

Advanced glycation end products-cytokine-nitric oxide sequence pathway in the development of diabetic nephropathy: aminoguanidine ameliorates the overexpression of tumour necrosis factor- α and inducible nitric oxide synthase in diabetic rat glomeruli

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Abstract

Aims/hypothesis. Advanced glycation end products are believed to contribute to diabetic microvascular complications by inducing glomerular damage but their role has not been fully clarified. In this study, we explain their central role in the induction of inducible nitric oxide synthase and production of nitric oxide (NO) in streptozotocin-induced diabetic rat glomeruli.

Methods. Localization of carboxymethyllysine, which is one of the chemical components of advanced glycation end products, glomerular expression of inducible nitric oxide synthase and urinary excretion and glomerular production of $\text{NO}_2^-/\text{NO}_3^-$ were examined at 0, 26, 51, and 52 weeks after the induction of diabetes. Therapeutic effects of aminoguanidine were also examined.

Results. Carboxymethyllysine was detected in the mesangial area in glomeruli and it progressively accumulated during 52 weeks of observation. Immunohis-

tochemistry and hybridization studies in situ showed that the number of inducible nitric oxide synthase-positive cells was notably increased in diabetic rat glomeruli at 52 weeks. Further, this augmented expression paralleled intraglomerular expression of TNF- α and $\text{NO}_2^-/\text{NO}_3^-$ in diabetic rat glomeruli. Treatment with aminoguanidine reduced the expression of TNF- α , inducible nitric oxide synthase and intraglomerular $\text{NO}_2^-/\text{NO}_3^-$ production. It also ameliorated proteinuria in diabetic rats.

Conclusion/interpretation. This study showed that carboxymethyllysine possibly enhances the expression of inducible nitric oxide synthase by stimulating the expression of TNF- α in diabetic rat glomeruli. The carboxymethyllysine-cytokine-NO sequence pathway could be one of the major mechanisms in the development of diabetic nephropathy. [Diabetologia (1999) 42: 878–886]

Keywords Advanced glycation end products, aminoguanidine, nitric oxide, diabetic nephropathy.

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Abbreviations: AGEs, Advanced glycation end products; CML, carboxymethyllysine; STZ, streptozotocin; NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial cell nitric oxide synthase; iNOS, inducible type nitric oxide synthase; nNOS, neuronal type nitric oxide synthase; SD rats, Sprague Dawley rats.

It is widely considered that persistent hyperglycaemia is the primary causal factor in the development of diabetic nephropathy [1]. Advanced glycation end products (AGEs) are believed to be one of the major factors which contribute to chronic diabetic complications. We previously reported that carboxymethyllysine (CML), which is one of the chemical components of AGEs, accumulates in human and rat diabetic glomeruli [2, 3].

In recent studies, it has been shown that nitric oxide (NO), a labile soluble compound, is important in cell signalling, vasodilatation and cell-mediated immunity [4]. Nitric oxide is synthesized from L-arginine and molecular oxygen by the enzyme NO syn-

Table 1. The number of rats killed each week

No of killed rats	0 wk	26 wk	51 wk	52 wk	Total
Control	5	5	5	5	20
STZ		5	5	5	15
STZ + AG		5		5	10
STZ + anti TNF			5	5	10
STZ + rabbit IgG			5	5	10

wk: week, AG: aminoguanidine, antiTNF: anti-TNF α antibody

these (NOS), of which there are several distinct isoforms. Endothelium-derived NOS (*e*NOS) and neuronal NOS (*n*NOS) are constitutive, Ca²⁺-dependent, agonist-triggered enzymes. The inducible type NOS (*i*NOS) is induced at the transcriptional and translational levels by endotoxin or various cytokines or both. Induction of this isoform has been shown in a variety of cells and tissues such as macrophages [5], vascular smooth muscle and mesangial cells [6]. It possibly has important pathophysiological activities, by generating large amounts of NO with cytotoxic and cytostatic effects.

It has recently been shown that AGEs stimulate the expression of IL-1 and TNF- α on macrophages and mesangial cells through binding to AGE receptors *in vitro* [7]. These cytokines are known to induce the expression of *i*NOS on glomerular mesangial cells and macrophages [8]. These findings suggest it is pos-

sible that AGEs induce the expression of *i*NOS by enhancement of cytokine expression. In our study using streptozotocin (STZ)-induced diabetic rats, we verify the hypothesis that CML contributes to glomerular damage by enhancement of *i*NOS expression through the overexpression of inflammatory cytokines in diabetic glomeruli. We examined the localization of CML and the expression of *i*NOS and TNF- α in glomeruli using an immunohistochemical method. In addition, mRNA expression of *i*NOS in glomeruli was examined by a nonradioactive *in situ* hybridization technique. Moreover, we explain the therapeutic effects of aminoguanidine on accumulation of CML and expression of *i*NOS and TNF- α in glomeruli and on urinary protein excretion in STZ-induced diabetic rats.

Materials and methods

Animals. The principle of laboratory animal care of the National Institute of Health (NIH) guideline was followed in all these experiments. Male Sprague Dawley (SD) rats were purchased from Charles River Japan (Yokohama, Japan). Male SD rats weighing about 120 g (4 weeks of age) were used in this study. These rats received a standard chow and water diet.

