CLINICAL STUDY

Increased N ϵ -(carboxymethyl)-lysine levels in cerebral blood vessels of diabetic patients and in a (streptozotocin-treated) rat model of diabetes mellitus

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Abstract

Objective: Non-enzymatic glycation of proteins and their end products (advanced glycation end products, AGE) have been implicated in the pathogenesis of diabetic complications. Our aim was to evaluate the association between diabetes mellitus (DM) and the accumulation of one of the most abundant AGEs, N ϵ -(carboxymethyl)-lysine (CML), in cerebral vessels.

Research design and methods: Brain tissue samples were obtained by autopsy from 20 DM patients and 13 age-matched controls. In addition, we investigated brain tissue samples of seven rats after induction of diabetes with streptozotocin (STZ) and six non-diabetic control rats. We used an immunohistochemical staining method to examine the CML immunoreactivity in the cerebral vessels.

Results: Staining intensity of CML was significantly higher in cerebral vessels of diabetic patients than in non-diabetic subjects (median of the immunohistochemical intensity score/cm² in the diabetic group of 0.85 (interquartile range (IQR) 0.66–1.52) vs 0.63 in the control group (IQR 0.44–0.70); $P=0.002$). Furthermore, there was a similar significant difference in CML staining intensity of cerebral vessels between STZ diabetic rats and non-diabetic control rats (median of the immunohistochemical intensity score/cm² in the diabetic group of 1.08 (IQR 0.73–1.43) vs 0.23 in the control group (IQR 0.12–0.43); $P=0.003$).

Conclusions: Accumulation of CML-modified proteins is significantly greater in the cerebral vessels of the diabetic patients than their age-matched controls. This association has been confirmed in the insulin-deficient diabetic rat model. It may be possible that the excessive accumulation of AGE-modified proteins in the cerebral vasculature alters the local environment and microcirculation and thereby contributes to the development of cognitive impairments in diabetes. Therefore, additional study on the causal link between AGE accumulation and cognitive dysfunction and the potential benefits of AGE-blocking and/or breaking compounds is indicated.

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Introduction

Prolonged exposure to chronic hyperglycaemia triggers abnormalities resulting in the occurrence of well-described diabetic complications, including nephropathy, retinopathy and peripheral neuropathy (1). Besides these long-term complications, diabetes mellitus (DM) can also affect the brain function. This may ultimately lead to a state of cognitive impairment, in particular, psychomotor slowing and reduced mental flexibility (2). In addition to this diabetes-associated cognitive decline (DACD) (3), DM increases the risk of dementia, cerebral atrophy and leukoencephalopathy not directly attributed to infarction (4). In recent years, it has become increasingly clear that vascular dysfunction plays a crucial role in the manifestation of long-term

diabetic complications. It is believed that chronic hyperglycaemia influences endothelial cell function and thereby causes vascular derangements. The precise mechanisms that link hyperglycaemia to vascular disease have not yet been established, although several hypotheses have been proposed. One such hypothesis focuses on non-enzymatic glycation and oxidation, suggesting that glycoxidation reactions and oxidative stress are important factors in the pathogenesis of vascular dysfunction and diabetic complications (5). In this process, amino groups in proteins, lipids and nucleic acids exposed to reducing sugars undergo a series of molecular rearrangements, resulting in the irreversible formation of a heterogeneous class of sugar-derived adducts. As a group, the late products of this cascade have been termed advanced glycation end

products (AGEs) (5). One of the most abundant and best characterized AGE is Nε-(carboxymethyl)-lysine (CML) (6) that can also be formed from oxidation of free fatty acids. AGE formation occurs during normal ageing, but is enhanced in DM, as a result of the associated hyperglycaemia (6). Since the formation of CML is irreversible, it has been suggested that CML may provide indirect evidence for the cumulative oxidative damage to the respective tissue. Of particular interest is the finding that CML levels in tissue and serum are related with the severity of several diabetic complications (7, 8).

