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Skin collagen pentosidine and fluorescence in diabetes were predictors of retinopathy progression and creatininemia increase already 6 years after punch-biopsy

Michel Sternberg [a,*], Jocelyne M'bemba [b], Paul Urios [a,c], Anne-Marie Borsos [a,d], Jean-Louis Selam [b], Jacques Peyroux [a], Gérard Slama [b]

[a] Équipe "Protéines Modifiées, Protéases et Physiopathologie de l'Endothélium Vasculaire", Laboratoire de Pharmacologie, Faculté de Pharmacie, Université René Descartes, Paris, France
[b] Centre de Diagnostic et de Thérapeutique, Diabétologie, Hôtel-Dieu de Paris, France
[c] Laboratoire de Biochimie, Hôpital Bichat, Paris, and Université Paris VI, France
[d] Laboratoire de Biochimie, Hormonologie et Génétique Moléculaire, Hôpital Ambroise-Paré, 92100 Boulogne and Université Paris VI, France

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ABSTRACT

Objective: Advanced glycation end products (AGEs) of collagens appear to contribute to microvascular complications in diabetes. Do high concentrations of AGEs in skin collagen predict accelerated progression of these complications after 6 years and indicate the need for tighter anti-diabetic treatment?

Design and methods: We measured two AGE parameters in collagen extracted from skin punch-biopsies: pentosidine and fluorescence at 370/440 nm, as markers and predictors of microvascular complications, in 30 patients with diabetes (14 type-1, 16 type-2) without renal insufficiency, and in age- and gender-matched normoglycemic controls, followed at Hôtel-Dieu in Paris.

Results: *At the time of biopsy*, marked increases in pentosidine ($p = 0.0014$) and fluorescence ($p = 0.0001$) expressed per collagen hydroxyproline, were found in the patients with diabetes versus the controls. A significant effect of age was found for pentosidine, but not fluorescence, measurements in the normoglycemic controls. Therefore pentosidine but not fluorescence results were corrected for age in the patients. Pentosidine and fluorescence were correlated with diabetes duration. Fluorescence was significantly dependent on retinopathy presence and score in type-1 and type-2 diabetes, whereas pentosidine was not. Fluorescence was correlated with microalbuminuria only in type-1 diabetes. Neither fluorescence nor pentosidine were correlated with creatininemia. *Already six years after biopsy*, retinopathy score progression and creatininemia increase were significantly correlated with initial pentosidine and fluorescence measurements.

Conclusions: These AGEs are good predictors of progression of microvascular complications and appear to be pathogenic. High skin concentrations of AGEs should induce tighter anti-diabetic treatment.

1. Introduction

Advanced glycation end products (AGEs) appear to contribute to the lesions characteristic of diabetic microvascular complications. Indeed inhibitors of advanced glycation, particularly aminoguanidine and pyridoxamine, have been shown to prevent development of retinopathy [9] and albuminuria [22] in diabetic animals [20]. Injection of AGE-Albumin (but not normal albumin) in rats induced albuminuria; this was prevented by aminoguanidine [26]. AGEs are formed particularly on long-lived collagens, in which they can cross-link peptidic chains.

Sell et al. [21] noted a significant relationship between skin collagen amounts of the AGE pentosidine adjusted for age and a score of complications in patients with type-1 diabetes. This was also similarly observed in type-1 diabetes by McCance et al. [10] and Beisswenger et al. [2]. Genuth et al. [7] showed the predictive value of skin collagen AGE carboxymethyllysine amounts for progress of complications after 10 years of type 1 diabetes evolution. We decided to study two AGE parameters of skin collagen: pentosidine (a specific cross-linking glycoxidation product formed by arginine and lysine linked by a carbohydrate-derived cycle) and fluorescence at 370–440 nm (characteristic of the presence of conjugated double bonds in various AGEs, constituting therefore a global parameter) in patients with diabetes followed at Hôtel-Dieu in Paris, in both type-1 and type-2 diabetes. The aims of our study were to test these AGE parameters as markers of microvascular complications, at the time of biopsy, but also as

* Corresponding author at: Equipe de Recherche sur la Biochimie et la Pharmacologie des Vaisseaux et du Rein, Laboratoire de Pharmacologie, Faculté de Pharmacie, Université René Descartes, Paris, France.
E-mail address: michel.sternberg@wanadoo.fr (M. Sternberg).

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predictors of the evolution of microvascular complications after 6 years, including the increase of creatininemia as a criterium of nephropathy. Positive correlations between AGE parameters and the degree or progression of complications would favor a pathogenic role of AGEs in the development of human diabetic microangiopathy. They could serve as an argument for tighter anti-diabetic treatment.

2. Subjects and methods

2.1. Subjects

The thirty patients with diabetes studied (including Caucasian and African-French patients), aged 26 to 62 years, were followed at the Diabetology and Ophthalmology Units of Hôtel-Dieu hospital in Paris, with duration of diabetes of 0.1 to 31 years. Diabetes had been formerly diagnosed according to the WHO criteria (fasting blood glucose >7 mmol/l or glycemia >11.1 mmol/l two hours after oral administration of 75 g glucose). Two subgroups of patients could be distinguished essentially on a clinical basis: 14 patients with type 1 diabetes and 16 patients with type 2 diabetes. Type 1 diabetes was generally discovered before 27 years of age, typically ketosis-prone with a BMI ≤26 kg/m^2 and required insulinotherapy within 3 months of diagnosis. Type 2 diabetes was generally discovered after 35 years of age with a BMI ≥27 kg/m^2 and did not require insulinotherapy at least during the first 8 years of evolution. Renal insufficiency (creatinine clearance <55 ml/min) was an exclusion criterium since it increases plasma and tissue AGE concentrations. Age- and gender-matched informed healthy volunteer controls (from 26 to 62 years old) from the Hospital and the University staff were studied in parallel.

