Amadori-configurated albumin induces nitric oxide-dependent apoptosis of endothelial cells: a possible mechanism of diabetic vasculopathy

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Abstract

Background. We have demonstrated previously that Amadori-configurated glycated albumin (GA) enhances nitric oxide synthase (NOS) activity, and this action may modulate glomerular hyperfiltration in early phases of diabetic nephropathy. Since the late stage of diabetic vasculopathy is characterized by reductions in viable cells within an expanded and disorganized matrix, we tested the hypothesis that GA enhances endothelial cell (EC) apoptosis.

Methods. Murine (t End.1) or human umbilical vein ECs (HUVECs) were incubated with graded GA concentrations (furosine 0.48–96 nmol/ml) at levels that approximated those reported in sera of diabetic patients (76 ± 0.02 nmol/ml). Apoptosis was evaluated using terminal uridine nick end labelling (TUNEL) to detect DNA fragmentation in gel electrophoresis and p53 expression in immunoperoxidase. Transcription of the inducible (i) and constitutive (c) isoforms of NOS was detected by northern analysis, and total NOS activity was measured as [3H]citrulline production from [3H]arginine. Cells were also incubated with the NOS inhibitors L-nitromethylarginine (L-NAME) at 0.01 M and aminoguanidine (AMG) at 0.01 M, the protein synthesis inhibitor cycloheximide (CHX) at 1 μg/ml, and the NO donor sodium nitroprusside (SNP) at 0.01 M.

Results. ECs cultured in the presence of GA at furosine concentrations corresponding to levels in diabetic patients showed a significant enhancement of apoptosis. GA also caused parallel dose-dependent increases in iNOS mRNA expression and total NOS activity. The pro-apoptotic effect of GA was inhibited by L-NAME, AMG and CHX, but enhanced by SNP.

Conclusions. We found that Amadori-configurated GA at furosine concentrations similar to those in diabetic patients favoured EC apoptosis through enhancement of iNOS activity. We propose that this process may be involved in the progressive cellular loss occurring in vascular and glomerular diabetic sclerosis.

Keywords: apoptosis; diabetes; furosine; glycated albumin; nitric oxide; vasculopathy

Introduction

A number of modifications in cell biology contribute to the pathogenesis of diabetic vasculopathy and glomerulopathy [1,2], which together result in reductions of viable cells scattered within an expanded and disorganized extracellular matrix. There has been increasing research over the last decade examining the progressive deposition of non-enzymatically glycated proteins, particularly advanced glycosylation end products (AGEs) within vascular and perivascular tissues [3]. However, the earlier formed Amadori adducts of proteins may deserve greater priority since they are present in the circulation at concentrations 2–10 times higher than AGEs [4]. In db/db mice, a genetic model of diabetes reproducing the glomerular lesions of human diabetic nephropathy, both histological and clinical features were prevented by injections of a specific monoclonal antibody against Amadori adducts of glycated albumin (GA) [5]. In this same study, incubation of early GA products with mesangial cells resulted in an antiproliferative effect, as well as increases in collagen type I transcription that were probably mediated by protein kinase Cβ stimulation [5].

We recently demonstrated that Amadori adducts of GA modulate nitric oxide synthase (NOS) in cultured endothelial cells (ECs), and speculated that this effect may be related to the haemodynamic changes that occur...
in the early phases of diabetic nephropathy [6]. Findings from this study also suggested that adducts stimulate de novo synthesis of the enzyme, which is possibly enhanced by tumour necrosis factor-α (TNF-α). We reasoned that Amadori adduct-induced elevations in NO release from ECs may favour hyperfiltration but also lead to oxidative damage and apoptosis in diabetic nephropathy. Several experiments have demonstrated that release of small amounts of NO from the constitutive isoform of NOS (cNOS) favours vasodilatation and cell proliferation, whereas high and sustained amounts of NO produced by the inducible isoform (iNOS) may be cytotoxic and pro-apoptotic [7–9]. NO can act as a free radical since it carries an unpaired electron that may induce modifications in molecules (such as Bel-2, Bad, Bax and p53) involved in programmed cell death [9,10]. Apoptosis, in turn, has been implicated in the ‘silent’ cell loss that occurs in different scarring processes, including renal fibrosis [9,10].

