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Possible role of tumor necrosis factor and interleukin-1 in the development of diabetic nephropathy

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Possible role of tumor necrosis factor and interleukin-1 in the development of diabetic nephropathy. The possibility that tumor necrosis factor (TNF) and interleukin-1 (IL-1) could participate in the development of diabetic nephropathy was evaluated in streptozocin (STZ)-treated diabetic rats. Diabetic rats were divided into two groups: aminoguanidine treated group (25 mg/kg body wt, daily i.p. injection; DM-AG group) and untreated group (DM group). Non-diabetic age-matched rats were also divided into two groups with the same manner and used as controls. After twelve weeks of treatment, glomerular basement membranes (GBM) were isolated from rats of each experimental group. When thioglycollate-elicited peritoneal macrophages (Mϕ) from normal rats were incubated with these GBM materials, GBM from DM group induced significantly greater levels of TNF and IL-1 production than did GBM from other three groups with at doses of 2.5 to 10 mg. The TNF and IL-1 production by stimulation of GBM from the DM-AG group were similar to those from each control group. Aminoguanidine treatment significantly decreased the accumulation of advanced glycation end-products (AGEs) in GBM of diabetic rats. These findings suggest that AGE-proteins may be involved in the production of TNF and IL-1 from Mϕ. AGE-induced cytokines may be implicated in the development of diabetic nephropathy.

Two major monocyte-macrophage derived cytokines, tumor necrosis factor (TNF) and interleukin 1 (IL-1), have broad biological activities on various target cells. Recently, a possible role of these cytokines in the pathogenesis of various disorders has been suggested [1, 2]. However, there have been no reports so far that associated diabetic nephropathy with these cytokines.

Non-enzymatic glycation of proteins, with subsequent formation of advanced glycation end-products (AGEs), is thought to play an important role in the development of diabetic complications [3, 4]. AGEs accumulate on long lived structural proteins such as collagen under diabetic hyperglycemia, through a complex series of chemical rearrangements of the nonenzymatic addition product of glucose with free amino groups, the Amadori product [5, 6]. It has been suggested that an accelerated formation of AGEs in diabetic patients could cause the structural and functional alterations in tissues involved in microvascular and macrovascular diabetic complications. Furthermore, binding of AGE-modified proteins to macrophages (Mϕ) was recently found to stimulate synthesis and release of TNF and IL-1 [7]. Other previous studies [8, 9] have documented an increase of glycation and AGE accumulation in glomerular basement membrane of streptozocin (STZ)-treated diabetic animals. These findings awake interest in the possibility that cytokines participate in the pathogenesis of diabetic nephropathy.

In this study, we evaluated the possibility that TNF and IL-1 could participate in the development of diabetic nephropathy, using streptozocin (STZ)-treated diabetic rats.

Methods

Animals

Male Lewis rats aged seven to eight weeks (Charles River Japan, Tokyo, Japan) were rendered diabetic by i.v. injection of STZ (60 mg/kg; Upjohn, Kalamazoo, Michigan, USA) dissolved in citrate buffer (pH 4.5). One week after injection, the animals with a blood glucose level above 350 mg/dl were selected for the present study. Then, these diabetic rats were divided into two groups of same blood glucose levels. One received daily i.p. injection of sterile saline alone (DM group). The another group was injected with sterile saline containing aminoguanidine (25 mg/kg body wt, Tokyo Kasei Kogyo Co., Tokyo, Japan), which has been reported to inhibit AGE formation and glucose-derived collagen cross linking in vivo (DM-AG group) [10]. Non-diabetic age-matched rats undergoing similar treatments were used as controls (C group and C-AG group).

Measurement of blood glucose levels and glycated hemoglobin (HbA_1)

Fed blood glucose levels (0900 to 1100 hr) were monitored every two weeks in whole blood obtained by tail snipping and measured by the glucose oxidase method (DRI-CHEM 2000; Fuji Medical System Co., Tokyo, Japan). HbA_1 was measured every four weeks by affinity chromatography using a commercially available kit (Glycohemoglobin-test Wako, Wako Pharmaceutical Co., Osaka, Japan).