At time 0 (T_0) a skin punch-biopsy was performed for collagen fluorescence, pentosidine and hydroxyproline determinations. All patients were followed for 6 years; the presence and importance of microvascular complications were evaluated regularly and particularly at T_0 and after 6 years (T_6) by retinopathy score, micro or macroalbuminuria, creatininemia, presence of peripheral neuropathy. For this study approval was given by the Hospital Ethics Committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale) and individual informed written consent, in accordance with the principles relating to the Declaration of Helsinki.

2.2. Skin collagen isolation and biochemical determinations

Skin punch-biopsy (4 mm diameter) was carried out only at T_0 from the buttocks, the specimen washed in saline, then frozen in liquid nitrogen. All patients and controls had negative tests for hepatitis B and C and for HIV. Collagen was extracted according to Sell et al. [21]: briefly, the skin specimen was homogenized, using an Ultra-Turax apparatus; the insoluble fraction was washed and delipidated, then submitted to hydrolysis by a specific collagenase (type VII collagenase catalog No. 0773, Sigma, St. Louis, USA); the soluble hydrolysate containing collagenic peptides was first assayed for fluorescence using a spectrofluorimeter (Kontron Instruments, Zurich, Switzerland) at an excitation wavelength of 370 nm and an emission wavelength of 440 nm; it was then submitted to further hydrolysis by 6 M HCl. In the final hydrolysate**,** pentosidine was measured by High-Performance Liquid Chromatography according to Sell et al. [21], using a Waters 600E apparatus with a fluorimetric detector Waters 474 (excitation wavelength 335 nm and emission wavelength 385 nm) and a Vydac C18 column type 218TP104; the pentosidine standard was given by Vincent Monnier (Case Western Reserve University, Cleveland, Ohio); hydroxyproline was determined photometrically [29]. Fluorescence intensity was expressed as arbitrary units/μmol hydroxyproline; pentosidine as pmol/μmol hydroxyproline (1 μmol hydroxyproline corresponding generally to 0.92 mg skin collagen).

2.3. Clinical parameters

Retinopathy score was determined according to the « Societé Francophone du Diabète» (SFD) recommendations [www.sfdiabete.org], using a simplified scoring scale inspired by the ETDRS research group classification [6]: 0 = no retinopathy; 1 = minimal background retinopathy; 2 = nonproliferative retinopathy; 3 = preproliferative retinopathy; 4 = proliferative retinopathy. Progressive retinopathy was defined in this study by an increase of the retinopathy score between T_0 and T_6; but patients presenting the maximal score of 4 already at T_0 were also included in this group. *Neuropathy* was searched according to the SFD recommendations based particularly on the use of Semmes–Weinstein monofilament.

2.4. Clinical biochemistry parameters

HbA1c was determined by HPLC (Diamat), micro-and macroalbuminuria using immunoturbidimetry (Image, Beckman), plasma creatinine by the Jaffé method. Creatinine clearance was calculated by the Cockroft equation [5]. The integrated mean of the trimestrial HbA1c determinations was calculated in all patients during a 2 year period preceeding the biopsy (this period was shorter for two patients whose type-2 diabetes was discovered 1 year and 1 mo respectively before the biopsy): the integrated mean will be presented as HbA1c [T_{-2},T_0].

2.5. Statistical methods

A result is generally presented as a mean (M) ± standard error or as M and (range). For comparisons between patients and their age-matched healthy controls, *paired Student t test* was performed; this test was also used for comparing quantitative parameters in patients with diabetes at T_0 and T_6; in this test each result is compared with that of the age-matched control (or that of the same subject 6 years before) which is subtracted and the difference tested versus zero. *Correlation coefficient* was determined between two quantitative parameters; if *r* is significant, the equation of the correlation straight line is given. *Analysis of variance* was carried out for studying association between a quantitative parameter and several qualitative scores; it was followed by comparison between groups using *Bonferoni-Student t test*; if only 2 qualitative scores were studied, then a simple *Student t test* was performed. Threshold of statistical significance was $p = 0.05$. Pentosidine was corrected for age in patients with diabetes as indicated in Results Section 3.2.

3. Results

3.1. Characteristics of the patients with diabetes and the controls

The characteristics of patients are presented in Table 1. At the time of biopsy (T_0) the patients with type-2 diabetes were treated with oral antidiabetic drugs (76%) or with insulin (24%); those with type-1 diabetes were all treated with insulin. After 6 years of follow-up, two patients with type-2 diabetes were deceased and five patients were lost to sight (3 with type-2 and 2 with type-1 diabetes).

HbA1c at T_0 varied from 6.0% to 15.3% (M = 8.9%). The integrated mean HbA1c during the 2 years preceeding the biopsy, HbA1c [T_{-2},T_0], was found to be 8.7 ± 0.4%. HbA1c at T_6 varied from 5.7% to 9.9% (M = 7.7%). The decrease in HbA1c percentage mean at T_6 was significant ($p = 0.035$ by Student paired t test).

Microvascular complications at T_0. The frequency of retinopathy as well as the mean values and ranges of creatininemia and creatinine clearance in type-1, type-2 and all diabetic patients are shown in Table 1. As designed in the inclusion criteria, creatinine clearance was >55 ml/min in all patients at T_0. Microalbuminuria (>30 mg/d and <300 mg/d) was present in 36% of type-1, 7% of type-2 and 21% of all diabetic patients; macroalbuminuria (>300 mg/d) was present only in