Induction of diabetes. Diabetes was induced in rats as described previously [9]. Five control rats (of total $n = 20$) were killed at 0, 26, 51 and 52 weeks after the injection with buffer.

Table 2. Changes in metabolic data after induction of diabetes

	Week 0	Week 26	Week 51	Week 52
(<i>n</i>)	(5)	(5)	(5)	(5)
Plasma glucose (mmol/l)				
Control	5.2 \pm 0.2	5.0 \pm 0.2	4.8 \pm 0.3	4.9 \pm 0.4
Diabetic		22.9 \pm 0.8 ^a	23.0 \pm 1.3 ^a	24.5 \pm 1.2 ^a
Diabetic + AG		22.6 \pm 1.0 ^a	23.3 \pm 0.4 ^a	23.3 \pm 0.6 ^a
Diabetic + Rabbit IgG		n. d.	22.9 \pm 0.5 ^a	22.7 \pm 0.7 ^a
Diabetic + Anti-TNF		n. d.	24.6 \pm 1.4 ^a	24.5 \pm 1.3 ^a
Fructosamine (μ mol/l)				
Control	222 \pm 19	253 \pm 15	317 \pm 15	321 \pm 17
Diabetic		525 \pm 39 ^a	729 \pm 13 ^a	721 \pm 17 ^a
Diabetic + AG		517 \pm 44 ^a	706 \pm 4 ^a	711 \pm 4 ^a
Diabetic + Rabbit IgG		n. d.	700 \pm 5 ^a	707 \pm 7 ^a
Diabetic + Anti-TNF		n. d.	700 \pm 5 ^a	707 \pm 9 ^a
Body weight (g)				
Control	117 \pm 2	510 \pm 14	675 \pm 16	681 \pm 17
Diabetic		278 \pm 10 ^a	305 \pm 7 ^a	301 \pm 14 ^a
Diabetic + AG		280 \pm 13 ^a	310 \pm 7 ^a	312 \pm 8 ^a
Diabetic + Rabbit IgG		n. d.	303 \pm 6 ^a	309 \pm 6 ^a
Diabetic + Anti-TNF		n. d.	294 \pm 8 ^a	294 \pm 8 ^a
Urinary Albumin Excretion (mg/day)				
Control	3.4 \pm 0.5	5.4 \pm 0.9	17.0 \pm 3.3	16.2 \pm 2.7
Diabetic		60.8 \pm 3.0 ^a	125.0 \pm 7.5 ^a	122.2 \pm 7.2 ^a
Diabetic + AG		60.6 \pm 3.6 ^a	16.5 \pm 1.1	21.8 \pm 4.9
Diabetic + Rabbit IgG		n. d.	105.0 \pm 14.0 ^a	105.8 \pm 10.0 ^a
Diabetic + Anti-TNF		n. d.	110.2 \pm 10.3 ^a	99.8 \pm 8.1 ^a

