Amino Acids (1993) 4: 111–119

Amino
Acids

In vivo monitoring of serum protein cross linking in patients with diabetes mellitus. Evidence for pharmacological modification of immunoglobulin G cross links

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Accepted July 3, 1992

Summary. It is well known that increased cross linking of proteins due to non-enzymatic glycosylation occurs in diabetic animals and humans leading to accumulation of proteins (e.g. collagen). This in turn is strongly associated with diabetic long term complications.

We developed a noninvasive method for studying in vivo cross linking and its pharmacological inhibition by L-arginine in a blind placebo controlled study with crossing over of two treatment periods of three months each.

Glycemic control was assessed by determining blood glucose, HbA1c, fructosamine, and total glycosylated hemoglobin. The patients were randomly assigned to two treatment groups A ($n = 14$) and B ($n = 16$). 20 healthy volunteers served as controls. Treatment consisted of two daily dosages of 1 g L-arginine free base. Cross linking of a human serum protein (IgG) was assessed by SDS polyacrylamide gel electrophoresis and subsequent Western blotting.

Diabetic patients showed a statistically increased number of cross links compared to normal controls (Group A: 3.6 vs 2.0 bands, group B: 3.8 vs 2.0 bands). L-arginine led to a significant reduction of cross links in both treatment groups (Group A: 3.6 to 2.1 bands, group B: 3.8 to 2.5 bands).

The described noninvasive method for assessing in vivo cross linking requires only μl amounts of serum and could serve to monitor protein cross linking in patients with diabetes mellitus.

Keywords: Amino acids – Western blot – Immunoglobulin G – Cross linking-polymerization – Diabetes mellitus – 3-Deoxyglucosone

Introduction

Long term complications of diabetes mellitus are strongly associated with advanced stage nonenzymatic glycosylation of proteins. Nonenzymatic glycosylation (Maillard reaction) is a reaction in which reducing carbohydrates are nonenzymatically and nonspecifically bound to proteins. Up to the formation of Amadori compounds, this process is regarded as early stage nonenzymatic glycosylation and leads to products such as hemoglobin A1c. The subsequent reactions are considered as advanced stage nonenzymatic glycosylation.

In the advanced stage the proteins become fluorescent and are modified in several respects: they show browning, loss of solubility, reduced susceptibility against proteolytic cleavage and increased cross linking (polymerization) (Schnider and Kohn, 1981; Monnier et al., 1984; Lubec and Pollak, 1980). This cross linking is of major interest in the pathogenesis of diabetic long term complications. It has been shown that cross linking of connective tissue proteins like collagens is responsible for changes of the vessel wall leading to micro – and macroangiopathy (Brownlee et al., 1986). Mediators of the cross linking process can be found within the advanced stage nonenzymatic glycosylation products (Kato et al., 1987; Igaki, 1990). One of the major compounds inducing cross linking of proteins is 3-deoxyglucosone which is able to polymerize proteins within a short period after incubation in vitro.

In vivo evaluation of glucose mediated abnormal cross linking of proteins would be of enormous interest for monitoring diabetic long term complications. To our knowledge this has not been reported in the literature. We therefore tried to develop a noninvasive method for studying the cross linking of proteins in patients with diabetes mellitus. Furthermore, we studied its inhibition by the oral administration of L-arginine in a placebo controlled study in human diabetics.

Patients and methods

Evaluation of protein cross links

We studied cross linking of serum IgG by SDS polyacrylamide gel electrophoresis and subsequent Western blotting.

Blood samples were centrifuged and the sera were kept frozen at −70°C. Electrophoresis was performed on 8% SDS polyacrylamide gel according to the principle of Lämmli (1970). Serum aliquots containing 1000 mg IgG/dl were used for electrophoresis. Sera were added to 40 μl of the stop solution. The stop solution was prepared from 1.2 ml dye (bromphenol blue), 0.6 ml β-mercapto-ethanol and 1.5 g sucrose and was heated in a water bath; 2 ml of this solution were added to 10 ml 10% SDS. The mixture of the sera and the stop solution were incubated for 30 minutes at 60°C. Low and high molecular weight marker proteins (Pharmacia) were used as internal standards. The electrophoresis was run at 135 mA and 320 V.