In the present work, we determined whether Amadori adducts of GA, measured as furosine, act as apoptotic agents via enhancement of NO production to instigate the progressive loss of cells which characterizes several diabetic vascular complications, including diabetic nephropathy.

Materials and methods

Early (Amadori-configured) glycation products of human serum albumin

Human serum albumin (HSA) (Farma-Biagini, Castelvecchio Pascoli, Lucca, Italy) was incubated under sterile conditions at 25°C, in 27.8 mmol D-glucose for 5 days. Free glucose (mol. wt 180 Da) was eliminated by 24 h dialysis at 4°C against phosphate-buffered saline (PBS), and GA was recovered and sterilized by filtration. This procedure allowed the production of large amounts of GA which were purified further by affinity chromatography on phenylboronate agarose (PBA), as previously described [11]. The purified GA was migrated on SDS-PAGE with an ~66 kDa band with greater electrophoretic mobility than HSA, consistent with glycation of lysine amino groups. The extent of glycation was kindly assessed by Dr D. Sell (laboratory of Professor V. Monnier, Case Western Reserve University, Cleveland, OH) by performing acid hydrolysis of the GA preparation in order to use HPLC to measure the stable product furosine (furoylmethyl imide). Results are expressed as furosine nmol/mg albumin [12]. The amount of furosine present in our probe of glycated albumin was 9.6 nmol/mg albumin.

The HSA preparation, used as a control, was subjected to the same in vitro incubation at 25°C without the high glucose, and then dialysed against sterile PBS.

The absence of endotoxins in GA or HSA was verified using the Limulus amoebocyte lysate (LAL) test at 405 nm (M.A. Bioproducts, Gaithersburg, MD and Kabi, Stockholm, Sweden).

Cells

Murine ECs, t End.1, derived from a thymic haemangioma were cultured as previously described [13]. The cells of this line retain the functional properties of normal ECs, proliferating at confluence without showing signs of overgrowth. They take up acetylated low-density lipoproteins and express CD31, vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin (13). Moreover, these cells respond to interleukin-1β (IL-1β), TNF-α and specific EC growth factors, and produce IL-6 and chemokines [8].

Transformed immortalized human umbilical vein endothelial cells (EC 304, HUVECs) (kindly provided by Professor M. Simonson, Medicine Department, Case Western Reserve University) have endothelium-specific Weibel–Palade bodies and show positive immunocytochemical staining for the lectin Ulex europeus I and the monoclonal anti-human endothelium antibody PHM5. These cells are negative for factor VIII-related antigen, alkaline or acid phosphatase, and for the epithelial marker keratin.

Culture media

ECs with t End.1 were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma Chemical Co., St Louis, MO) (1.8 mM glucose) supplemented with 10% fetal calf serum (FCS; Sigma), penicillin, streptomycin and amphotericin (Sigma). HUVECs were cultured in 199 growth medium supplemented with Earle’s salts (Sigma), 10% FCS, penicillin, streptomycin and amphotericin.

Experimental design

Murine and human ECs were seeded in different culture devices, were attached and allowed to rest for 24 h in serum-free medium, and finally were grown for 12 h (from 4 to 24 h in the time course experiments) under experimental conditions that mimicked the diabetic milieu. Because the amount of furosine in diabetics was reported to be 76 ± 0.02 nmol/ml of plasma [14], we used graded concentrations of furosine (from 0.48 to 96 nmol/ml), which included diabetic levels. Cells were incubated in medium alone or with added native HSA or GA at concentrations ranging from 0.05 to 10 mg/ml (containing furosine from 0.48 to 96 nmol/ml) for 12 h at 37°C in a 5% CO₂ humidified atmosphere.

