed as mean±SE (n=10).

Figure 4. Immunostaining of CEdG, CD-68, α-smooth muscle actin and CD-31 in the aortaic walls

A-D) Diabetic hemodialysis (a 50-year-old man diagnosed diabetic mellitus 18 years ago and treated on hemodialysis for 4 years); Immunostaining with antibodies against CEdG (A), CD-68 (B), α-smooth muscle actin (C), and CD-31 (D);

E-H) Control (a 71-year-old non-diabetic non-hemodialysis woman), Immunostaining with antibodies against CEdG (E), CD-68 (F), α-smooth muscle actin (G), and CD-31 (H); (x 100)

Figure 5. Number of CEdG-positive cells in aortic walls of patients with non-diabetic non-hemodialysis (control), diabetes (DM), hemodialysis (HD) and diabetic hemodialysis (DM+HD). Data are expressed as mean±SE (n=5).



D-glucose Deoxyguanosine CEdG

Fig. 1

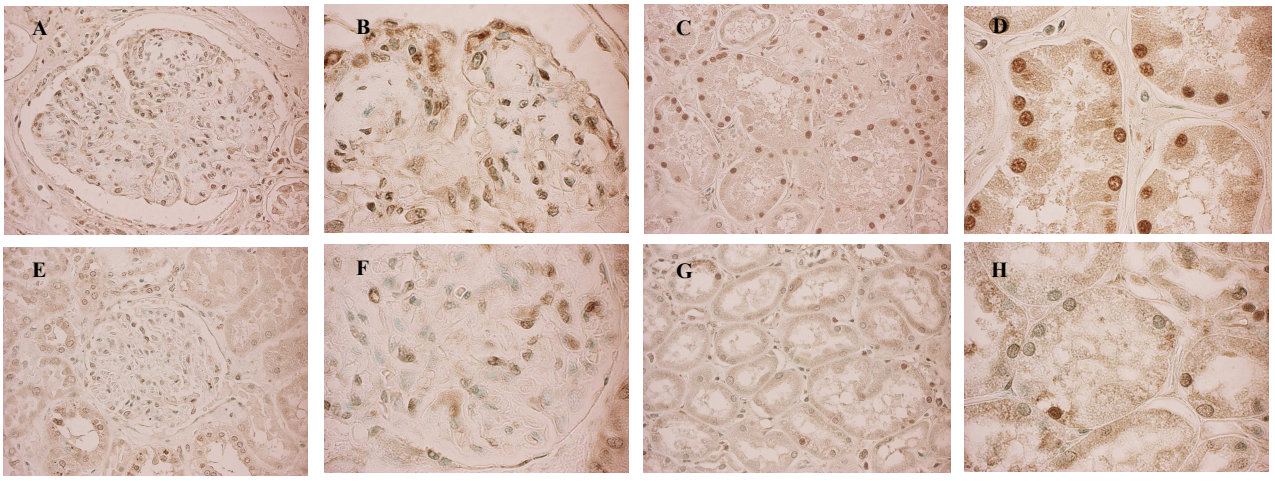


Fig. 2

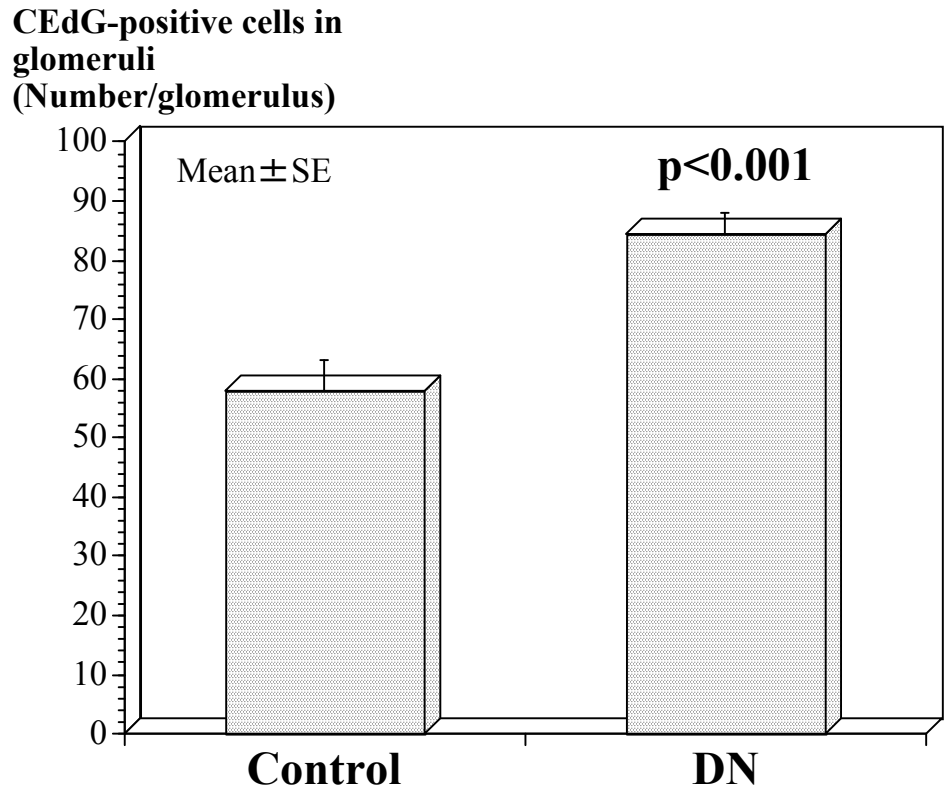

CEdG-positive cells in glomeruli (Number/glomerulus)
100
90
80
70
60
50
40
30
20
10
0
Mean±SE
p<0.001
Control
DN

