Diabetologia (1999) 42: 603–607

Diabetologia

Short communication

Diabetic retinopathy risk correlates with intracellular concentrations of the glycoxidation product N^{ε}-(carboxymethyl) lysine independently of glycohaemoglobin concentrations

H.-P. Hammes[1], M. Brownlee[2], J. Lin[1], E. Schleicher[3], R. G. Bretzel[1]

[1] 3rd Medical Department, Centre of Internal Medicine, Justus-Liebig-University, Giessen, Germany
[2] Diabetes Research Center, Albert Einstein College of Medicine, Bronx, New York, USA
[3] 4rd Medical Department, Eberhard-Karl-University, Tübingen, Germany

Abstract

Aims/hypothesis. We investigated whether either the amount of diabetes-induced intracellular oxidative stress or the concentration of hyperglycaemia-induced advanced glycation endproducts is associated with the risk of diabetic retinopathy.
Methods. We measured concentrations of the glycoxidation product N^{ε}-(carboxymethyl)lysine and two non-oxidation-dependent advanced glycation endproducts (methylglyoxal-derived and 3-deoxyglucosone-derived) in $CD45RA^{+}$ T-cells from 21 Type I (insulin-dependent) diabetic patients with and without diabetic retinopathy and from age-matched nondiabetic control subjects.
Results. Intracellular concentrations of both oxidation-dependent N^{ε}-(carboxymethyl)lysine and oxidation-independent advanced glycation endproducts were increased in memory T-cells from diabetic patients. N^{ε}-(carboxymethyl)lysine: diabetic median-24176 arbitrary units/mg protein (95% confidence interval 18690–34099 arbitrary units/mg protein); nondiabetic-9088 arbitrary units/mg protein (confidence interval 6994–10696 arbitrary units/mg protein; $p < 0.0001$). Methylglyoxal-derived advanced glycation end products: diabetic-5430 arbitrary units/mg protein (confidence interval 3458–13610); nondiabetic-271 arbitrary units/mg protein (confidence interval 61–760 arbitrary units/mg protein; $p < 0.0001$). 3-Deoxyglucosone-derived advanced glycation end products: diabetic-8070 arbitrary units/mg protein (confidence interval 7049–16551 arbitrary units/mg protein); nondiabetic-1479 arbitrary units/mg protein (confidence interval 1169–3170; $p < 0.0001$). Only N^{ε}-(carboxymethyl)lysine concentrations, however, inversely correlated with the duration of retinopathy-free diabetes ($r = -0.51$; $p < 0.02$). Diabetes-dependent N^{ε}-(carboxymethyl)lysine accumulation did not correlate with age, diabetes duration, or averaged glycohaemoglobin concentrations. In vitro experiments wih menadione and lymphocytes confirmed that N^{ε}-(carboxymethyl)lysine concentrations reflect intracellular oxidative stress.
Conclusion/interpretation. Monitoring intracellular concentrations of increased oxidative stress in long-lived $CD45RA^{+}$ lymphocytes by markers such as N^{ε}-(carboxymethyl)lysine possibly identifies a subgroup of patients at high risk for microvascular complications. [Diabetologia (1999) 42: 603–607]

Keywords Diabetic retinopathy, prediction, lymphocytes, glycation.

Received: 9 November 1998 and in revised form: 18 December 1998

Corresponding author: Dr. H.-P. Hammes, 3rd Department Internal Medicine, Justus-Liebig-University, Rodthohl 6, D-35392 Giessen, Germany
Abbreviations: CML, N^{ε}-(carboxylmethyl)lysine; AGE, advanced glycation endproducts; 3-DG, 3-deoxyglucosone; MG, methylglyoxal; AU, arbitrary units.