After electrophoresis the gel was transferred to nitrocellulose using a Trans Blot Cell (BioRad). IgG was immunostained by Western blotting with grade affinity purified goat anti human ($H + L$) IgG – horse radish peroxidase conjugate (BioRad 172-1050)

All 120 samples were analyzed as described. 24 samples were also run without β-mercapto-ethanol in order to analyze differences under nonreducing conditions. The same 24 samples were also run in the presence of 8 M urea in order to rule out nonspecific noncovalent binding. Sera of 20 healthy adults were used as normal controls.

Incubation of serum IgG with 3-deoxyglucosone (DOG)

We compared our in vivo results of cross linking with the in vitro model of Igaki et al. (1990) for non-enzymatic glycosylation mediated cross linking. For this purpose we incubated 6 sera of healthy adults containing 1000 mg/dl IgG with DOG (5 mmol/L) for a period of 28 days and analyzed these sera as described above.

Determination of IgG fluorescence

In order to study the influence of free radical mediated cross linking we determined specific IgG fluorescence at 360 nm excitation and 454 nm emission according to the method described by Jones et al. (1988). The fluorescence of IgG was expressed as the ratio of fluorescence to its UV absorption at 280 nm reflecting free radical oxidation of IgG.

Assessment of glycemic control

Blood glucose was determined by glucoseoxydase method. HbA1c was determined according to a chromatographic standard method. Fructosamine was assessed by a commercially available photometric kit (Roche), total glycosylated hemoglobin by a commercially available affinity chromatographical assay (Isolab).

Placebo controlled study

Patients

40 patients with diabetes mellitus (age 56–78 years) were recruited from the outpatient clinic of the Ist Department of Medicine, University of Vienna. Criteria for inclusion into the study were the diagnosis of diabetes mellitus, the willingness to participate and to accept to take medications and to take part in the follow-up investigations. Exclusion criteria were other connective tissue disorders, glomerulonephritis and a medication known to influence cross linking such as *d*-penicillamine or cortisone.

Study design

We performed a blind placebo controlled study with crossing over of two treatment periods of three months. Patients were randomly assigned to treatment groups A or B.

At the beginning of the study there were no significant differences between the two groups concerning sex, age, weight, type and duration of diabetes, associated conditions and their treatment. At enrollment, a first blood sample was taken from both groups for evaluation of glycemic control ($t1$). After a baseline period without treatment of one month, a second blood sample was taken ($t2$). Thereafter the patients of group A were treated with L-arginine and the patients of group B with placebo for a period of three months. Then, after drawing a third blood sample ($t3$), the treatment was crossed over so that group B received *L*-arginine and group A the placebo for a period of three months. Thereafter, the fourth blood sample ($t4$) was taken.

The study protocol was evaluated and approved by the Ethical Committee of the University of Vienna. Written informed consent was obtained from all patients.

Treatment

Treatment consisted of two daily dosages of 1 g L-arginine free base (sugar, salt and starch free, Solgar Inc., Lynbrook, N.Y., U.S.A., FDA approved) in capsules of 500 mg each. Patients were asked to take the capsules at meals (breakfast and dinner) or thereafter with plenty of fluid. Intake of L-arginine was controlled by interviewing the patients regularly.

Concomitant diseases

Concomitant diseases observed were hypertension ($n = 5$), cirrhosis of the liver ($n = 2$), steatosis of the liver ($n = 1$), asymptomatic cholelithiasis ($n = 1$), osteoporosis ($n = 1$), glaucoma ($n = 1$), cardiac arrhythmias ($n = 1$), stenocardic symptoms ($n = 1$), and bronchial asthma ($n = 1$).

Statistical analysis

Data were analyzed by Statistical Analysis System (SAS Institute, Cary NC) (1985). Differences between groups were analyzed by unpaired t-test if appropriate (Shapiro-Wilk statistic), or by Wilcoxon test. Differences within groups were analyzed by paired t-test if appropriate (Shapiro-Wilk statistic), or by Friedman's two-way analysis for block design. The number of bands was correlated with the intensity of fluorescence and with parameters of glycemic control.

Results

From the initially enrolled 40 patients, 30 (14 of group A and 16 of group B) terminated the study. Eight patients did not reappear, two patients stopped L-arginine medication at $t3$.

Western blots of IgG

IgG Western blots of the sera of the diabetic patients showed an increased number of bands in contrast to the Western blots of the normal controls who never had more than two bands. The number of the clearly visible bands of the diabetic patients are listed in Table 1. Under L-arginine therapy the number of bands (representing cross linking of IgG) was significantly reduced. An example of the typical Western blot of a diabetic patient is shown in Fig. 1.

