



Genotoxicity of advanced glycation end products in mammalian cells

Helga Stopper^{a,*}, Reinhard Schinzel^b, Katarina Sebekova^c, August Heidland^d

^aDepartment of Pharmacology and Toxicology, University of Würzburg, Versbacher Strasse 9, D-97078 Würzburg, Germany

^bDepartment of Physiological Chemistry I, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany

^cInstitute of Preventive and Clinical Medicine, Bratislava, Slovak Republic

^dDepartment of Internal Medicine, University of Würzburg, Josef-Schneider-Strasse 2, 97080 Würzburg, Germany

Received 6 September 2002; received in revised form 24 October 2002; accepted 28 October 2002

Abstract

In patients with chronic renal failure, cancer incidence is enhanced. Since levels of advanced glycation end products (AGEs) are markedly elevated in renal insufficiency, we investigated potential effects of various AGEs on structural DNA integrity in tubule cells. The comet-assay was employed, a method based on the computer-aided microscopic analysis of single cells after electrophoretic separation of their nuclear DNA. Incubation of pig kidney LLC-PK1-cells for 24 h with AGE-BSA (AGE-bovine serum albumin), carboxymethyllysine-BSA as well as methylglyoxal-BSA resulted in a significant increase in DNA damage. Pretreatment of the cells with the proteases trypsin and bromelain abolished the AGE-induced comet-formation. This is in agreement with the idea that the observed genotoxicity of AGEs could be receptor-mediated and that proteases inactivate the extracellular domain of the receptor for AGEs. Binding of AGEs to the RAGE receptor leads to an increased intracellular formation of active oxygen species, which are known to induce DNA damage. It is concluded that AGEs induce genotoxicity in tubule cells, which may be involved in the enhanced cancer development in advanced kidney diseases.

© 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Comet assay; DNA damage; Advanced glycation end product

1. Introduction

One consequence of chronic renal disease is an elevated cancer risk [1,2]. However, the underlying mechanism is unclear. The observed increased genomic damage in peripheral lymphocytes of kidney disease patients may be indicative for their increased cancer susceptibility [3,4]. The involvement of

enhanced oxidative stress and increased levels of various uremic toxins are discussed. One possible candidate are advanced glycation end products (AGEs), which are strongly elevated in patients with renal insufficiency. AGE formation arises from a nonenzymatic reaction of a sugar ketone or aldehyde group with a free amino group of proteins, lipids, or amino acids. After a complex cascade of dehydration, condensation, fragmentation, oxidation and cyclization reactions, a diverse and largely undefined group of compounds termed AGEs are formed [5]. Only few

* Corresponding author. Tel.: +49-931-201-48427; fax: +49-931-201-48446.

E-mail address: stopper@toxi.uni-wuerzburg.de (H. Stopper).

AGEs have been characterized chemically and identified in **tissues**, the most investigated of which has been **carboxymethyllysine** (CML). In renal disease AGE accumulation is ascribed both to the impaired elimination of AGEs and to enhanced formation due to oxidative stress [6]. Accumulation of AGEs in tissues is a common phenomenon of normal aging and occurs at accelerated rates in patients with diabetes **mellitus** [7,8]. While there is increasing evidence that AGEs were implicated in the **pathogenesis** of complications in diabetes (**nephropathy**, **retinopathy**, neuropathy, atherosclerosis) [9], **genotoxic** effects of AGEs are not conclusively shown. In *in vitro* studies **mutagenic** effects of **DNA-AGE** such as **deletions**, insertions and **transposon** activation were shown in bacterial model systems [10]. Furthermore it is shown that DNA can be glycosylated *in vitro* yielding **carboxyethylguanosine** as major products [11]. In this study a mammalian cell culture model was used to investigate whether AGEs could contribute to the development of cancer in renal disease by causing genotoxic effects. The porcine cell line **LLC-PK1** was chosen because it exhibits properties of proximal tubules. The alkaline comet assay (single cell gel **electrophoresis**), a **well-established** method for quantifying DNA damage [12] was used to analyze **AGE-induced genotoxicity**.