Data are means \pm SEM, n. d.: not done, AG: aminoguanidine

^a $p < 0.05$ vs control rats

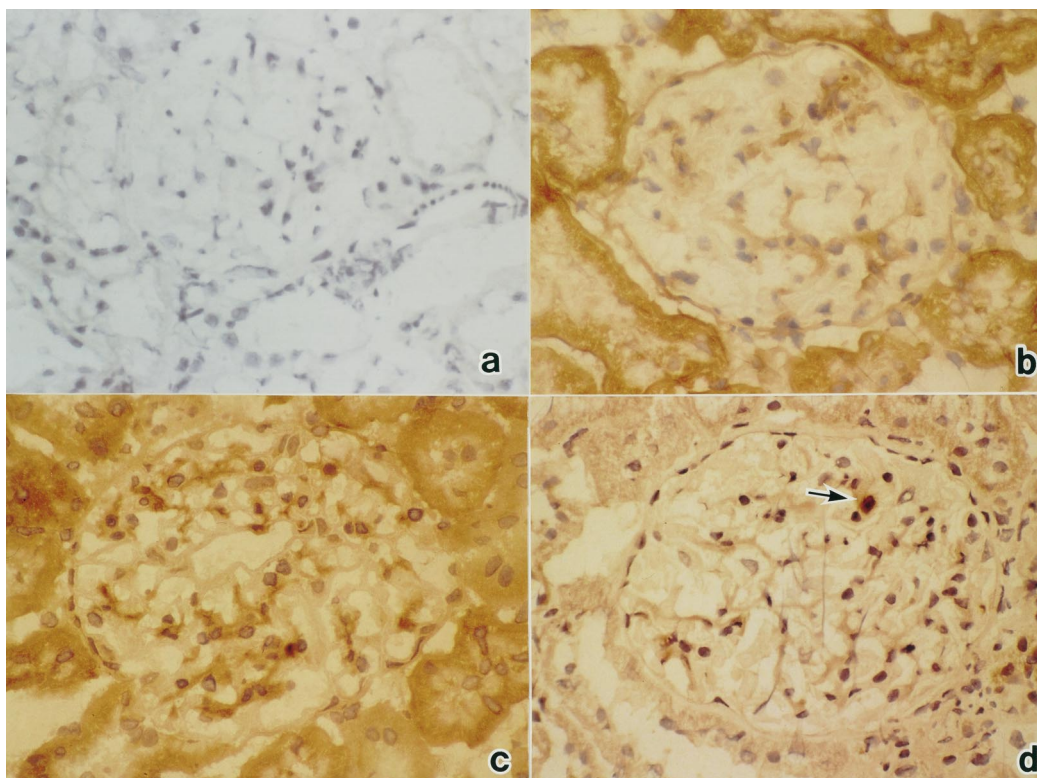


Fig. 1.a–d Immunoperoxidase staining for CML with monoclonal anti-CML antibody in normal-rats at 0 weeks (**a**) and 52 weeks (**b**) and in STZ-induced diabetic rats at 52 weeks (**c**) and in diabetic rats treated with aminoguanidine at 52 weeks (**d**). (**c**) Note strong staining in the mesangial regions. (**d**) Note some positive staining in the cytoplasm of the macrophage (arrows). Magnification: $\times 400$

Experimental rats were killed after injection with streptozotocin (STZ) or buffer and processed for immunohistochemical study. To study the therapeutic effects of aminoguanidine treatment, 26 weeks after the injection of STZ ($n = 10$), half ($n = 5$) of the diabetic rats were further selected at random to receive 1 g/l aminoguanidine (Fluka Chemica, Buchs, Switzerland) in the drinking water. Since preliminary studies showed that iNOS expression was not apparent at 26 weeks but was increased at 52 weeks, aminoguanidine was started at 26 weeks to test the therapeutic effects of aminoguanidine. All these rats were killed at 52 weeks and processed for immunohistochemical study. The number of rats killed each week are summarised in Table 1. Metabolic data were measured as described previously [9]. Urine samples were assayed for the stable NO metabolic end products, NO_2 and NO_3 with a nitrate/nitrite assay kit (Cayman Chemical, Ann Arbor, Mich., USA) [10].

Effects of anti-TNF- α antibody. To study the effects of anti-rat TNF- α antibody (Innogenetics, Zwijndrecht, Belgium), 10 diabetic rats of 51 weeks after the injection of STZ ($n = 10$) were divided equally between a treatment group and a control group. From our previous (unpublished) data we know that 3 μg of rat recombinant TNF (1×10^7 U/mg) amplifies to develop proteinuria in rats with nephrotoxic nephritis. The treatment group were given 0.5 mg of this antiserum, which neutralizes about 10 μg of the TNF. The control group were given the

equivalent dose of purified rabbit IgG (Jackson Immuno-research Laboratories, West Grove, Pa., USA). The antibodies were injected subcutaneously every other day for 7 days at which point all rats were killed and processed for immunohistochemical study.

Antibodies. As primary antibodies, we used polyclonal antibodies (mAbs) against rat iNOS (Calbiochem, San Diego, Calif., USA) and rat eNOS (Transduction Laboratory, Ky., USA), biotinylated-anti-CML antibody (6D12) [11] and polyclonal antibody against rat TNF- α (Innogenetics, Zwijndrecht, Belgium). As secondary antibodies, biotinylated goat anti-mouse IgG and biotinylated goat anti-rabbit IgG, both of which lack cross-reaction with rat IgG, were obtained from Jackson Immuno-research Laboratories (West Grove, Pa., USA).

Immunohistochemical procedure and scoring of immunostaining for CML, TNF- α . Immunoreactivity of CML, TNF- α and iNOS was studied with immunoperoxidase techniques by the labelled streptavidin-biotin method [2]. We evaluated the scoring of immunostaining for CML and TNF- α according to a method described previously [12].

Quantification of iNOS-positive cells. Intraglomerular iNOS-positive cells were counted in 50 glomeruli per animal and expressed as the average number per glomerulus.

Histochemical staining procedure for NADH dehydrogenase. The catalytic activity of NOS was shown by enzymatic reduction of nitro blue tetrazolium (NBT) in the presence of NADPH [11].

Oligonucleotide probe. Synthetic antisense and sense oligonucleotide probes (40 bases) of rat iNOS were purchased from Takara Biochemicals (Osaka, Japan). According to the computer-based homology similarity search program (Soft Ware Developing, Toko, Japan) of all nucleic acid sequences in

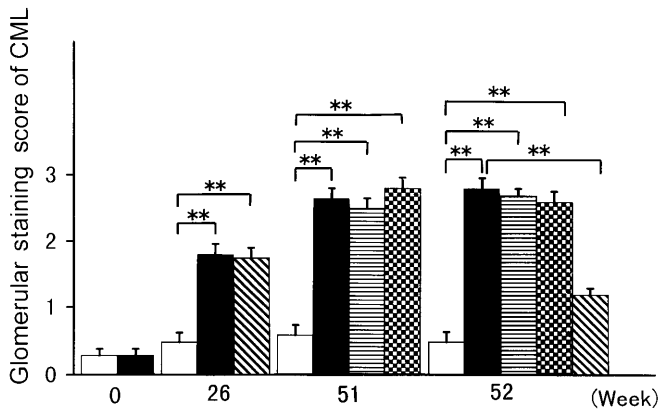


Fig. 2. Glomerular immunohistochemical staining scores for CML at 26, 51 and 52 weeks after STZ injection (closed bars) as compared with age-matched controls (open bars), aminoguanidine-treated diabetic rats (hatched bars), and anti-TNF- α antibody-treated diabetic rats (latticed bars). ** $p < 0.01$; \square CONT, \blacksquare STZ, ▨ STZ + AG, ▩ STZ + rabbit IgG, ▧ STZ + Anti-TNF

dents stored in the GenBank, these oligomers have no homology with any other known nucleic acid sequences. Each probe was labelled with digoxigenin-labelled dUTP using a Dig Oligonucleotide Tailing Kit (Boeringer Mannheim Biochemica, Indianapolis, Ind., USA) according to the manufacturer's instructions.