A linear relation exists between time-averaged blood glucose concentrations and the development and progression of microvascular complications in diabetic patients [1]. Some individual patients, however, with low average blood glucose concentrations develop severe complications, while some with high mean blood glucose concentrations do not. Overall, only one-third of Type I diabetic patients develop clinical nephropathy, usually after 12 to 15 years of

diabetes [2], and approximately 65 % develop proliferative diabetic retinopathy [3]. Familial clustering suggests that a genetic component is involved in the susceptibility to both diabetic nephropathy and retinopathy [4]. To date, no parameter has been found that can predict the clinical course of an individual patient. Such a test would allow special intensive treatment of the high risk subgroup of diabetic patients. The mean concentration of blood glucose is currently assessed by the glycohaemoglobin test which is a measure of glucose-derived Amadori products on free amino groups of haemoglobin [5]. Amadori products give rise to a diverse series of lysine or arginine-linked adducts, called advanced glycation endproducts (AGE), or both, which integrate glycaemia over longer periods of time than glycohaemoglobin [6]. Non-oxidative rearrangement yields 3-deoxyglucosone (3-DG), a dicarbonyl compound that reacts with arginine to form a hydroimidazolone [7]. The dicarbonyl methylglyoxal (MG), formed by non-oxidative fragmentation of glycolysis-derived triose phosphates, reacts with lysine residues to form carboxyethyllysine and 4-methylimidazolium, and with arginine residues to form argpyrimidine and methylimidazolone [8]. MG is a major source of intracellular AGEs in endothelial cells [9]. Oxidation of Amadori products gives rise to protein-bound N^{ε}-(carboxymethyl-)lysine (CML) and erythronic acid [10]. CML can also form as a result of lipid peroxidation [11]. CML concentrations reflect integrated oxidative stress over long periods of time. To investigate whether either the amount of diabetes-induced intracellular oxidative stress or the concentration of hyperglycaemia-induced AGEs is associated with the risk of diabetic retinopathy, we measured concentrations of the glycoxidation product CML and both 3-DG- and MG-derived AGEs in $CD45RA^+$ lymphocytes from diabetic patients with and without diabetic retinopathy, and from age-matched non diabetic control subjects. AGE concentrations in $CD45RA^+$ T-cells reflect integrated values of long-term hyperglycaemia or oxidative stress or both, since these cells have a half-life of more than 700 days [12].

Subjects and methods

Patients. We included 21 Type I diabetic patients and 21 non-diabetic subjects in the study (Table 1). Patients with a diabetes duration of more than 9 years and two or more measurements of glycated haemoglobin per year over a period of 9 years were enrolled. Glycohaemoglobin was measured by high pressure liquid chromatography (HPLC) (Biorad, Munich, Germany) and values were given as total HbA1. Retinopathy was assessed by direct funduscopy (until 1985; written report) and fundus photography (after 1985). Retinopathy was graded according to the Early Treatment Diabetic Retinopathy Study (ETDRS) protocol [13] in which the presence of diabetic retinopathy is defined as an ETDRS level equal or greater than 20/10. Retinopathy-free diabetes duration was defined as the interval between the onset of diabetes and the first identification of retinopathy ETDRS level equal or greater than 20/10. All subjects involved in the study gave informed consent. The study was approved by the institutional ethics committee.

Table 1. Clinical and metabolic characteristics of Type I diabetic patients and control subjects

	Nondiabetic	Diabetic
Sex (female/male)	10/11	11/10
Mean age (SD) in years	40 (17)	40 (12)
Mean diabetes duration (SD)	–	20 (8)
Mean % HbA1 (SD)	6.4 (0.4)	9.5 (1.6)
With retinopathy *(n)*	–	15

Lymphocyte isolation from peripheral blood. Venous blood (18 ml) was drawn into a syringe prefilled with heparin (2 ml Vetren 200, Promonta, Hamburg, Germany). Samples were diluted (20 ml PBS, pH 7.4), gently mixed and 20 ml of the diluted samples were overlayed with 10 ml of Lymphoprep (Nycomed, Oslo, Norway). After centrifugation (800 *g,* 20 min) lymphocytes were isolated by aspiration, washed in PBS and cells were pelleted by centrifugation (10 min at 250 *g*). After repeated washing (PBS), cells were resuspended in 1 ml PBS containing 30 % FCS (Gibco BRL, Eggenstein, Germany).