In an attempt to elucidate whether the AGE accumulation in the cerebral vessels of diabetic patients is significantly higher than in non-diabetic individuals, we investigated the immunohistochemical distribution of CML in post-mortem brain tissue from subjects with DM and age-matched controls. The study was extended by studying the CML distribution in the brains of hyperglycaemic streptozotocin (STZ)-treated diabetic rats and normoglycaemic rats as controls.

Materials and methods

Subjects

Representative brain tissue samples from the cortex (one region per patient) were obtained by autopsy from 20 DM patients and 13 age-matched controls. None of the controls had a clinical history of DM or elevated blood glucose levels. The mean ages of the DM cases and the controls were 61.9 years (range 9–86 years) and 66.9 years (range 7–92 years) respectively. The DM cases and controls were clinically and historically without neurological disorders, including acute disorders such as cerebrovascular events and brain oedema caused by head injury. Details of the patients are given in Table 1.

Animal model

Two groups of adult male Wistar rats (Charles River, Maastricht, The Netherlands), weighing ~250 g, were used (as described previously, (9)), namely a control group (n=6) and a diabetic group (n=7). Diabetes was induced by a single i.p. administration of STZ (60 mg/kg; Sigma). STZ diabetes was confirmed by measuring the blood glucose level 3 days after the induction. Diabetes was verified by a serum glucose level >13.9 mmol/l. After 6 weeks of STZ diabetes, animals were killed by decapitation, followed by harvesting of brain tissues. In the end, we obtained brain tissue from seven diabetic rats and six non-diabetic controls.

The protocol was performed in compliance with the European Community guidelines for the use of experimental animals and approved by the National Authorities for Animal Experiments.

Table 1 Characteristics of study subjects.

Age (years)	Type of DM	Clinical diagnosis, cause of death	Brain weight (g)
DM patients (n=20)			
9	1	Arrhythmia	1342
22	1	DKA	1470
25	1	Opioid abuse	1500
25	1	Arrhythmia	1500
41	1	CVA	1510
52	2	CMV pneumonia, ARDS	1690
55	2	Acute renal failure, myocardial infarction	1146
59	2	Myocardial infarction	1133
67	2	Ruptured AAA	1120
69	2	Pericarditis, broncho-pneumonia	1475
73	2	Myocardial infarction	1530
76	2	Chronic B-lymphocytic leukaemia, pneumonia	1355
78	2	Adenocarcinoma of the lung, broncho-pneumonia	1457
81	2	Myocardial infarction	1360
81	2	Pneumonia, sepsis	1290
84	2	Myocardial infarction	1225
84	2	Pulmonary thrombo-embolism	1220
85	2	Pneumonia, sepsis	1230
86	2	Sepsis	1115
86	2	Bronchopneumonia	1100
Controls (n=13)			
7		Arrhythmia	1157
40		Small cell carcinoma, bronchopneumonia	1279
55		Small cell carcinoma, bronchopneumonia	1650
59		Chronic alcohol abuse, hepatic insufficiency	1064
66		Sepsis	1401
68		Hypopharynx carcinoma, rupture of common carotid artery	1450
72		Adenocarcinoma of the lung, broncho-pneumonia	1515
83		Myocardial infarction	1182
87		Pancreatic ductal carcinoma, broncho-pneumonia	1190
92		Sepsis	1193
73		Myocardial infarction	1356
78		Myocardial infarction	1297
89		Bronchopneumonia, myocardial infarction	1324

DM, diabetes mellitus; DKA, diabetic ketoacidosis; CVA cerebrovascular accident; ARDS, acute respiratory distress syndrome; CMV, cytomegalovirus; AAA, abdominal aortic aneurysm.

Immunohistochemical staining methods

To investigate the presence and accumulation of CML in tissues, a monoclonal anti-CML antibody was used as described recently (10).