In situ hybridization. In situ hybridization was done according to a previous report [9].

Glomerular NO₂/NO₃ production. Diabetic rats including those treated with aminoguanidine were anaesthetized with diethylether and glomeruli were isolated by mechanical sieving

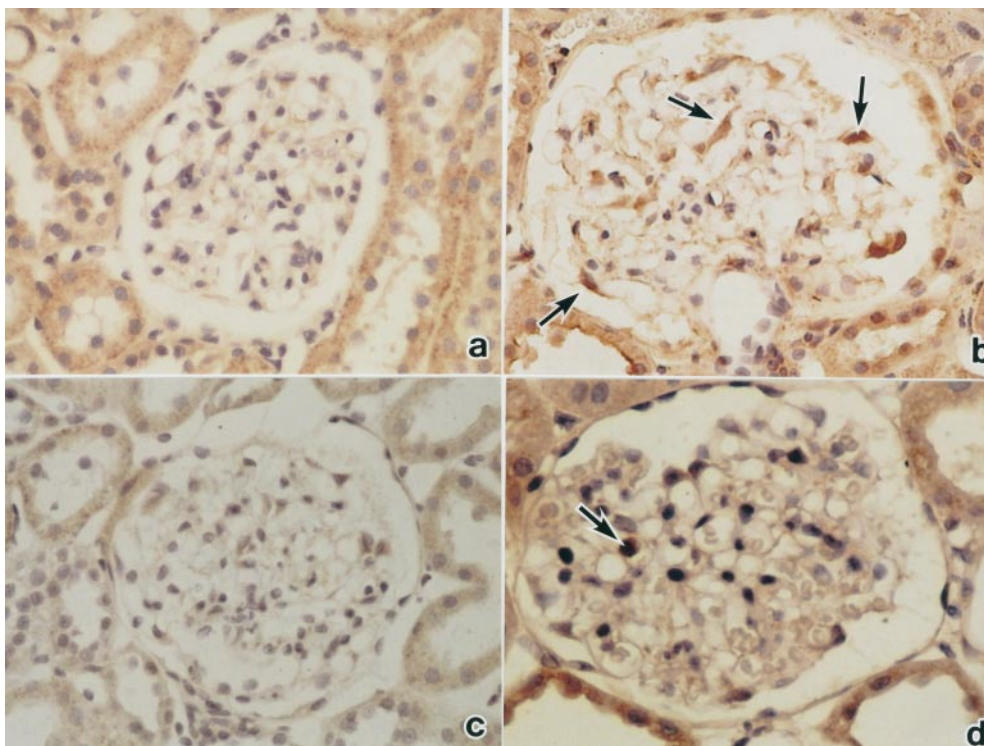
in sterile conditions and plated out in 4X6-well plates at a concentration of 7000 glomeruli per well. Glomeruli were incubated at 37°C in endotoxin-free RPMI 1640 culture medium (Gibco, Rockville, Md., USA) supplemented with 10% fetal calf serum. Nitrite production was determined in the supernatant for 24-h incubation with a nitrate/nitrite assay kit.

Statistical analysis. Values are expressed as means \pm SEM. The significance of the differences between two groups was analysed by Wilcoxon's test. Comparisons among three groups were done by two-way analysis of variance (ANOVA) followed by Scheffe's test to evaluate the significance of the differences between any two groups. A p value of less than 0.05 was considered as statistically significant.

Results

Metabolic data. Body weight, blood glucose, fructosamine are shown in Table 2. All diabetic rats were moderately hyperglycaemic and had lower body weights. An increase in urinary protein in diabetic rats was noted at 26 and 52 weeks. Proteinuria was ameliorated by aminoguanidine at 52 weeks ($p < 0.05$).

Fig. 3. a-d Immunohistochemical localization of iNOS in (a) a control rat kidney at 52 weeks, (b) STZ-induced diabetic rat kidney at 52 weeks, (c) an aminoguanidine-treated diabetic rat kidney at 52 weeks, (d) an anti-TNF- α antibody-treated diabetic rat kidney at 52 weeks. (d) Note that the iNOS-positive cell (arrow) is morphologically macrophage. Magnification: $\times 400$