Preparation of magnetic beads. We added 50 µl beads (Immunotech, Fisher Scientific, Nidderau, Germany) to 1 ml PBS containing 0.2 % BSA in a 4 ml plastic tube. Beads were separated by incubation with a Cobalt-Samarium magnet for 5 min. The supernatant was discarded, 20 µl of $CD45RA^+$ antibody (Immunotech) were added and incubated for 30 min at room temperature. Beads were washed twice with 2 ml PBS/BSA.

Separation of $CD45RA^+$ T-cells. Isolated lymphocytes were incubated with the immunobeads for 10 min at room temperature. Complexes were washed twice with PBS. Cells were separated by $CD45RA^+$-coated beads. Isolated cells were resuspended in 0.5 ml PBS. Lymphocytes were lysed by freezing/thawing in liquid nitrogen and subsequent sonication (3 times 30 s on ice). Samples were centrifuged (5 min 1300 *g*) and stored at – 20 °C until use.

Quantitative immunoblotting. One µl of lymphocyte lysate solution was loaded onto nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany). Membranes were dried for 10 min at room temperature and subsequently blocked with 5 % nonfat dry milk in PBS-T (0.5 % tween 20 in PBS, pH 7.4) for 1 h. After repeated washings (PBS-Tween 1 × 15 min, 2 × 5 min), they were incubated using the following antibodies: a polyclonal anti-CML antibody (diluted 1 : 10000) [14], a monoclonal anti-methylglyoxal-derived AGE antibody (1 µg/ml) [10] and a monoclonal antibody directed against a 3-DG-derived AGE epitope (1 µg/ml) [15].

After extensive washing in PBS-T, membranes were exposed to peroxidase(POD)-anti-mouse IgG or POD-anti-rabbit IgG antibodies (DAKO, Hamburg, Germany), respectively, for 1 h. Membranes were washed again and exposed to the enhanced chemoluminescence (ECL) detection system (Amersham Buchler, Braunschweig, Germany). Membranes were exposed to X-ray film (Sterling, Newark, Del., USA)

and films were scanned into a image analysis system (CUE-2, Olympus Opt. Europe, Hamburg, Germany). Optical density and areas of the dots were quantitated. Results were obtained from three independent experiments and three independent determinations of each individual AGE value. Results were expressed as arbitrary units (arbitrary units/mg protein), which means optical density × area per mg of protein; protein concentrations of lysates measured by a standard photometric assay (Biorad, Munich, Germany). Immunoblots with the polyclonal antibody CML showed a linear dependence on both CML-modified key hole limpet haemocyanin (KLH)- and BSA-concentrations (data not shown). Preincubation of the primary antibody with CML-modified BSA yielded negative results.

Menadione in-vitro experiments. CD45RA$^+$ lymphocytes were isolated as described above, washed with PBS, and incubated with increasing concentrations of menadione (0–1.5 μmol/l) for 2 min at 37 °C or with 2 μmol/l menadione for 0–2 min [16]. The reaction was terminated by freezing the cells in liquid nitrogen, followed by three cycles of thawing and freezing and subsequent sonication. The formation of CML was followed by dot blot analysis, using the polyclonal CML-antibody as described.

Statistical analysis. Data are expressed as median and confidence intervals unless otherwise stated. Differences between groups were tested using the alternative Welsh *t*-test (Instat, GraphPad Software Inc., San Diego, Calif., USA). Differences in group proportions were tested using Fisher's exact test and correlations were tested using linear regression analysis. A p value of less than 0.05 was regarded as significant.