2. Materials and methods

2.1. Generation and *characterization* of glycosylated proteins

Bovine serum **albumin** (1 mM BSA Fraction V, **Sigma**, **Deisenhofen**, Germany) solution was maximally glycosylated by incubation with 0.5 M glucose in 50 mM potassium phosphate, pH 7.4, 1 mM EDTA under sterile conditions at 50 °C for 40 days following a modified protocol of Schmidt et al. [13]. The AGE-BSA solution was then **lyophilized** and **redissolved** in 50 ml **bidistilled** water. Glucose and other compounds were removed by extensive dialysis against 5 × 5 l bidistilled water for 72 h. Residual glucose was less than 0.1 mM **after this** procedure. The **dialyzed** AGE-BSA was again lyophilized resulting in a brownish powder. **Methylglyoxal-modified** albumin (MGO-BSA) was produced by reacting BSA with **methyl-**

glyoxal in 0.1 M phosphate buffer (pH 7.4). The concentrations of BSA and **methylglyoxal** were 20 mg/ml (0.3 mM) and 30 mM, respectively. After modification, samples were purified as described before. Shortly before use, modified albumins were redissolved in 50 mM PBS. Control BSA was incubated under the same conditions but in the absence of glucose. CML content was measured by **HPLC** and **ELISA** and showed to be approximately 10 **mol CML/mol** albumin. **Fructosyl-lysine** content was found to be low (approximately 0.3 mol **FL/mol** albumin) under these conditions as determined by the reduction of **nitroblue tetrazolium** at alkaline pH using **dihydroacetone** as a standard. **CML-modified** albumin was prepared by a modified protocol of **Reddy et al.** [14] (**Faist**, personal communication). Albumin (1 mM BSA Fraction V, Sigma) was dissolved in 0.2 M phosphate buffer (pH 7.4) containing 0.05 M glyoxylic acid (Sigma). After adjustment of the pH value the solution was pre-incubated at room temperature for 2 h, then solid **NaBH₃CN** was added to reach a concentration of 0.15 M. After incubation for 16 h at room temperature, CML-modified albumin was purified as described above. The yield was approximately 22 mol **CML/mol** albumin.

2.2. Cell culture

LLC-PK1 cells, a porcine cell line, which exhibits properties of proximal tubules, were grown at 37 °C in a humidified atmosphere of 5% **CO₂** in **Dulbecco's** modified Eagle's medium (**DMEM**) with 1000 mg/l glucose supplemented with 10% fetal calf serum (**FCS**; **Gibco-BRL**), 25 mM **HEPES**, 1% **glutamine** and antibiotics. Cells were routinely split twice a week to keep the **under-exponential-growth** conditions. They were cultured for no more than 20 passages after thawing them from stock. Stock was obtained from the American Type Culture Collection (**ATCC**; No. **CL-101**). For experiments, 1 × 10⁶ cells in 5 ml medium were treated with test compounds in culture medium for 24 h, and then harvested for the comet assay. For experiments applying protease **pretreatment**, cells were treated in serum-free medium with protease (concentrations as indicated in figure legends) for 2 h. After that, the medium containing proteases was removed, and the test compounds were added in rich culture medium (10% serum). The

positive control substance **ethylmethanesulfonate** (200 $\mu\text{g/ml}$) was added at this time and remained in the treated culture until harvest. After 24 h, cells were harvested for the comet assay.