In the absence of β-mercapto-ethanol, i.e. under nonreducing conditions, numerous bands appeared indicating the presence of reducible IgG and aggregates of IgG (Fig. 2).

The addition of 8 M urea, a chaotropic agent, had no effect on the aggregation of IgG in the diabetic sera, ruling out non specific, non covalent electrostatic binding.

Table 1. Results of in vivo cross linking of IgG expressed by number of bands on the Western blots

	Number of bands (mean ± SD)	
Blood sample	Group A $N = 14$ (therapy period $t2$–$t3$)	Group B $N = 16$ (therapy period $t3$–$t4$)
$t1$	3.6 ± 0.9	3.8 ± 1.1
$t2$	3.5 ± 0.8	3.4 ± 0.9
$t3$	2.1 ± 0.4*	3.7 ± 1.0
$t4$	3.2 ± 0.7	2.5 ± 0.6*

* $p < 0.001$

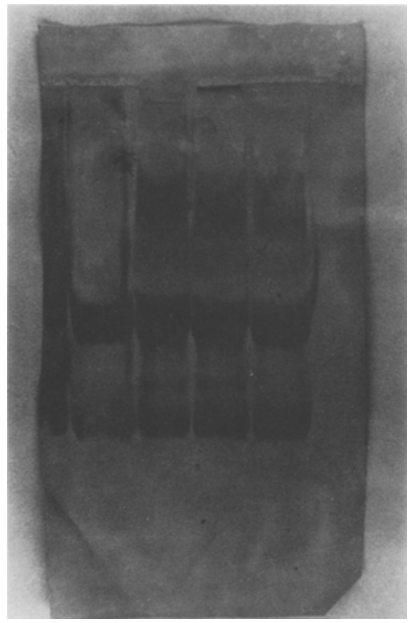

Fig. 1. IgG bands on Western blot of a diabetic patient under reducing conditions. Increased number of bands indicates cross linking (polymerization) of IgG

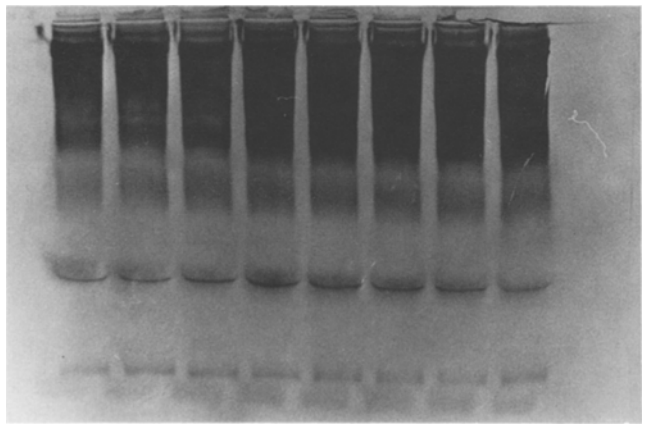

Fig. 2. IgG bands on Western blots of diabetic sera analyzed under nonreducing conditions

Incubation of normal sera with 3-deoxyglucosone led to the formation of multiple bands on the Western blot and resembled those found in the diabetic sera (Fig. 3).

Measurement of the specific fluorescence of IgG in diabetic sera showed no significant differences between the different treatment periods (Table 2).

The results of the assessment of glycemic control in the diabetic patients are listed in Table 3.

There was no correlation between the number of bands in the Western blot and the fluorescence intensity or parameters of glycemic control.

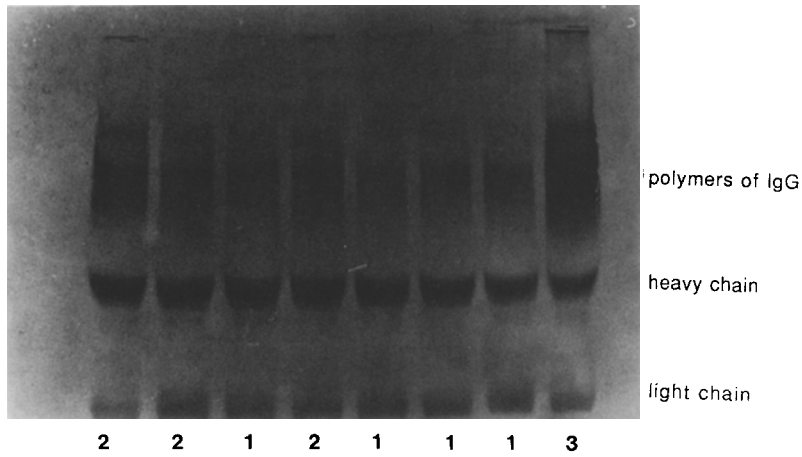

Fig. 3. Western blots of normal sera incubated with 3-deoxyglucosone (*1*) and of sera of diabetic patients (*2*). The molecular weights were identical in both groups. Western blot of normal serum long term incubated with 3-deoxyglucosonet (*3*)