Results

Diabetes was associated with a threefold increase of CML concentration in circulating CD45RA$^+$ memory T-cells [diabetic group median 24176 arbitrary units (AU), CI 18690–34099 AU; nondiabetic 9088 AU, CI 6994–10696 AU; $p < 0.0001$]. The relative mean increase of MG-derived AGEs in the same diabetic population was 20-fold, and the median increased to 5430 AU, CI 3458–13610 AU; nondiabetic 271 AU, CI 61–760 AU; $p < 0.0001$ and the relative increase of 3-DG-derived AGEs was 5.4-fold, diabetic 8070 AU, CI 7049–16551 AU nondiabetic 1479 AU, CI 1169–3170 AU; $p < 0.0001$). The relation of accumulated AGE-concentrations in memory T-cells to retinopathy susceptibility was evaluated by comparing intracellular concentrations of CML, MG-derived AGEs and 3-DG-derived AGEs to the known retinopathy-free diabetes duration. A notable correlation was found only for CML and the retinopathy-free diabetes duration (Fig. 1). CML accumulation in CD45RA$^+$ T-cells did not correlate with age, diabetes duration or glycohaemoglobin concentration ($p > 0.05$ for each test). There was no correlation between the non-oxidatively generated AGE (MG-derived and 3-DG-derived) and age, diabetes duration or glycohaemoglobin ($p > 0.05$ for each test).

To study the generation of CML reactive material in lymphocytes in vitro and to establish that CML does reflect increased intracellular oxidative stress independent of glycaemia, we tested the effect of menadione on lymphocyte-CML formation as a function of concentration and time. CML reactivity increased linearly with time after exposure to 2 μmol/l menadione. The total increase over baseline was 300 % after 90 s. (Fig. 2 A). A similar relation was shown when the cells were exposed to 0–2 μmol/l menadione for 2 min (Fig. 2 B).

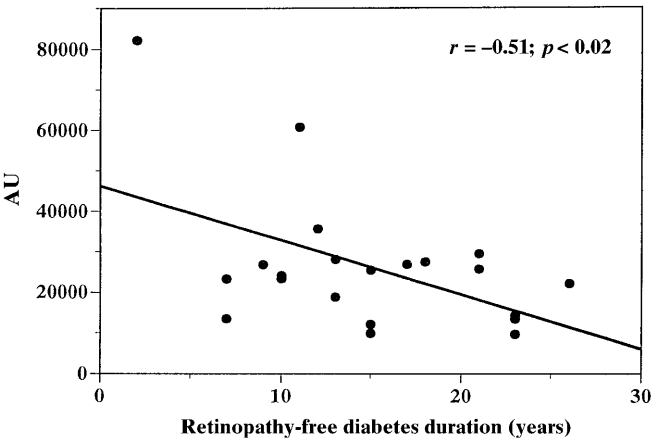

Fig. 1. Correlation of the retinopathy-free diabetes duration, defined as the disease duration minus years of known diabetic retinopathy, with the concentration of CML in memory T-cells. AU, arbitrary units

Discussion

Intracellular concentrations of both oxidation-dependent (CML) and oxidation-independent (MG-derived and 3-DG-derived) AGEs are increased in long-lived CD45RA$^+$ lymphocytes from diabetic patients. Our data suggest that diabetes-induced intracellular oxidative stress has a critical role in the pathogenesis of diabetic retinopathy. There was no correlation of intracellular CML concentrations with mean glycohaemoglobin concentrations, suggesting that the level of intracellular oxidative stress is rate-limiting for CML formation. This was confirmed by in vitro experiments with cultured lymphocytes. These results indicate that intracellular concentrations of oxidation-dependent AGEs in long-lived CD45RA$^+$ lymphocytes reflect relative risk for diabetic retinopathy independently of glycohaemoglobin concentrations.