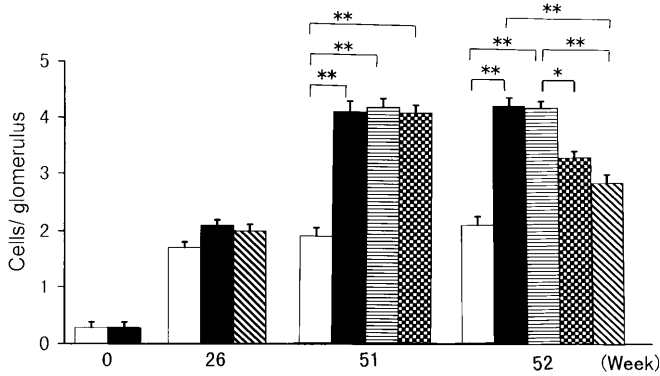
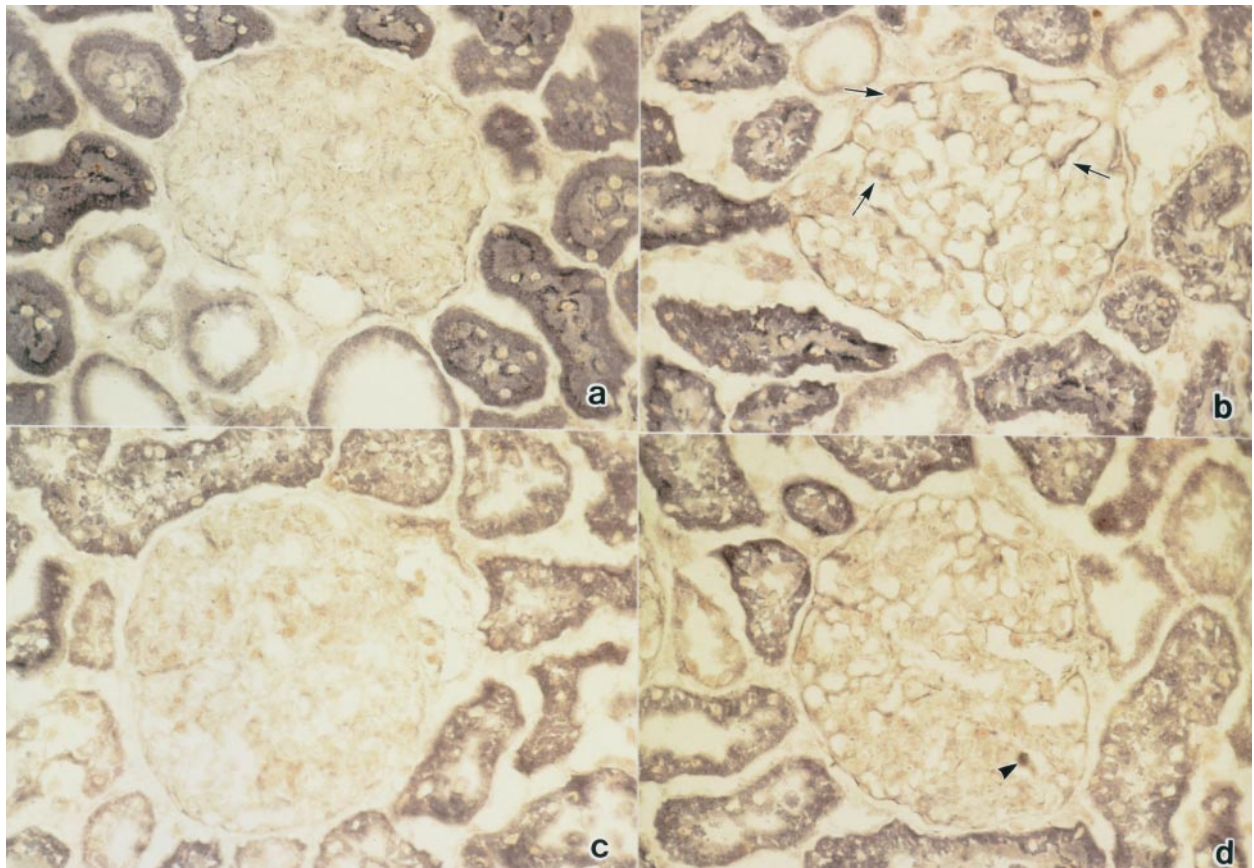


Fig. 4. The number of *i*NOS-positive cells in control rat glomeruli was gradually increased with ageing, which paralleled the CML staining. The number of *i*NOS positive cells in glomeruli of STZ-induced diabetic rats was increased compared with that in control rat glomeruli at 52 weeks. Treatment of diabetic rats with aminoguanidine or anti-TNF- α antibody reduced the number of *i*NOS-positive cells in glomeruli at 52 weeks. * $p < 0.05$, ** $p < 0.01$; □ CONT, ■ STZ, ▨ STZ + AG, ▩ STZ + rabbit IgG, ▩ STZ + Anti-TNF

Fig. 5. a-d NADPH diaphorase staining in (a) a control rat kidney at 52 weeks, (b) a STZ-induced diabetic rat kidney at 52 weeks, (c) an aminoguanidine-treated diabetic rat kidney at 52 weeks, (d) anti-TNF- α antibody-treated diabetic rat kidney at 52 weeks. Magnification: $\times 200$. Arrows indicate NADPH diaphorase activity. (d) Note that the NADPH diaphorase staining-positive cell (arrow head) is morphologically macrophage



CML staining. In normal rat glomeruli, CML staining was barely detected and was weak in the tubules at 0 weeks (Fig. 1a). From 26 to 52 weeks, very faint staining was detected in the glomerular mesangium. The intensity of tubular staining progressively increased with time, i.e., ageing (Fig. 1b). In STZ-induced diabetic rats glomerular staining was evident at 26 weeks and increased at 52 weeks. The glomerular staining score was higher in diabetic rats than age-matched control rats throughout the experimental period. (Fig. 2).