2.3. Comet assay

The comet assay was performed according to Singh et al. [15] with slight modifications. In this test, cells are embedded in **agar** and exposed to an electrical field. The presence of single or double strand breaks, alkali-labile sites and relaxed chromatin **causes** the resulting DNA fragments or loops to **move** ahead of the intact nuclear DNA. A comet-like structure is formed because smaller fragments and relaxed loops move **faster** than larger fragments and intact DNA. Comets are quantified microscopically after DNA staining. Fully frosted microscope slides were coated with **agarose** (1.5% in Ca- and Mg-free PBS buffer). Forty-five **microliters** of cell suspension (1:10 in 0.5% low melting point agarose, diluted in Ca- and Mg-free PBS) were layered on top. Slides were then immersed in a jar containing **cold lysing** solution (1% Triton X-100, 10% DMSO and 89% of 10 mM Tris/1% Na-lauryl-sarcosine/2.5 M NaCl/100 mM Na₂EDTA, pH 10) for lysis at 4 °C (1 h). Then, slides were **pre-treated** for 20 min in **electrophoresis** buffer (300 mM NaOH/1 mM Na₂EDTA, pH 10) and after that exposed to 1.14 V/cm and 300 mA for 20 min. **Preincubation** and electrophoresis were performed in an ice **bath**. Slides were neutralized for 5

min in 0.4 M Tris, pH 7.5, and washed with distilled water (1 min). DNA was stained by adding 20 μl of **ethidium bromide** (50 $\mu\text{g/ml}$) onto each slide. Cells were analyzed using a fluorescence microscope at a **500-fold** magnification and a computer-aided image analysis. Images of at least 50 cells (25 from each of two slides) were evaluated by the use of the software program NIH Image 1.54 (NIH, USA). Two areas were selected by the investigator in each picture: the whole cellular DNA including the tail region of the comet and a region containing only the tail region of the comet. The integrated densities (sum of the gray values of all pixels in the selection) were measured in each selection and the percentage in the tail region of the comet was calculated. This number represents the amount of DNA in the tail. In the figures, the average of the 50 measurements of one experiment is given and is referred to as '**DNA in tail (%)**'. All experiments were repeated at least twice and repeat experiments yielded consistent results.

3. Results

The incubation of **LLC-PK1-cells** with 50–400 $\mu\text{g/ml}$ CML-BSA led to the formation of comets (Fig. 1). A **dose-dependency** was observed between 50 and 200 $\mu\text{g/ml}$, and a saturation type response was seen at higher (200–400 $\mu\text{g/ml}$) concentrations. Ethylmethanesulfonate (EMS) was used as a positive control for **genotoxicity**. Comet formation after EMS treatment is **due** to the **compound's** direct action on DNA (and the following cellular repair attempts). While the cell proliferation as assessed by Coulter Counter cell counting was reduced to 91% of the control with EMS, no reduction was observed with any applied concentration of CML-BSA. A similar dose response with a saturation-type shape at more than 200 $\mu\text{g/ml}$ was observed with AGE-BSA (data not shown). A dose of 200 $\mu\text{g/ml}$ was chosen for further experiments. When we **pretreated** the cells with the protease **trypsin** (2.5 $\mu\text{g/ml}$), the AGE-BSA-induced comet formation was abolished (Fig. 2). BSA alone or trypsin by itself had no influence on comet damage (BSA/trypsin-treatment), and comets induced by the genotoxic positive control EMS were not reduced after **protease-pretreatment**. EMS induced a decreased cell proliferation (66% of control), while AGE-BSA

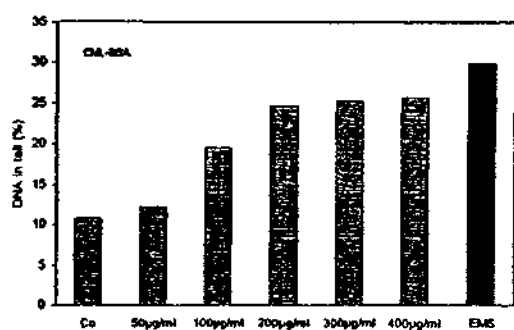


Fig. 1. Assessment of DNA damage (% DNA in tail) in the comet-assay in LLC-PK1-cells after treatment with the indicated concentrations of **carboxymethyllysine-BSA** (CML-BSA). EMS: **ethylmethanesulfonate**, 200 $\mu\text{g/ml}$, positive control for **genotoxicity**. Untreated cells are shown as control.