The diabetes-induced increase in intracellular AGEs described in this report are consistent with observations described previously [9]. Our finding of a 20-fold increase of MG-derived AGEs in long-lived lymphocytes is consistent with both the 10-fold increase in flux through the MG-pathway in endothelial cells incubated in 30 mmol/l glucose for 7 days, and the long integrated exposure time of CD45RA$^+$

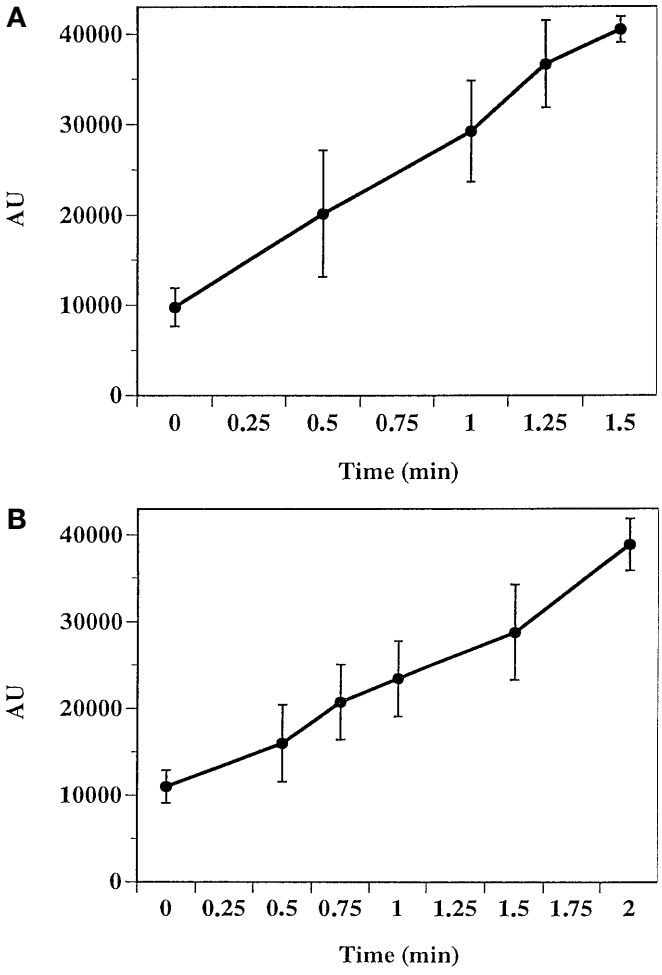

Fig. 2 A, B. Intracellular CML formation by menadion exposure. **A** Time-dependent increase of CML formation in lymphocytes exposed to 2 μmol/l menadione. Values are mean (± SD) of three independent experiments. **B** Concentration-dependent increase of CML formation in lymphocytes exposed for 2 min to menadione. Values are mean (± SD) of three independent experiments. AU, arbitrary units

cells to hyperglycaemia. 3-DG-derived AGEs are also found in a variety of diabetic tissues [15] and increase in diabetic erythrocytes 1.8-fold over normal.

Quantitatively, CML accumulates threefold in dermal skin collagen of diabetic patients, compared with age-matched control subjects [14].

Diabetes-induced CML formation on extracellular proteins correlates weakly with glycohaemoglobin [14] suggesting in diabetes no increase in oxidative stress in the extracellular space, while our data suggest that diabetes does increase oxidative stress in the intracellular space. In support of this hypothesis are recent reports of no change in oxidized amino acids in skin collagen from diabetic patients and of hyperglycaemia-induced increases in reactive oxygen species inside cultured endothelial cells [17, 18].

Diabetic retinopathy shows familial clustering [4], suggesting genetic control of individual patients' susceptibility to hyperglycaemia-induced tissue damage. Our study is consistent with the hypothesis that genes governing susceptibility to microvascular complications are possibly involved in the generation or prevention or both of intracellular reactive oxygen species [19]. A role for intracellular oxidative stress in the pathogenesis of diabetic retinopathy is supported by the recent observation of a positive effect of vitamin E treatment on early diabetic retinopathy [20]. Despite the obvious limitations of a cross-sectional study, we believe that monitoring intracellular concentrations of increased oxidant stress in long-lived $CD45RA^+$ lymphocytes by markers such as CML might identify a subgroup of patients at high risk for microvascular complications.