Staining for *i*NOS protein. There was no difference in the extraglomerular distribution of *i*NOS protein by immunohistochemical staining between STZ diabetic and control rats from 0 to 52 weeks (Fig. 3). In control rats, there was only weak staining for *i*NOS within the glomeruli throughout the experimental period (Figs. 3, 4). The number of *i*NOS-positive cells in control rat glomeruli gradually increased with ageing, which paralleled the CML staining. There were more *i*NOS-positive cells in the glomeruli of STZ-induced diabetic rats than in those of the control rats at 52 weeks (Figs. 3, 4). Treatment of diabetic rats with aminoguanidine or anti-TNF- α antibody reduced the number of *i*NOS-positive cells in glomeruli at 52 weeks.

NADPH diaphorase activity. In the glomeruli of STZ-induced diabetes, staining intensity of NADPH dia-

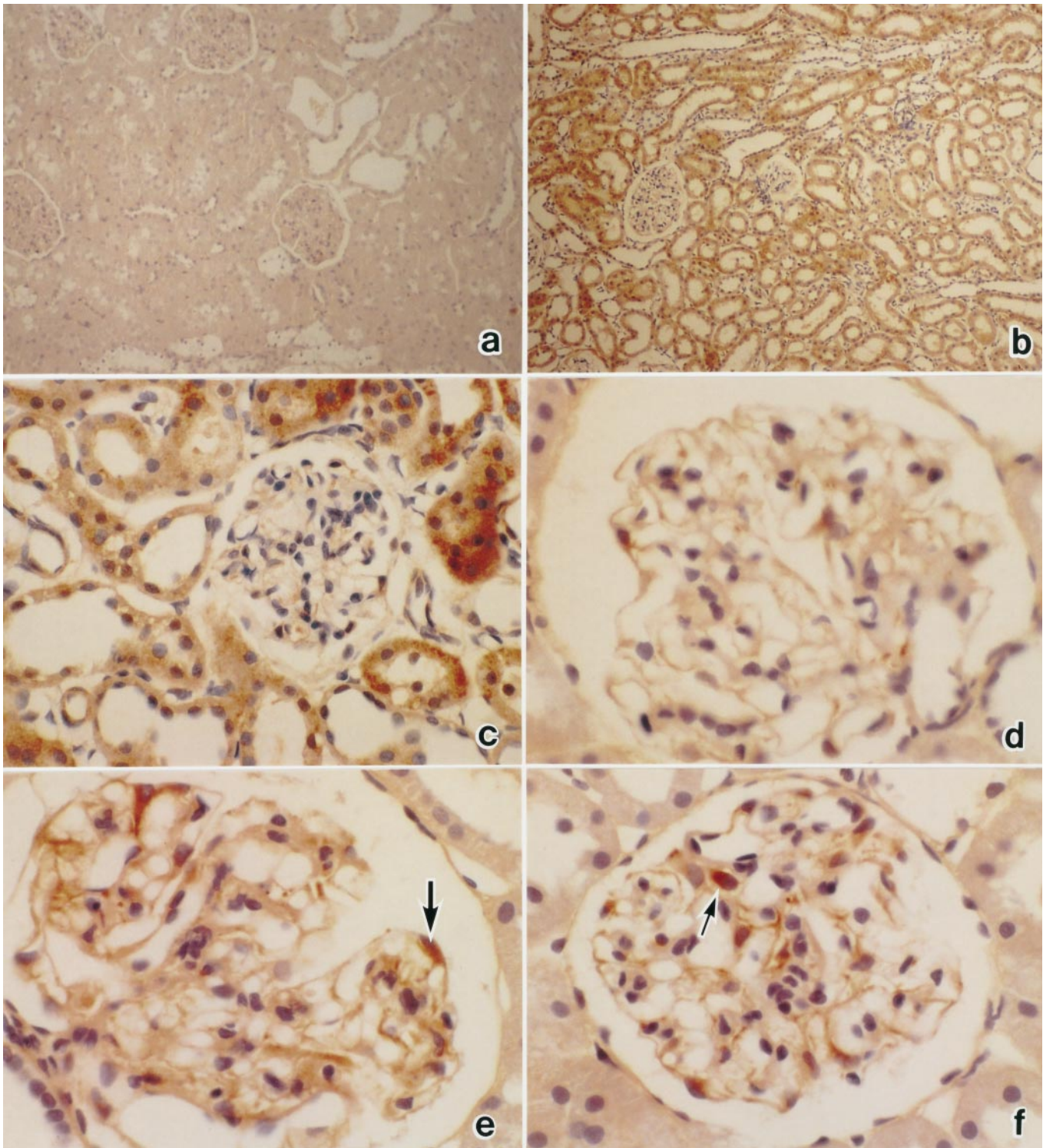


Fig. 6. a–f Localization of iNOS mRNA (a) In STZ-induced diabetic rats at 52 weeks hybridization with the sense probe did not label any renal structures. (b) Hybridization with anti-sense probe yielded positive labelling of cortical thick ascending limb (TAL), proximal tubules, collecting ducts in control rats at 52 weeks. The glomeruli and vasculature of control rats were not significantly labelled. (c) Nearly identical labelling

pattern of the renal tubules was observed in diabetic rats at 52 weeks. (d) iNOS positive cells were rarely seen in control rat glomeruli at 52 weeks. (e, f) In contrast to the control rats, diabetic rats at 52 weeks displayed a strong hybridization signal in the podocytes (e, arrow) and glomerular macrophages (f, arrow). Magnification: a, b $\times 100$, c $\times 400$, d–f $\times 1,000$