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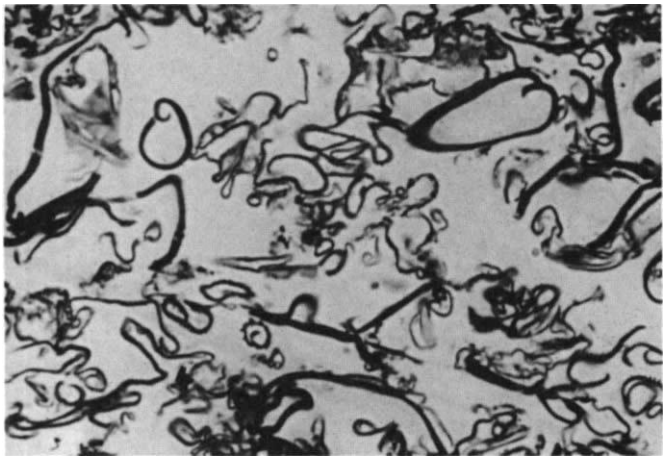

Fig. 1. *Light micrograph of the GBM material.* GBM was fixed in cold 2% glutaraldehyde buffered in sodium cacodylate pH 7.4, embedded in vestpol. One μm sections were stained with Toluidine blue. GBM appears as loops or plates with no cells attached. ×500.

Measurement of urinary albumin

Twenty-four-hour urine collections were obtained every four weeks for the measurement of urinary albumin content. Urinary albumin levels were measured by radioimmunoassay, using rabbit anti-rat albumin (Cappel, West Chester, Pennsylvania, USA) as a first antibody and sheep anti-rabbit IgG (Pharmacia, Uppsala, Sweden) as a second antibody.

Isolation of glomerular basement membranes (GBM)

GBM were isolated according to Cohen and Carlson [11] with minor modification. After twelve weeks of the treatment, rats from each of the four groups were lightly anesthetized with ether and killed by decapitation. The kidneys were rapidly removed from each rat and placed on ice. The decapsulated cortices were separated, and pooled for each group of four to five rats. Glomeruli were isolated by serial sieving and retained on 63 μm nylon screens. Tubular contamination was consistently less than 5%. The glomeruli were suspended in 20 ml of ice-cold 1 M sodium chloride, buffered with 25 mM Tris to pH 7.2, and containing protease inhibitors. They were sonicated in an ice bath using ASTRASON W-380 (Heat System-Ultrasonics, Farmingdale, New York, USA). The sonicator was set at power output "9", cycle rate "5", % duty cycle "50", and used a one minute burst with a one minute cooling time. After total sonication time of 12 minutes, the sonicated materials were passed over a 63 μm nylon screen. The basement membranes was settled by centrifugation at 800 g for 15 minutes at 4°C. GBM fractions were washed several times with 1 M NaCl containing protease inhibitors, followed by distilled water alone. Then, the GBM fractions were weighed and suspended in RPMI medium at a known concentration.

In this procedure, the mean phosphorous content, an indicator of the purity of the preparation, was 0.58 ± 0.052 μg/mg of dry weight GBM (mean ± SEM, $N = 12$), which was measured according to Bartlett [12]. Furthermore, light microscopy indicated that these GBM preparations were free of cells or recognizable cell fragments with a little amount of debris (Fig. 1). These results indicate that this preparation is equally pure when compared with the GBM materials used in previous studies [13, 14].

Preparation of Mϕ

Thioglycollate (TG)-elicited peritoneal Mϕ were obtained according to the method of Kumagai et al [15] from normal Lewis rats (aged 7 to 8 weeks) injected i.p. with 5 ml of 3% TG broth (Difco, Detroit, Michigan, USA) four days before harvest. The purity of Mϕ by this procedure was more than 95%, which was confirmed by cytochemical elastase staining.