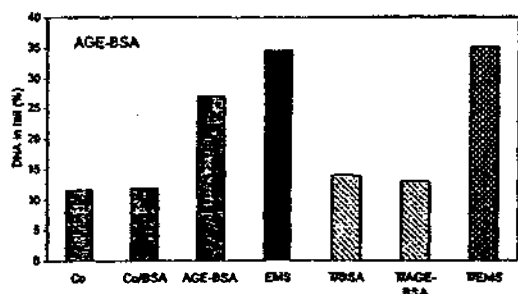


Fig. 2. DNA damage (% DNA in tail) in the comet assay in LLC-PK1-cells after treatment with 200 µg/ml AGE-BSA with (T/AGE-BSA) and without pretreatment of the cells with the protease trypsin (2.5 µg/ml). Co/BSA: treatment with 200 µg/ml non-glycated BSA was performed as additional control. EMS: ethylmethanesulfonate, 200 µg/ml, positive control for genotoxicity. T/BSA: trypsin-pretreated BSA-control. T/EMS: trypsin-pretreated EMS positive control.

did not (94%) and AGE-BSA/trypsin only slightly (80%) reduce cell proliferation. MGO-BSA also induced the formation of comets, which were similarly abolished by a pre-treatment with trypsin (Fig. 3). Cell proliferation was reduced to 77% of control after treatment with MGO-BSA and to 67% with EMS. Another protease, bromelain (2.5 µg/ml), also abolished AGE-BSA-induced comets, while EMS-induced comets were again not influenced (data not shown). The protease-pretreatment was performed in serum-free medium. While the addition of 0.5 and 1% PCS to the treatment medium did not

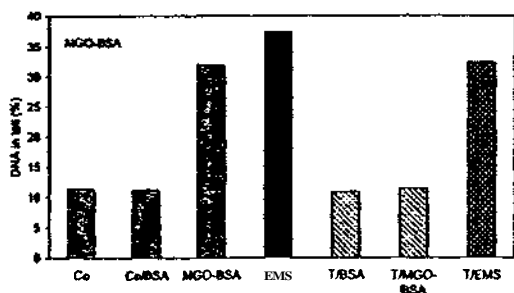


Fig. 3. DNA damage (% DNA in tail) in the comet-assay in LLC-PK1-cells after treatment with 200 µg/ml methylglyoxal-BSA (MGO-BSA) with (T/MGO-BSA) and without pretreatment of the cells with the protease trypsin (2.5 µg/ml). Co/BSA: treatment with 200 µg/ml non-glycated BSA was performed as additional control. EMS: ethylmethanesulfonate, 200 µg/ml, positive control for genotoxicity. T/BSA: trypsin-pretreated BSA-control. T/EMS: trypsin-pretreated EMS positive control.

influence the results to a detectable degree, the addition of 5% FCS reduced the effect of trypsin and only a minor decrease of AGE-BSA-induced genotoxicity was observed (data not shown).

4. Discussion

In vitro studies have shown modification of DNA by reducing sugars as well as increased mobility of a transposable element after modification of DNA by advanced glycation in mammalian cells [16]. However, the genotoxicity of AGE proteins has not been shown before. Using the comet-assay as an endpoint for DNA damage it could clearly be shown here that CML-BSA as well as AGE-BSA and MGO-BSA were genotoxic in cultured LLC-PK1 epithelial pig kidney cells. This genotoxicity was abolished after pre-treatment of the cells with proteases. The genotoxicity of the DNA-damaging agent EMS was not reduced by protease-pretreatment. These findings as well as the saturation of the effect in dose-response experiments is in agreement with the idea that AGE-induced genotoxicity could be mediated by receptor binding. In support of this, it has been shown that protease treatment of cells markedly reduces the amount of AGE-BSA uptake into LLC-PK1 cells [17]. A given number of receptors per cell may limit the maximum amount of achievable damage explaining saturation in the dose response. AGE receptors have been found on the surface of multiple cells (endothelial, mesangial cells, monocytes, macrophages, neurons, renal cells). The best characterized receptor for AGE is RAGE, a member of the immunoglobulin superfamily [18]. CML adducts are one of the most relevant ligands for RAGE and mediate NF-κB pathways [19]. Binding of AGEs to RAGE generates intracellular reactive oxygen species [20]. This enhanced oxidative stress activates and translocates the transcription factor NF-κB [21]. Reactive oxygen species are known to attack DNA [22,23] and may thus be involved in the observed genotoxicity. Another possibility of a pathway leading to the induction of DNA damage upon uptake of AGE-BSA into the cell lies in the prooxidative activity of AGE-BSA due to metal-binding [24].