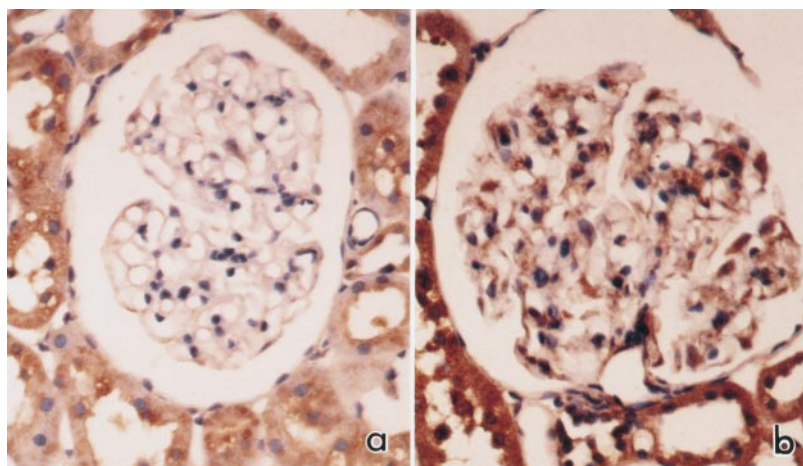


Fig. 7. a, b Immunohistochemical localization of TNF- α in (a) a control rat glomerulus at 52 weeks, (b) a STZ-induced diabetic rat glomerulus at 52 weeks

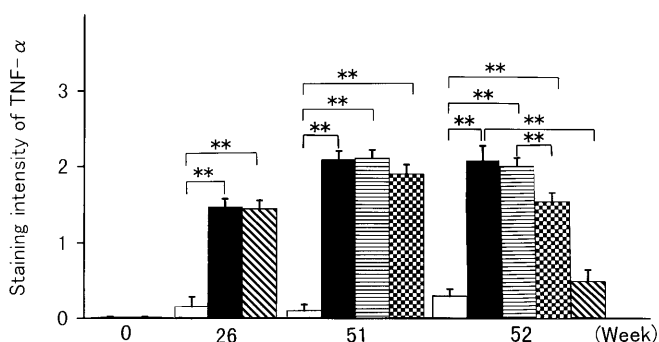


Fig. 8. Expression of TNF- α in STZ-induced diabetic rat glomeruli. The staining intensity of TNF- α in glomeruli of STZ-induced diabetic rats was increased compared with that in control rats at 52 weeks. Aminoguanidine therapy ameliorated its staining intensity. ** $p < 0.01$; □ CONT, ■ STZ, ▨ STZ + AG, ▩ STZ + rabbit IgG, ▤ STZ + Anti-TNF

phorase was higher than in the control animals, thus confirming the results of the immunohistochemical studies for *i*NOS (Fig. 5).

Expression of *i*NOS mRNA in the kidney. In control experiments, hybridization with the sense probe did not show any notable signals both in the normal and STZ-induced diabetic rats (Fig. 6a). The hybridization of kidney sections of the normal rats with the antisense *i*NOS probe yielded positive signals of cortical thick ascending limb (TAL), proximal tubules and collecting ducts (Fig. 6b). The glomeruli and vasculature of control rats showed no signals (Fig. 6b). Similar *i*NOS mRNA expression was detected in the renal tubules of diabetic rats. Diabetic rats at 52 weeks displayed, however, strong hybridization signals in the podocytes (Fig. 6e, arrow) and glomerular macrophages compared with the control rats (Fig. 6f, arrow).

Staining for TNF- α . In control rats from 0 to 52 weeks, there was almost no staining for TNF- α in the glomeruli (Figs. 7a, 8). The increased expression of TNF- α in the glomeruli of the diabetic group was noted at 52 weeks (Figs. 7b, 8). Treatment with aminoguanidine decreased the staining intensity of TNF- α .

Urinary NO_2/NO_3 and glomerular NO_2/NO_3 production. From 26 to 52 weeks, in STZ-induced diabetic rats the urinary NO_2/NO_3 excretion showed no significant difference from the control animals (Fig. 9). At 52 weeks diabetic rats treated with aminoguanidine showed decreased urinary NO_2/NO_3 excretion compared with control and diabetic rats. Although intraglomerular *i*NOS expression was increased at 52 weeks in the diabetic group, urinary NO_2/NO_3 excretion showed no significant differences from the control animals. Therefore, we measured the glomerular NO_2/NO_3 production (Fig. 10).

Diabetic rats had increased glomerular NO_2/NO_3 excretion at 52 weeks compared with control rats (Fig. 10). Aminoguanidine therapy prevented an increase in NO_2/NO_3 in diabetic rats and decreased nitrite concentrations to lower concentrations than those found in control rat glomeruli.