Incubation of Mϕ with GBM

Mϕ (1×10^6 cells) were resuspended in 1.5 ml of RPMI supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), and 5% FCS in 24-well tissue culture plates. Time-course of GBM-induced TNF and IL-1 production by Mϕ was evaluated by adding 10 mg (wet weight) of GBM from DM or C group to each well. The samples were collected after 2 to 48 hours of incubation at 37°C in a humidified 5% CO_2 atmosphere. As shown in Figure 4, major peaks of IL-1 and TNF activities were at 24 hours. Therefore, 24 hours of incubation was applied to the comparison among DM, DM-AG, C, and C-AG group. Then, 1.25, 2.5, 5 or 10 mg GBM (wet weight) from each group of rats were added to each well. Control wells contained either Mϕ or 10 mg of GBM alone. After incubation for 24 hours, the supernatants were harvested and stored −20°C before assay for TNF and IL-1. All reagents contained less than 0.050 ng of endotoxin/ml as quantified by the limulus amebocyte lysate assay (LIMUTESTER; Funakoshi, Tokyo, Japan).

TNF and IL-1 assay

TNF levels were assayed by monitoring the lysis of LM cells in the presence of actinomycin D, using human TNFα (Dainippon Pharmaceutical Co., Osaka, Japan) as the standard [16]. IL-1 levels were assayed by the enhancement of C3H/Hej mouse thymocyte proliferation in response to PHA (Difco), using human IL-1α (Dainippon Pharmaceutical Co.) as the standard [17]. The specificities of assays of TNF and IL-1 were studied in the selected samples by blocking experiments with rabbit anti-mouse TNFα (provided by Suntory Laboratory Center, Osaka, Japan) or rabbit anti-mouse IL-1α (kindly provided from Dr. Katsuji Nakano, Dainippon Pharmaceutical Co., Osaka, Japan). Equal volumes of test samples were mixed with anti-mouse TNFα serum (at a dilution of 1:100) or anti-mouse IL-1α serum (at a dilution of 1:200). After incubation at 37°C in 5% CO_2 for 30 minutes, residual TNF or IL-1 activity was determined. Furthermore, to determine whether de novo protein synthesis was required for GBM-induced TNF and IL-1 secretion of Mϕ, cells were treated with cycloheximide (0.2 μg/ml) for 24 hours in the presence of GBM. This condition did not affect the viability of cells as determined by Trypan blue exclusion

Measurement of AGE and nonenzymatic glycation

In a separate experiment, GBM samples were obtained from each group of rats after 12 weeks of aminoguanidine or saline treatment as described above. We used four to five rats per group for the preparation of GBM samples and made five sets of GBM samples in order to derive the mean values. Freeze dried GBM samples were solubilized by incubation for 72 hours at