The GA and HSA dilutions were prepared in culture media in the presence of identical FCS concentrations. Since GA acts through specific receptors, we expected no competition with bovine albumin.

Cells for in situ DNA chain elongation and immunoperoxidase experiments were grown in 8-well chamber slides (Lab-Tek, Miles Scientific Inc., Naperville, IL) and cells for DNA and RNA extraction were grown in 75 cm² plastic flasks (Falcon, Becton Dickinson, Franklin Lakes NJ). As internal controls, cells were co-incubated with 0.01 M L-nitromethyl-arginine (L-NAME), 0.01 M aminoguanidine (AMG) as iNOS activity-specific inhibitor, 1 μg/ml cycloheximide (CHX) or 0.01 M sodium nitroprusside (SNP) (all reagents from Sigma), with or without added HSA or GA. A time course experiment was performed by incubating cells with 96 nmol furosine/ml (corresponding to 10 mg/ml GA) or 10 mg/ml native HSA, and harvesting cells at 4, 8, 12 or 24 h. Cell viability was ensured by trypan blue and lactate dehydrogenase (LDH) release, both measured according to the manufacturer’s instructions (Roche Molecular Biochemicals, Monza, Italy).
Detection of apoptosis by terminal uridine Nick 3' end labelling (TUNEL)

TUNEL was performed according to the manufacturer’s instructions (Boehringer, Mannheim, Germany) as described previously [13]. Apoptotic cells were quantified by counting 200 cells in at least 10 microscopic fields.

DNA extraction and assessment of DNA fragmentation

DNA was extracted from cells in culture by standard methods as previously described [13]. Equal quantities of DNA from each sample were fractionated on a 1% agarose gel at 120 V for 1 h in a Supersub Gel Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA). As positive and negative controls, we used DNA extracted from CTL4 (IL-2-dependent) lymphocytes that was incubated in the presence or absence of IL-2.

Immunoperoxidase staining for p53

Cells that were grown in chamber slides were stained according to the manufacturer’s instructions (Vector Laboratories Inc., Burlingame, CA) as described in previous studies [13]. Rabbit IgG anti-p53 (Dako, Milano, Italy) or normal non-immune rabbit IgG (Vector), both diluted 1:40 in PBS containing goat serum, were incubated for 30 min. After washing, the slides were developed with the Vectastain ABC rabbit IgG detection kit (Vector). The sections were counterstained with haematoxylin, and mounted under coverslips, and were evaluated qualitatively for intensity of staining.

RNA extraction and northern analysis of mRNA encoding iNOS and cNOS

Total RNA was extracted from cells using the RNA STAT-60 (TEL-TEST ‘B’, Inc., Friendswood, TX) kit according to the manufacturer’s instructions as described in previous work [13].

The concentration and purity of RNA were determined by the absorbance at 260 and 280 nm.

Northern blotting was performed according to standard methods [13]. The 32P-labelled cDNA probes (cNOS or iNOS) (Cayman Chemical Company, Ann Arbor, MI) were prepared by random priming of the appropriate cDNA fragment (Oligolabelling Kit, Pharmacia Biotech, Piscataway, NJ). The membranes were exposed to X-OMAT AR X-ray films (Eastman Kodak, Rochester, NY) at −80°C for 1–3 days, then stripped and re-hybridized with a 32P-labelled glyceraldehyde phosphate dehydrogenase (GAPDH) probe (a kind gift from Professor J. R. Sedor, Case Western Reserve University) which was used as a control for equal loading. Autoradiographs were obtained and the bands were quantified by laser densitometry. Four experiments for each condition were performed to allow statistical analysis of the densitometry data.

Measurement of NOS activity

We used, without modification, a protocol that was previously described in detail [13]. NOS activity, measured as pmol of citrulline generated/min of incubation/mg cell protein, was expressed as fold increase from the activity of cells under basal conditions.

Statistical analysis

The reported values represent means ± SD from four pooled experiments, each performed in triplicate (except northern blot, see above). Statistical significance was determined by one-way analysis of variance (ANOVA), followed by post hoc analysis with Dunnet’s multiple comparison t-tests when appropriate. Values of P < 0.05 were considered statistically significant.