Acknowledgements. This study was partly supported by the German Research Council grant Ha 1755/1-3 (H-PH), by National Institutes of Health grant DK-33861 (MB), and by a grant from Alteon, Inc., Ramsey, NJ, USA. The anti-imidazolone antibody was kindly provided by Dr. T. Niwa, Nagoya, Japan. We acknowledge the constant help of E. Mäser and we acknowledge gratefully the participation of the patients in this study.

References

1. Klein R, Klein BE, Moss SE (1996) Relation of glycemic control to diabetic microvascular complications in diabetes mellitus. Ann Intern Med 124: 90–96
2. Kofoed-Enevoldson A, Borch-Johnson K, Kreiner S, Nerup J, Deckert T (1987) Declining incidence of persistent proteinuria in type 1 (insulin-dependent) diabetic patients in Denmark. Diabetes 36: 205–209
3. Klein R, Klein BEK, Moss SE et al. (1984) The Wisconsin epidemiologic study of diabetic retinopathy: II. Prevalence and risk of diabetic retinopathy when the age at diagnosis is less than 30 years. Arch Ophthalmol 102: 520–526
4. The Diabetes Control and Complications Trial Research Group (1997) Clustering of long-term complications in families with diabetes in the diabetes control and complications trial. Diabetes 46: 1829–1839
5. American Diabetes Association (1998) Tests of Glycemia in Diabetes. Diabetes Care 21 [Suppl 1]: S69–S71
6. Brownlee M (1994) Lilly lecture 1993. Glycation and diabetic complications. Diabetes 43: 836–841
7. Kato H, Shin DB, Hayase F (1987) 3-Deoxyglucosone crosslinks proteins under physiological conditions. Agricultural Biological Chemistry 51: 2009–2011
8. Thornally PJ (1990) The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. Biochem J 269: 1–11
9. Shinohara M, Thornalley PJ, Giardino I et al. (1998) Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endoytosis. J Clin Invest 101: 1142–1147
10. Ahmed MU, Thorpe SR, Baynes JW (1986) Identification of N^{ε}-(carboxymethyl-)lysine as a degradation product of fructose lysine in glycated proteins. J Biol Chem 261: 4889–4894
11. Fu M-X, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR (1995) The advanced glycation end product,

N^{ε}-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. J Biol Chem 271: 9982–9986
12. Michie CA, McLean A, Alcock C, Beverly PCL (1992) Lifespan of human lymphocyte subsets defined by CD45 isoforms. Nature 360: 264–265
13. Davis MD, Hubbard LD, Tarutman J (1985) Studies of retinopathy; methodology assessment and classification with fundus photographs. Diabetes 35 [Suppl 3]: 42–49
14. Schleicher ED, Wagner E, Nerlich AG (1997) Increased accumulation of the glycoxidation product N^{ε}-(carboxymethyl)lysine in human tissues in diabetes and aging. J Clin Invest 99: 457–468
15. Niwa T, Katsuzaki T, Miyazaki S et al. (1997) Immunohistochemical detection of imidazolone, a novel advanced glycation end product, in kidneys and aortas of diabetic patients. J Clin Invest 99: 1272–1280
16. Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75: 241–251
17. Wells-Knecht MC, Lyons TJ, McCance DR, Thorpe SR, Baynes JW (1997) Age-dependent increase in ortho-tyrosine and methionine sulfoxide in human skin collagen is not accelerated in diabetes. J Clin Invest 100: 839–846
18. Giardino I, Edelstein D, Brownlee M (1996) BCL-2 expression or antioxidants prevent hyperglycemia-induced formation of intracellular advanced glycation endproducts in bovine endothelial cells. J Clin Invest 97: 1422–1428
19. Kowluru RA, Kern TS, Engerman RL, Armstrong D (1996) Abnormalities of retinal metabolism in diabetes or experimental galactosemia. III. Effects of antioxidants. Diabetes 45: 1233–1237
20. Kunisaki M, Bursell SE, Clermont AC et al. (1995) Vitamin E prevents diabetes-induced abnormal retinal blood flow via the diacylglycerol-protein kinase C pathway. Am J Physiol 269: E239–E246