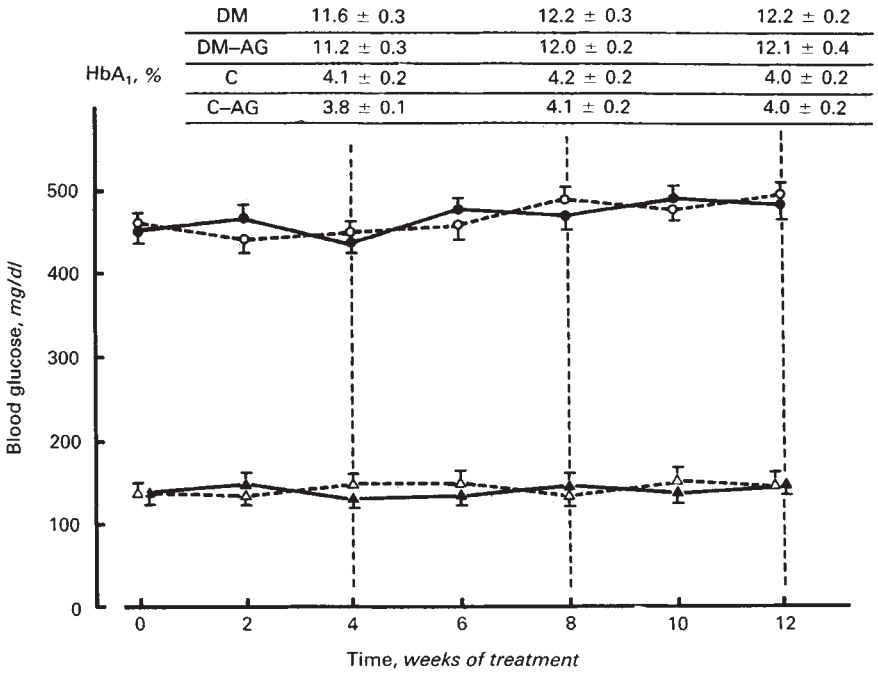

Fig. 2. *Fed blood glucose and HbA_1 profiles in the four experimental groups.* HbA_1 levels in DM and DM-AG rats were significantly elevated ($P < 0.01$) than those in two control groups. Data are shown as means ± SEM. Symbols are: (●—) DM ($N = 15$); (○---) DM-AG ($N = 15$); (▲—) C ($N = 15$); (△---) C-AG ($N = 15$).

37°C with highly purified collagenase (Advance, Linbrook, New York, USA) (1000 U/mg GBM in 0.5 M phosphate buffer containing 1 mM sodium azide, pH 7.4). The solubilized GBM fraction was analyzed by measuring the fluorescence at 440 nm upon excitation at 370 nm, using a Hitachi 650-40 model fluorometer (Hitachi, Tokyo, Japan) [18]. An aliquot of solubilized sample was hydrolyzed with 6 N HCl for 22 hours at 110°C. Then, it was placed on an amino acid analyzer (Shimadzu LC-6A Amino Acids Analysis System, Shimadzu, Kyoto, Japan) for determination of hydroxyproline levels. The results were expressed as fluorescent intensity/μmol hydroxyproline. Portions of the GBM samples were solubilized with 1 N NaOH and nonenzymatic glycation of protein was estimated by a thiobarbituric color assay [19]. Protein was measured by the method of Lowry et al [20].

Statistics

Data are presented as mean ± SEM. Statistical analysis was performed by analysis of variance with significance at $P < 0.05$. Further specific group differences were determined by Duncan's multiple range test.

Results

Fed blood glucose and HbA_1 levels

Fed blood glucose levels in DM and DM-AG groups at the beginning of the treatment were 451.9 ± 14.5 and 455.3 ± 11.4 mg/dl, respectively, (Fig. 2). Thereafter, high blood glucose levels were maintained in these two groups, and aminoguanidine treatment did not affect the blood glucose levels in either diabetic or normal rats. Similarly, DM and DM-AG groups maintained high HbA_1 levels throughout the study, and there were no significant differences between the two groups.

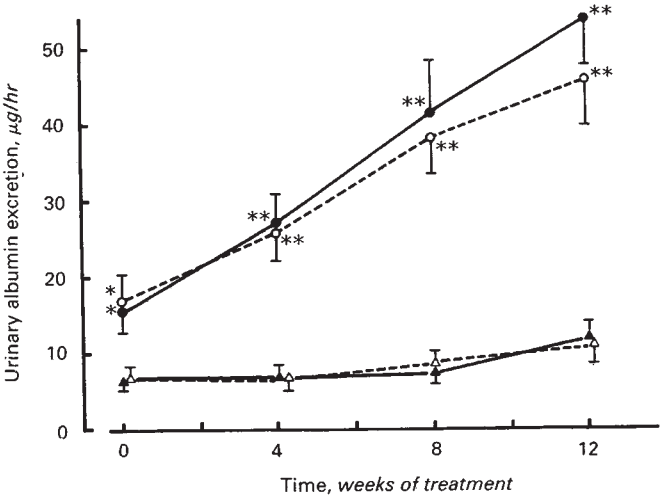

Fig. 3. *Urinary albumin excretion profiles in the four experimental groups.* The urinary albumin excretion in DM and DM-AG rats were significantly elevated at all points. Aminoguanidine treatment did not significantly suppress diabetes induced albuminuria. Data are shown as means ± SEM. Symbols are (●—) DM ($N = 15$); (○---) DM-AG ($N = 15$); (▲—) C ($N = 15$); (△---) C-AG ($N = 15$). *$P < 0.05$, ** $P < 0.01$ vs. C and C-AG group.