Table 2. Measurement of the specific fluorescence of IgG in diabetic sera

	Arbitrary units (mean ± SD)	
Blood sample	Group A $N = 14$	Group B $N = 16$
$t1$	4.8 ± 1.2	4.4 ± 1.0
$t2$	4.2 ± 0.9	3.9 ± 0.7
$t3$	4.3 ± 1.2	3.9 ± 1.0
$t4$	4.3 ± 1.2	3.9 ± 1.0

Discussion

We describe for the first time the evidence of cross linked IgG in sera of patients with diabetes mellitus. These cross links were significantly suppressed under L-arginine therapy. The cross links were stable to urea thus ruling out electrostatic noncovalent binding. As β-mercapto-ethanol was used in the experiment, the bonds can be regarded as nonreducible cross links.

In a recent study we investigated the effect of L-arginine on glucose mediated cross links in diabetic animals. In this in vivo model we found an inhibitory effect of this amino acid on glucose mediated cross links in vivo. L-arginine rendered collagens soluble, reduced cross linking and advanced stage nonenzymatic glycosylation product mediated fluorescence (Lubec et al., 1990a and b). These observations stimulated us to investigate the influence of L-arginine on the reduction of cross linking in humans. Our placebo controlled study (Lubec et al., 1991, and yet unpublished data) revealed a reduction of cross links after a three months treatment period. Clinically, cross linking of collagen proteins may be studied in repeated skin punch biopsies. However, the invasiveness of

Table 3. Glycemic control (blood glucose, fructosamine, HbA1c, total glycosylated hemoglobin) in diabetic patients

Blood sample		Group A (mean ± SD) $N = 14$	Group B (mean ± SD) $N = 16$
*t*1	Blood glucose	168.4 ± 50.5	212.9 ± 83.6
	Fructosamine	366.2 ± 83.4	353.0 ± 96.0
	HbA1c	7.5 ± 1.6	8.0 ± 2.2
	Tot. glycos. Hb*	10.0 ± 2.2	10.9 ± 3.5
*t*2	Blood glucose	227.1 ± 100.0	193.1 ± 54.6
	Fructosamine	355.0 ± 55.3	364.5 ± 96.9
	HbA1c	7.1 ± 1.4	8.1 ± 2.2
	Tot. glycos. Hb	10.0 ± 2.5	10.5 ± 3.6
*t*3	Blood glucose	207.0 ± 61.0	184.4 ± 97.3
	Fructosamine	329.1 ± 70.4	321.0 ± 88.6
	HbA1c	7.4 ± 1.5	8.2 ± 1.7
	Tot. glycos. Hb	9.7 ± 2.1	10.6 ± 3.3
*t*4	Blood glucose	200.0 ± 89.0	183.9 ± 62.0
	Fructosamine	316.4 ± 45.1	290.5 ± 74.9
	HbA1c	7.8 ± 1.4	7.8 ± 2.2
	Tot. glycos. Hb	9.8 ± 2.3	9.2 ± 3.1

* Total glycosylated hemoglobin

this procedure limits its frequent use. Therefore, evaluation of serum protein cross linking could be used easily in clinical studies. However, it remains to be proven that cross linking of IgG correlates with long term complications of diabetes mellitus as it has been shown for the cross linking of connective tissue proteins. (Monnier et al., 1986).

Our study suggests that low dose L-arginine reduced IgG polymerization. The mechanism responsible for this inhibitory effect of glucose mediated cross links seems to be related to that of aminoguanidine (Brownlee et al., 1986). Aminoguanidine inhibits cross linking by reacting with carbonyl residues of nonenzymatically glycosylated proteins. Igaki showed in vitro that aminoguanidine is able to prevent deoxyglucosone mediated cross linking of Lysozyme (Igaki et al., 1990). We have performed similar studies with L-arginine which was also able to prevent deoxyglucosone mediated cross linking of IgG in vitro (unpublished data). Therefore inhibition of deoxyglucosone mediated cross linking in vivo may explain the positive effect of L-arginine on IgG cross linking in this study. As macrophages and Lymphocytes may be activated by L-arginine (Barbul et al., 1985), the reduction of cross links could also be explained by an activation of macrophages bearing a receptor for nonenzymatically glycosylated products (Vlassara et al., 1984).