All brain tissue samples, both humans and rats, were fixed in 4–6% buffered formaldehyde, pH 7.4,

followed by processing and embedding in paraffin wax, as routinely performed. From each block ($\sim 5 \text{ cm}^2$ brain tissue), $4 \mu\text{m}$ sections were cut and placed on silanized slides. Subsequently, the formalin-fixed and paraffin-embedded tissue sections were deparaffinized, incubated with $0.3\% \text{ H}_2\text{O}_2$ in methanol for 30 min and enzymatically pre-treated with 0.1% pepsin. Sections were not heated to prevent artificial heat-induced CML formation (11). After washing steps with PBS (pH 7.4) and further blocking by normal bovine serum (1:50; Dako, Glostrup, Denmark), sections were incubated with anti-CML (1:500, Dako) for 60 min. After further washings, sections were incubated for 30 min with a rabbit anti-mouse biotin-labelled secondary antibody (1:500; Dako), rinsed with PBS and incubated with streptavidin–horseradish peroxidase (1:200; Dako) at room temperature for 60 min. The colour reaction was carried out by incubating the slides with 3,3-diamino-benzidine-tetrahydrochloride/ H_2O_2 (Sigma Chemical Company) for 5 min. After a final washing, counterstaining of the nuclei with haematoxylin was performed.

Immunoscoreing

Quantification of the immunohistochemical staining was determined visually in a microscopic study. In each slide, the positively stained vessels were scored for the intensity of CML staining. Therefore, each positive vessel was given a score of 1 (meaning weak positive staining), 2 (moderate staining) or 3 (strong positive staining). Next, each intensity score was multiplied by the amount of vessels positive for this score. Each multiplication score (for 1, 2 and 3) was then added and the sum score was divided by the total slide area (in cm) giving an immunohistochemical score per cm^2 .

Statistical analysis

Data are presented as median (interquartile range, IQR). Comparison of two study groups was performed using Mann–Whitney U test. A P value <0.05 was considered statistically significant. The relationship between age and staining intensity was evaluated using regression analysis. All analyses were performed with the statistical package SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Human tissue

The presence of CML in human brain tissue, obtained from DM and non-DM patients, was investigated. Immunohistochemical analysis showed CML staining in the astrocytes (not shown) and the endothelial cells of the cerebral vasculature (Fig. 1), as described previously (12). We now focused on and quantified the intensity of the CML staining in the cerebral vessels in DM and non-DM brain tissues. None of the tissue samples showed morphological damage of the vessels or any sign of inflammation. Significant differences in the immunohistochemical intensity score of CML staining were evident between DM patients and age-matched controls (Fig. 2). The CML immunoreactivity of the cerebral vessels was significantly more intense in the DM group than in the controls (median of the immunohistochemical intensity score/ cm^2 in the DM group of 0.85 (IQR 0.66–1.52) vs 0.63 (IQR 0.44–0.70) in the control group; $P=0.002$). In controls, the CML immunoreactivity was characterized by numerous weak positive-stained vessels (no difference in the minor (score 1) and moderate (score 2) intensity scores for CML in controls and diabetes), whereas the tissue samples of DM patients showed more pronounced staining (median of the

Figure 1 CML staining of human cerebral blood vessels in a patient with diabetes. Arrows indicate CML staining of endothelial cells of cerebral blood vessels.

Figure 2 Semiquantitative intensity scoring of CML immunoreactivity of cerebral vessels in diabetes patients and age-matched controls. The horizontal lines represent the median of the immunohistochemical score per cm² (defined in Materials and methods) for CML in control patients (squares) and DM patients (triangles). The difference in immunoreactivity between the control and DM groups was statistically significant (*P*=0.002).

immunohistochemical intensity score/cm² in the DM group of 0.67 (IQR 0.49–1.28) vs 0.29 (IQR 0.18–0.46) in the control group; *P*=0.002), especially in the severe intensity score (score 3) (Table 2). Furthermore, five of the DM patients (four with diabetic nephropathy and one with glomerulosclerosis caused by haemolytic uraemic syndrome) and four of the patients (from which two had end-stage renal disease, one with chronic pyelonephritis and one with nephrolithiasis) in the control group were known with kidney abnormalities. Interestingly, the immunohistochemical intensity scores of the DM patients were around the median. In addition, the kidney function was not statistically different in both groups: the median plasma creatinine level in the DM group was 124 µmol/l (IQR 101–180) and the control group was 140 µmol/l (IQR 75–259), *P*=0.87.