Previously, it was shown that an in vitro AGE-modified plasmid transformed into *Escherichia coli*

resulted in an increased mutation frequency [10]. This yields the idea that **glycating** agents may directly exert modification of DNA and thus induce **DNA-damaging** or **mutagenic** effects. In **fact**, Seidel et al. [25] have shown that **glycation** of DNA can lead to **DNA-bound** advanced glycation end products, resulting in **depurination** and **abasic** sites. **However**, in our experiments only **BSA-bound** AGEs were applied, ruling out this pathway for the **induction** of DNA damage. It will be interesting to investigate the relative contributions of glycation of DNA and of receptor-mediated effects to the overall achievable mutagenic damage by AGEs.

Acknowledgements

We thank Mrs. ML Scheurich for her expert technical assistance. The financial support of MUCOS Pharma GmbH & Co is gratefully acknowledged.

References

- [1] P. Maisonneuve, L. Agodoa, R. Gellert, J.H. Stewart, G. Buccianti, A.B. Lowenfels, R.A. Wolfe, E. Jones, A.P. Disney, D. Briggs, M. McCredie, P. Boyle, Cancer in patients on dialysis for end-stage renal disease: an international collaborative study, *Lancet* 354 (1999) 93–99.
- [2] A. Heidland, U. Banner, S. Vamvakas, Incidence and spectrum of dialysis-associated cancer in three continents, *Am. J. Kidney Dis.* 35 (2000) 347–351.
- [3] H. Stopper, T. Meysea, A. Bockenforde, U. Bahner, A. Heidland, S. Vamvakas, Increased genomic damage in lymphocytes of patients before and after long-term maintenance hemodialysis therapy. *Am. J. Kidney Dis.* 34 (1999) 433–437.
- [4] H. Stopper, F. Boullay, A. Heidland, J. Vienken, U. Bahner, Comet-assay analysis identifies genomic damage in lymphocytes of uremic patients. *Am. J. Kidney Dis.* 38 (2001) 296–301.
- [5] E.D. Schleicher, A. Bierhaus, H.U. Haring, P.P. Nawroth, R. Lehmann, Chemistry and pathobiology of advanced glycation end products, *Contrib. Nephrol.* 131 (2001) 1–9.
- [6] A. Heidland, K. Sebekova, R. Schinzel, Advanced glycation end products and the progressive course of renal disease, *Am. J. Kidney Dis.* 38 (2001) S100–S106.
- [7] J.W. Baynes, The role of AGEs in aging: causation or correlation, *Exp. Gerontol.* 36 (2001) 1527–1537.
- [8] E.D. Schleicher, E. Wagner, A.G. Nerlich, Increased accumulation of the glycoxidation product N(epsilon)-(carboxymethyl)lysine in human tissues in diabetes and aging, *J. Clin. Invest.* 99 (1997) 457–468.
- [9] M. Brownlee, Biochemistry and molecular cell biology of diabetic complications. *Nature* 414 (2001) 813–820.
- [10] M. Pischetsrieder, W. Seidel, G. Munch, R. Schinzel, N(2)-(1-carboxyethyl)deoxyguanosine, a nonenzymatic glycation adduct of DNA, induces single-strand breaks and increases mutation frequencies, *Biochem. Biophys. Res. Commun.* 264 (1999) 544–549.
- [11] W. Seidel, M. Pischetsrieder, DNA-glycation leads to depurination by the loss of N2-carboxyethylguanine in vitro, *Cell. Mol. Biol. (Noisy-le-Grand)* 44 (1998) 1165–1170.