Urinary albumin excretion

Diabetic rats already had values significantly elevated ($P < 0.05$) over the normal rats at one week after the injection of STZ (Fig. 3). The excretion rates in DM and DM-AG groups progressively increased until 12 weeks of the study. DM-AG group had slightly lower value than DM group at 12 weeks, however, there were no significant differences.

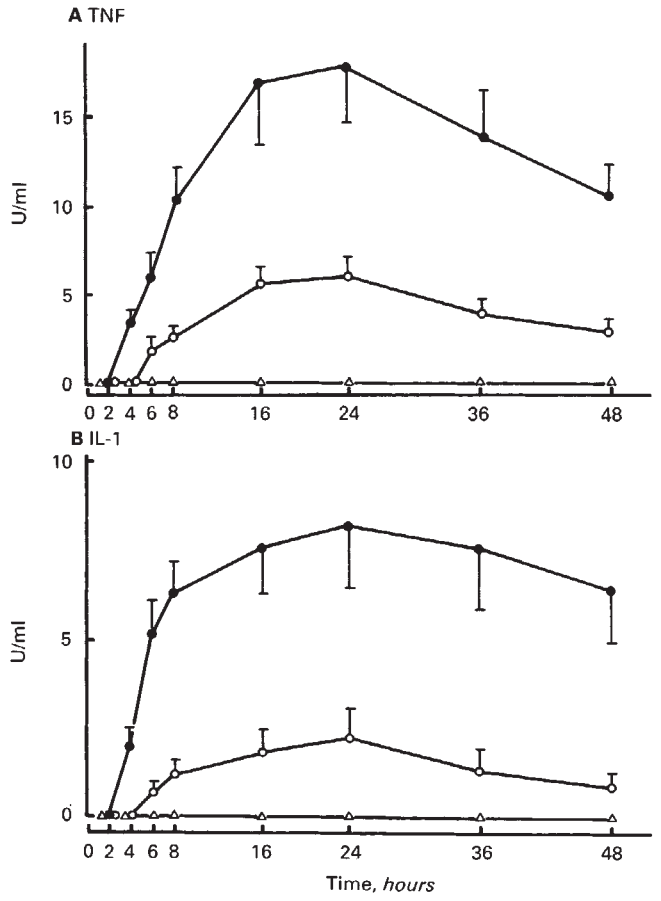

Fig. 4. *Time course curves of TNF and IL-1 production from TG-elicited Mϕ induced by GBM from DM and C group.* Mϕ were incubated in the presence of 10 mg of GBM from each group and the supernatants were collected at the times indicated. Data are shown as means ± SEM of four experiments, with triplicate determination. Symbols are: DM (●—); C (○—); Mϕ alone (△—).

TNF and IL-1 production by Mϕ in response to GBM

The time course for the production of TNF and IL-1 from Mϕ in response to diabetic or normal GBM is shown in Figure 4. A slight TNF activity was detectable after four hours of incubation in the presence of 10 mg of diabetic GBM. The levels increased for up to 24 hours. IL-1 activity was also detectable after four hours of incubation. A major peak of activity was detected at 24 hours, and the rising of the kinetic curve during first eight hours was steeper than that of TNF activity. Identical results were obtained in the presence of normal GBM, although the levels were low and a longer period was needed to detect the activity.

The GBM-induced TNF activity was completely blocked by rabbit anti-mouse TNFα. The IL-1 activity was also completely neutralized by rabbit anti-mouse IL-1α. Activities of both cytokines became undetectable by treating the Mϕ with cycloheximide, indicating that de novo protein synthesis was required.

TNF and IL-1 productions from Mϕ after 24 hours' incubation with GBM from the DM, DM-AG, C, and C-AG groups are shown in Figure 5. GBM from DM rats induced significantly greater levels of TNF and IL-1 production from Mϕ than did GBM from other three groups at doses of 2.5 to 10 mg. Dose-dependent productions of both cytokines were also observed. On the other hand, TNF and IL-1 productions from Mϕ induced by GBM from DM-AG rats were similar to those induced by GBM from C and C-AG rats. No activities of either cytokine was detected when GBM, regardless of diabetic or normal, was incubated alone.