We also evaluated whether CML immunoreactivity correlated with the individual's age. Simple regression analysis, however, found no such association in either group (data not shown).

Table 2 Semiquantitative intensity scoring of *N*ε-(carboxymethyl)-lysine (CML) immunoreactivity of human cerebral vessels.

	Controls <i>n</i> = 13	Diabetes mellitus <i>n</i> = 20	<i>P</i> value
Immunohistochemical score per cm ²			
Minor	0.01 (0.00–0.04)	0.00 (0.00–0.00)	0.07
Moderate	0.19 (0.13–0.34)	0.19 (0.04–0.22)	0.20
Severe	0.29 (0.18–0.46)	0.67 (0.49–1.28)	<0.001

Immunohistochemical scores per cm² for the three different intensity scores for CML (minor, moderate, severe; defined in detail in Materials and methods) are expressed as median (interquartile range, IQR). Significance was determined with the Mann–Whitney test.

Rat tissue

The immunohistochemical staining observed in the rat model resembles that in human tissue. Again, all the brain tissue slides showed a distinct staining for CML, but with a marked difference in mean immunohistochemical intensity score between STZ-induced diabetic rats and non-diabetic control rats (Fig. 3). Non-diabetic rats showed only minor staining of cerebral vessels. In these rats, the stained vessels were mostly weak positive, with no strong staining of any cerebral vessel (Table 3). The tissue samples of the diabetic rats, however, had a more pronounced staining pattern, with a significant higher score of CML in DM compared with the non-diabetic controls (median of the immunohistochemical intensity score/cm² in the diabetic group of 1.08 (IQR 0.73–1.43) vs 0.23 in the control group (IQR 0.12–0.43); *P*=0.003).

Discussion

In this study, we demonstrated the deposition of CML-modified proteins in the cerebral vessels of diabetic and non-diabetic subjects. The overall intensity of the CML immunoreactivity was significantly increased in the vessels of diabetic patients compared with non-diabetic controls, although some positive staining pattern was also observed in each of the controls. In DM patients, the vessels defined as strongly positive for CML staining (severe/score 3) were more than twofold increased compared with the controls. In a previous study, we had no indication for ongoing oxidation in post-mortem human intramyocardial blood vessels (13). Therefore, we think that the increased CML staining in the DM patients compared with the age-matched control group

Figure 3 Semiquantitative intensity scoring of CML immunoreactivity of cerebral vessels in streptozotocin-induced diabetic rats (DM rats) and non-diabetic controls (non-DM rats). The horizontal lines within the bars represent the median of the immunohistochemical score per cm² (defined in Materials and methods) for CML in control rats (squares) and DM rats (triangles). The difference in immunoreactivity between the control and DM groups was statistically significant (*P*=0.003).

Table 3 Semiquantitative intensity scoring of N ϵ -(carboxymethyl)-lysine (CML) immunoreactivity of rat cerebral vessels.

	Controls <i>n</i> =6	Diabetes mellitus <i>n</i> =7	<i>P</i> value
Immunohistochemical score per cm ²			
Minor	0.23 (0.08–0.35)	0.53 (0.40–0.75)	0.01
Moderate	0.04 (0.00–0.08)	0.40 (0.16–0.67)	0.003
Severe	0.00 (0.00–0.00)	0.10 (0.00–0.25)	0.036

Immunohistochemical scores per cm² for the three different intensity scores for CML (minor, moderate, severe; defined in detail in Materials and methods) are expressed as median (interquartile range, IQR). Significance was determined with the Mann–Whitney test.

should be attributed to CML accumulation in the cerebral vasculature during life.