Another possible explanation could be an activation of alpha-ketoaldehyde dehydrogenase by L-arginine leading to degradation of cross linkers like deoxyglucosone (Oimomi et al., 1989).

In parallel experiments by fluorescence studies we evaluated free radical mediated autooxidation IgG in order to investigate the role of oxidative stress

which is known to occur in diabetes mellitus (Jones et al., 1988; Lunec, 1983). The absence of a correlation between the number of bands and the fluorescence intensity, i.e. free radical autooxidation, suggests that the cross linking process in the diabetic patients was not mediated by free radicals. On the other hand, the absence of a correlation between the number of IgG bands and the parameters of glycemic control possibly rules out that early stage nonenzymatic glycosylation was the cause of IgG cross linking.

Incubation of human serum with 3-deoxyglucosone resulted in a pattern comparable to that found in the serum of diabetic patients. The similarity of the cross linking pattern in vivo and in vitro points for a role of 3-deoxyglucosone in the cross linking reaction.

We conclude from this study that IgG cross linking may be considered a simple but sensitive and valuable non-invasive tool for the monitoring of nonenzymatic glycosylation of body proteins. Its clinical significance and applicability deserves further studies.

References

Barbul A, Fichell RS, Shimadzu S, et al (1985) Intravenous hyperalimentation with high arginine levels improved wound healing and immune functions. J Surg Res 38: 328–334

Brownlee M, Vlassara H, Kooney T, Ulrich P, Cerami A (1986) Aminoguanidine prevents diabetes induced arterial wall protein cross linking. Science 232: 1629–1632

Igaki N, Sakai M, Hata H, Oimomi M, Baba S, Kato H (1990) Effects of 3-deoxyglucosone on the Maillard reaction. Clin Chem 36: 631–634

Jones AF, Jennings PE, Wakefield A, Winkles J, Lunec J, Barnett AH (1988) The fluorescence of serum proteins in diabetic patients with and without retinopathy. Diab Med 5: 547–551

Kato H, Cho RK, Okitani A, Hayase F (1987) Responsibility of 3-deoxyglucosone for the glucose induced polymerization of proteins. Agric Biol Chem 51: 683–689

Lämmli UK (1970) A cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–682

Lubec G, Bartosch B, Mallinger R, et al. (1990a) The effect of substance L on glucose mediated cross links of collagen in the *db/db* mouse. Nephron 86: 678–694

Lubec G, Bartosch B, Mallinger R, et al. (1990b) The effect of substance L on glucose mediated cross links of collagen in the *kk*-mouse. In: Lubec G, Rosenthal GA (eds) Amino acids. Escom, Leiden, pp 670–675

Lubec G, Pollak A (1980) Reduced susceptibility of nonenzymatically glycosylated glomerular basement membranes to proteases. Renal Physiol; 3: 4–9

Lunec J (1983) Free radical mediated aggregation of IgG. Agents Actions 15: 37–38

Lubec G, Vierhapper H, Bailey AJ, Damjancic P, Fasching P, Sims TJ, Kampel D, Popow C, Bartosch B (1991) Influence of L-arginine on glucose mediated collagen cross links precursors in patients with diabetes mellitus. Amino Acids 1: 35–41

Monnier VM, Kohn R, Cerami A (1984) Accelerated age related browning of human collagen in diabetes mellitus. Proc NAS (USA), 81: 583–587

Monnier VM, Vishwanath V, Frank KE, et al (1986) Relation between complications of type I diabetes mellitus and collagen linked fluorescence. N Engl J Med 314: 402–408

Oimomi M, Hata F, Igaki N, Nakamichi T, Baba S, Kato H (1989) Purification of alpha-ketoaldehyde dehydrogenase from human liver and its possible significance in the control of glycation. Experientia 45: 463–466

SAS User's guide: Statistics (1985) Version 5 th edn. SAS Institute, Cary, NC

Schnider GL, Kohn RR (1981) Effects of age and diabetes mellitus on the solubility and nonenzymatic glycosylation of human skin collagen. J Clin Invest 67: 1630–1635
Vlassara H, Brownlee M, Cerami A (1984) Accumulation of diabetic rat peripheral nerve myelin by macrophages increases with the presence of advanced glycosylation end products. J Exp Med 160: 197–207

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Received January 2, 1992