- [12] F. Kassie, W. Parzefall, S. Knasmüller, Single cell gel electrophoresis assay: a new technique for human biomonitoring studies, *Mutat. Res.* 463 (2000) 13–31.
- [13] A.M. Schmidt, M. Hasu, D. Popov, J.H. Zhang, J. Chen, S.D. Yan, J. Brett, R. Cao, K. Kuwabara, G. Costache, N. Simionescu, D. Stern, Receptor for advanced glycation endproducts (AGEs) has a central role in vessel wall interactions and gene activation in response to circulating AGE proteins, *Proc. Natl. Acad. Sci. USA* 91 (1994) 8807–8811.
- [14] S. Reddy, J. Bichler, K.J. Wells-Knecht, S.R. Thorpe, J.W. Baynes, N epsilon-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins, *Biochemistry* 34 (1995) 10872–10878.
- [15] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, *Exp. Cell Res.* 175 (1988) 184–191.
- [16] R. Bucala, A.T. Lee, L. Rourke, A. Cerami, Transposition of an Alu-containing element induced by DNA-advanced glycosylation endproducts, *Proc. Natl. Acad. Sci. USA* 90 (1993) 2666–2670.
- [17] G. Xiang, R. Schinzel, A. Simm, G. Munch, K. Sebekova, M. Kasper, T. Niwa, C. Schmitz, A. Heidland, Advanced glycation end products (AGEs)-induced expression of TGF-beta 1 is suppressed by a protease in the tubule cell line LLC-PK1, *Nephrol. Dial. Transplant* 16 (2001) 1562–1569.
- [18] A.M. Schmidt, D.M. Stern, RAGE: a new target for the prevention and treatment of the vascular and inflammatory complications of diabetes, *Trends Endocrinol. Metab.* 11 (2000) 368–375.
- [19] T. Kislinger, C. Fu, B. Huber, W. Qu, A. Taguchi, S. Du Yan, M. Hofmann, S.F. Yan, M. Pischetsrieder, D. Stern, A.M. Schmidt, N(epsilon)-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression, *J. Biol. Chem.* 274 (1999) 31740–31749.
- [20] S.D. Yan, A.M. Schmidt, G.M. Anderson, J. Zhang, J. Brett, Y.S. Zou, D. Pinsky, D. Stern, Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins, *J. Biol. Chem.* 269 (1994) 9889–9897.
- [21] A. Bierhaus, T. Illmer, M. Kasper, T. Luther, P. Quehenberger, H. Tritschler, P. Wahl, R. Ziegler, M. Mueller, P.P. Nawroth, Advanced glycation end product-mediated induction of tissue

- factor in cultured **endothelial** cells is dependent on **RAGE**, *Circulation* 96 (1997) 2262–2271.
- [22] L.J. Marnett, **Oxylradicals** and DNA damage, *Carcinogenesis* 21 (2000) 361–370.
- [23] B. Epc, J. Hegler, D. Wild, **Identification** of ultimate DNA damaging oxygen **species**, *Environ. Health Perspect.* 88 (1990) 111–115.
- [24] A.K. Saxena, P. Saxena, X. Wu, M. Obrenovich, W.F. Weiss, V.M. Monnier, Protein aging by **carboxymethylation** of **lysines** generates sites for divalent metal and **redox** active copper binding: **relevance** to diseases of **glycoxidative** stress, *Biochem. Biophys. Res. Commun.* 260 (1999) 332–338.
- [25] W. Seidel, M. Pischetsrieder, **Immunochemical** detection of **N2-[1-(1-carboxy)ethyl]guanosine**, an advanced **glycation** end product **formed by** the reaction of **DNA** and reducing sugars or **L-ascorbic** acid in vitro, *Biochim. Biophys. Acta* 1425 (1998) 478–484.