To further explore this finding of CML accumulation, we compared the CML staining intensity of cerebral vessels in STZ-induced diabetic rats and non-diabetic control rats. This model confirmed our findings with human cerebral vessels, as more extensive staining was seen in the cerebral vessels of the diabetic rats. In contrast to the situation in human brain tissues, the rat study showed significant differences in CML immunoreactivity in all three intensity scores for CML.

Previous studies have emphasized a possible link between the accumulation of AGEs in the vessel wall and the diabetic late complications accompanying chronic hyperglycaemia. Indeed, the CML immunoreactivity has been found in the vasculature of the tissues prone to diabetic late complications, such as kidney, retina, heart (13) and peripheral nerves (14). There are several mechanisms by which increased AGE deposition may damage the organs and the vasculature. In general, AGE modification of proteins can alter vascular cell function, interfere with normal cellular interactions and pathologically modify gene expression (15). The consequences of these AGE-induced changes in vascular function are the generation of damaging free radicals, focal thrombosis, impaired vasodilatation, luminal narrowing and excessive vascular inflammation (16).

Several systemic reviews have unequivocally shown that DM is associated with accelerated cognitive decline and dementia (2, 17, 18). Cognitive impairments have been described in both children and adults, as well as in both type 1 and type 2 diabetes. This DACD may be due to cerebral microvascular damage. Analogous to well-known diabetes-related (micro)vascular complications, such as peripheral diabetic neuropathy and diabetic retinopathy, chronic hyperglycaemia may result from a cascade of events in DACD. Moreover, retinopathy and peripheral neuropathy in type 1 diabetes patients have been shown to be predictive of DACD (19, 20). We have shown that type 1 diabetes patients with proliferative retinopathy showed a different cerebral activation pattern, using functional MRI, than those without retinopathy to a cognitive test (21). Reske-Nielsen *et al.* described, more than 30 years

ago in autopsy studies, the changes in the cerebral microvasculature derived from diabetes patients (22). Since then, not much additional data have been published concerning structural abnormalities in the diabetic brain and the cerebral microvasculature.

In our work and in much of the cited studies, it is acknowledged that AGE modification of proteins is relevant to the pathophysiology of these vascular changes. AGE distribution in the vasculature of the kidney, retina and peripheral nerves has extensively been investigated. In addition, Gironès *et al.* (12) have examined the topographical distribution of CML reactivity in the brain of patients with Alzheimer's disease and diabetes, but they did not quantify this. In our study, we have focused on and quantified the AGE accumulation in the vasculature of the diabetic brain, both in humans and rats. We concluded that DM is associated with excessive accumulation of AGE-modified proteins in the cerebral vasculature. Because vascular function may be adversely affected by AGE, it may be possible that AGEs contribute to the development of cognitive impairments in diabetes.

The cognitive impairment in young and old type 1 diabetes patients is only modest, whereas the cognitive impairment in older type 2 diabetes patients is more pronounced (17, 23). This might be due to differential effects of type 1 and type 2 diabetes on the brain.

The major limitation of our study is that no relation has been made between the intensity of AGE accumulation and the severity of the cognitive decline. Therefore, the future studies with a rat model of (type 1 or 2) diabetes should focus on cognitive function and the association with AGE accumulation. The data reported here emphasize the need of further study into a causal link between AGE accumulation in cerebral vessels and cognitive dysfunction. Understanding the contribution of AGEs should provide insight into the complex aetiology of any diabetes-related cognitive disorder. Moreover, it is clear that studies should be developed to find out the effect of AGE inhibitors as aminoguanidine (24) on AGE accumulation in cerebral vessels and cognitive impairment.

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