Nonenzymatic glycation and AGE accumulation in GBM

Nonenzymatic glycation of proteins developed in GBM from DM rats (Table 1). Treatment with aminoguanidine did not modify the level of nonenzymatic glycated proteins. Furthermore, the accumulation of fluorescent AGEs in GBM from DM rats was significantly greater than those in GBM from other three groups ($P < 0.01$). In aminoguanidine-treated diabetic animals exposed to identical levels of hyperglycemia, however, the level was nearly normal.

Discussion

Glomerulus consists of cellular and matrix (GBM and mesangial matrix) component. In the isolation procedure for GBM, regardless of sonic disruption or osmotic lysis-detergent treatment, the contamination with mesangial matrix cannot be excluded. We showed that these GBM materials from diabetic rats stimulated Mϕ to produce greater levels of TNF and IL-1 than did normal GBM. In the preliminary study, there were no differences in TNF and IL-1 production in response to diabetic GBM between Mϕ of diabetic rats (13 weeks after STZ injection) and those of normal rats (data not shown).

Aminoguanidine is thought to bind to glycated proteins, thus preventing further progression to AGEs [10]. It has been reported that this agent prevents accumulation of AGEs in aortic collagen or GBM in chemically-induced diabetic animals [9, 10]. Our studies also demonstrated that aminoguanidine treatment prevented the accumulation of AGEs in GBM of the diabetic rats. Furthermore, these GBM materials did not stimulate Mϕ to produce TNF and IL-1. Therefore, AGE-proteins, which were accumulated on collagen or other structural proteins of diabetic GBM, could stimulate Mϕ to produce TNF and IL-1 [7]. Brownlee et al [21, 22] reported that IgG was covalently attached to AGE-collagen or AGE-GBM in proportion to AGE concentration. In addition, this binding IgG retained the ability to form immune complexes in situ. Their results suggest the possibility that, other than AGEs, immune complexes could become one of stimulatory factors for Mϕ [23].

GBM from normal and aminoguanidine-treated normal rats also stimulated Mϕ to produce TNF and IL-1, although the levels were low. Deposition of plasma proteins, such as IgG or complement, in the kidneys of normal rats with an older age has been indicated [24]. Unknown factors which were deposited or trapped in GBM from plasma might stimulate Mϕ to release the cytokines. Vissers et al [23] found no productions of TNF and IL-1 by monocytes from peripheral blood upon stimulation by normal GBM. The difference between the two sets of data may result from the functional characteristics of effector cells used in each experiment, that is, we used nonspecifically-induced peritoneal exudate macrophages, to which Adams et al [25] referred as responsive macrophages.

The glomerular mesangium has been shown to contain bone marrow derived Mϕ [26], and a recent study has shown that