Discussion

Our study shows that intraglomerular AGE (CML) accumulation precedes the increase in the expression of TNF- α and *i*NOS and the production of NO_2/NO_3 in diabetic rat glomeruli. Treatment with aminoguanidine, which prevents AGE formation, reduced the glomerular expression of TNF- α and *i*NOS, and glomerular NO_2/NO_3 production. These data suggest that overexpression of intraglomerular *i*NOS could be caused by a CML-induced cytokine, i.e. TNF- α , in diabetic glomeruli (CML-cytokine-NO sequence pathway). Moreover, intraglomerular expression of *i*NOS paralleled glomerular NO_2/NO_3 production

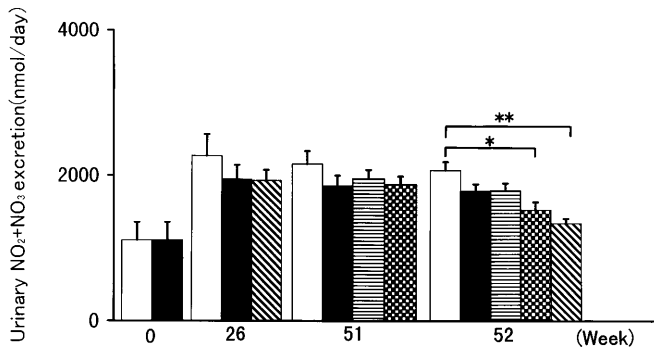


Fig. 9. From 0 to 52 weeks, in STZ-induced diabetic rats the urinary NO₂/NO₃ excretion showed no significant difference from the control animals. At 52 weeks, diabetic rats treated with aminoguanidine showed decreased urinary NO₂/NO₃ excretion compared with control and diabetic rats. * $p < 0.05$, ** $p < 0.01$; □ CONT, ■ STZ, ▨ STZ + AG, ▩ STZ + rabbit IgG, ▪ STZ + Anti-TNF

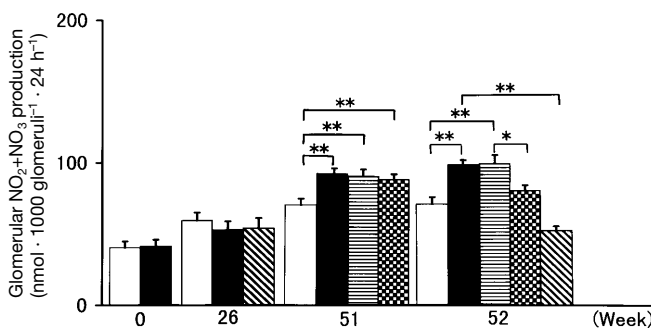


Fig. 10. Diabetic rats had increased glomerular NO₂/NO₃ concentrations at 52 weeks compared with control rats. Treatment with aminoguanidine or anti-TNF- α antibody prevented the increase in NO₂/NO₃ concentrations in diabetic rats and in aminoguanidine-treated diabetic rats decreased these concentrations to even less than that in control rat glomeruli. * $p < 0.05$, ** $p < 0.01$; □ CONT, ■ STZ, ▨ STZ + AG, ▩ STZ + rabbit IgG, ▪ STZ + Anti-TNF

and urinary protein excretion suggesting that overproduction of NO contributes to the development of diabetic nephropathy. Recently, it has been shown that AGE receptors are present on both macrophages and mesangial cells and that certain cytokines, such as TNF- α and interleukin-1 α (IL-1 α) are produced after monocytic uptake and breakdown of AGEs. Treatment of mesangial cells with IL-1 or TNF- α has been shown to induce iNOS [13]. It has been reported that AGE-proteins are possibly involved in the production of TNF- α and IL-1 from macrophages in diabetic nephropathy [14]. These data support our findings in vivo.

Receptors for AGEs provide the cell surface binding sites for AGEs. These receptors are localized on macrophages and mesangial cells [7, 8]. Many macrophages are found in the glomeruli of streptozotocin-induced diabetic rats [15]. These findings suggest

that the AGEs observed in the cells of our study may be explained by those phagocytosed by macrophages in the mesangial regions.

At 52 weeks, diabetic rats had more iNOS-positive cells in their glomeruli and increased nitrite/nitrate production in glomerular supernatant after 24-h incubation than control rats. In the rats with STZ-induced diabetes, staining intensity of NADPH diaphorase was higher than in the control animals, thus supporting the results of the immunohistochemical studies for iNOS. Glomerular AGE staining and the number of iNOS-positive cells correlated with proteinuria. Increased urinary nitrite/nitrate excretion has recently been reported in diabetic rats even though no changes in iNOS protein expression were detected [16]. This difference can possibly be explained by the duration of diabetes in the rats examined. Since an increase of iNOS-positive cells was observed at 52 weeks and not at 26 weeks in our study, the observation period in that study (32 weeks) might have been insufficient to detect the increased expression of iNOS.

Nitric oxide has been implicated in the inhibition of mitochondrial respiration, specifically by knocking out iron containing enzymes such as aconitase, NADH-ubiquinone and succinate-ubiquinone oxidoreductase [17]. Inhibition of glycolysis in parallel with inhibition of the respiratory chain and citric acid cycle will severely impair the cellular energy supply and contribute to the cytotoxic action of NO.

Our study shows that CML may enhance the expression of iNOS by stimulating the expression of TNF- α in diabetic rat glomeruli. The CML-cytokine-NO sequence pathway could be one of the major mechanisms in the development of diabetic nephropathy.

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