Fig. 3

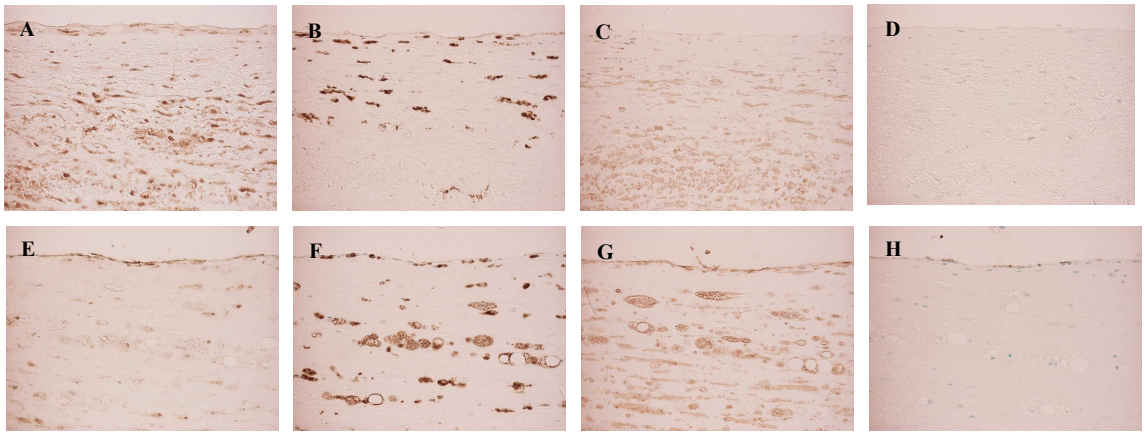


Fig. 4

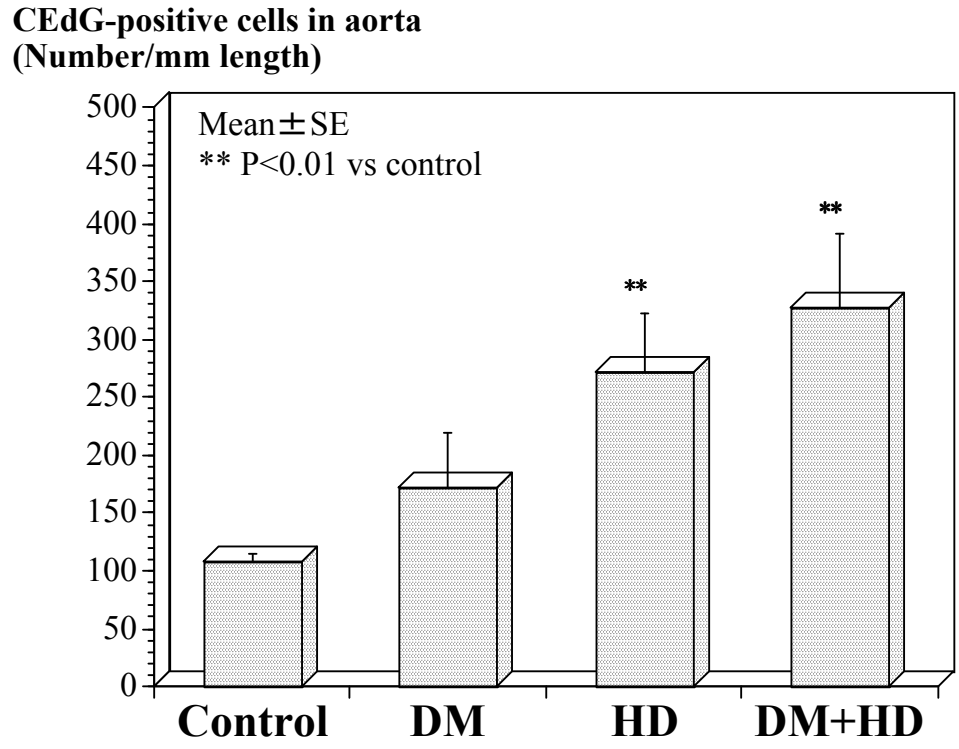

CEdG-positive cells in aorta
(Number/mm length)
Mean±SE
** P<0.01 vs control
500
450
400
350
300
250
200
150
100
50
0
**
**
Control
DM
HD
DM+HD

Fig. 5