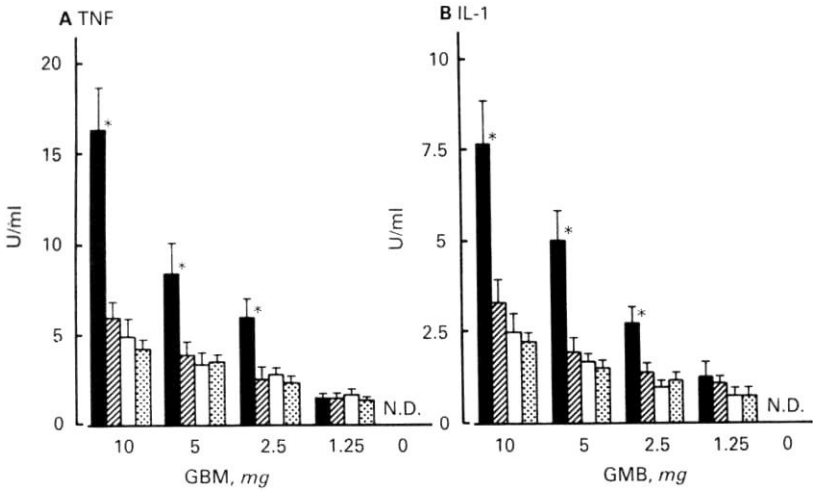

Fig. 5. *TNF and IL-1 production by TG-elicited peritoneal macrophages in response to GBM from DM (solid bar), DM-AG (hatched bar), C (open bar), and C-AG (stippled bar) groups.* Cells are incubated with GBM as detailed in the text. Diabetic GBM induced significantly greater levels of TNF and IL-1 production than GBM of the other three groups. Data are shown as means ± SEM of six experiments, with a triplicate determination. *$P < 0.01$ vs. GBM from the other three groups. N.D.; not detected.

Table 1. Contents of AGE and non-enzymatic glycated proteins in GBM of the four experimental groups.

Groups	*N*	AGE content[a]	Non-enzymatic glycation[b]
DM	5	3.43 ± 0.36[c]	19.2 ± 1.9[d]
DM-AG	5	1.92 ± 0.17	18.4 ± 2.0[d]
C	5	1.68 ± 0.16	9.2 ± 0.5
C-AG	5	1.70 ± 0.22	8.9 ± 0.9

Data are presented as means ± SEM.
[a] Specific fluorescence per μmol hydroxyproline
[b] nmoles 5-hydroxymethylfurfuralaldehyde per mg protein
[c] $P < 0.01$ vs. DM-AG, C, and C-AG groups
[d] $P < 0.01$ vs. C and C-AG group

cultured mesangial cells produce TNF and IL-1 [27, 28]. These findings suggest that glomerular mesangial cells can act as effector cells.

TNF and IL-1, produced by Mϕ in response to AGE-proteins, are considered to account for the normal tissue remodeling with the removal and replacement of extracellular matrix components [7]. In the diabetic state, it has been proposed that this normal tissue homeostasis may be disturbed by increased AGE-protein cross linkings, which reduce the susceptibility to digestion by nonspecific proteases, leading the synthetic and proliferative responses to be enhanced [4]. The contribution of TNF and IL-1 to the pathogenesis of diabetic nephropathy is suggested by the following observations: a) IL-1 can stimulate total protein and collagen synthesis by mesangial cells, which would lead to expansion of the mesangium and thickening of the GBM [29]. b) IL-1 and TNF stimulate prostaglandin production by mesangial cells, which may be responsible for the alteration of glomerular microcirculation [30]. c) These cytokines induce endothelial procoagulant activity and also increase endothelial permeability [1, 31].

In a similar comparative study [23], GBM containing immune complexes induced monocytes to secrete maximum levels of TNF and IL-1. However, in the present study, TNF and IL-1 activities induced by diabetic GBM were low levels and they were only two- to threefold increased in comparison with the levels induced by normal GBM. This difference may be reflected in the pathological features of immune-mediated glomerulonephritis and diabetic nephropathy, that is, in diabetic nephropathy severe proliferative lesions, such as crescent formation, are not seen. These findings would suggest that cytokines are not a pathogenetic determinant of diabetic nephropathy. The mechanism by which diabetic nephropathy occurs is most likely multifactorial based on the chronic metabolic disturbance. In combination with these factors, locally released TNF and IL-1 could act to accelerate the progression of nephropathy.

Aminoguanidine treatment could not significantly suppress the increase of urinary albumin excretion in the early stages of STZ-treated diabetic rats, although the value after 12 weeks of treatment was slightly lowered. This result suggests that hemodynamic factors may be predominant for the increase of urinary albumin excretion in these stages of experimental diabetes [32, 33]. The longer periods of study would be needed for the evaluation of effect of aminoguanidine on nephropathy, because AGEs are accumulated in tissue in time-dependent manner [5, 6].

In conclusion, our data indicate that TNF and IL-1 may participate in the development of diabetic nephropathy. AGE-proteins, which were accumulated on diabetic GBM, may stimulate effector cells to produce these cytokines. Further analysis of the effects of cytokines would increase our understanding of the pathogenesis of diabetic nephropathy.

Acknowledgments

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