Results

Apoptosis in endothelial cell cultures

Under basal conditions, only 0.3–0.6% of either HUVECs or t End.1 ECs showed positive staining with the TUNEL method. The incubation of both EC cell lines with graded concentrations of Amadori adducts of GA induced a dose-dependent increase in the fraction of apoptotic cells. Conversely, native HSA failed to exert a significant pro-apoptotic effect on cultured ECs. Differences between GA and HSA probes were significant for concentrations ≥1.2 mg/ml GA that contained ≥11.52 nmol/ml furosine (Figures 1, and 2A and B). CHX almost completely blocked this effect at each concentration of GA, and a similar inhibition was obtained by co-incubating treated cells with the NOS inhibitors L-NAME or AMG (Figure 1). Neither CHX, L-NAME nor AMG altered the percentage of apoptotic cells when GA was not present (the values, not shown, were superimposable on cells in the basal unconditioned state). When the NO donor SNP (0.001 M) was added alone to the cells, it significantly enhanced apoptosis (t End.1 45 ± 5%; HUVEC 39 ± 4%, P < 0.0001 vs unconditioned cells). Time course studies of apoptosis in cells incubated in media supplemented with 96 nmol/ml furosine (detectable in 10 mg/ml of our GA preparation) revealed a peak in TUNEL-positive cells after 8–12 h of incubation. Conversely, 10 mg/ml HSA had almost no effect (Figure 3).

There were no toxic effects of GA on ECs, as indicated by staining with the vital dye trypan blue, the absence of significant LDH release in t End.1 or HUVECs treated with GA or HSA (data not shown), and the effective gene transcription of the housekeeping gene GAPDH.

DNA fragmentation

The addition of Amadori adducts, in the form of GA, to either t End.1 or HUVECs caused a typical laddering of genomic DNA (Figure 4). Although this DNA fragmentation was inhibited by L-NAME, AMG or CHX, these agents had no effect on the cells by themselves. Native HSA failed to modify the integrity of DNA, whereas addition of SNP alone caused an enhancement of DNA fragmentation.
Nuclear expression of the tumour suppressor protein p53 was not detectable in unconditioned cells and was not modified by native HSA. However, nuclear staining for p53 was strongly enhanced by Amadori adducts of GA (Figure 5A). This increased staining was significantly inhibited by co-incubating ECs with L-NAME, AMG or CHX (Figure 5B, C and D).

**cNOS and iNOS mRNA expression and NOS activity**

Northern analysis revealed that cNOS mRNA expression was unmodified by incubation with various concentrations of Amadori adducts of GA, whereas expression of iNOS transcripts was significantly increased. Native HSA did not modify iNOS or cNOS (Figure 6A and B).
Whereas native HSA was ineffective in modulating in vitro NOS activity in HUVECs or in t End.1 ECs, Amadori adducts comprised of furosine at 96 nmol/ml contained in 10 mg/ml of the GA probe. Incubation of cells with 10 mg/ml native albumin (HSA) had no effect. Lane 1, molecular weight markers; lane 2, negative control; lane 3, positive control; lane 4, HSA 10 mg/ml; lane 5, GA 10 mg/ml (furosine 96 nmol/ml); lane 6, GA 10 mg/ml + 0.01 M L-NAME; lane 7, GA 10 mg/ml + 1 μg/ml cycloheximide; lane 8, 0.01 M sodium nitroprusside.

Fig. 4. EC apoptosis detected by DNA fragmentation in agarose gel electrophoresis. Agarose gel electrophoresis shows laddering of DNA extracted from t End.1 and HUVECs incubated for 12 h with Amadori adducts comprised of furosine at 96 nmol/ml contained in 10 mg/ml of the GA probe. Incubation of cells with 10 mg/ml native albumin (HSA) had no effect. Lane 1, molecular weight markers; lane 2, negative control; lane 3, positive control; lane 4, HSA 10 mg/ml; lane 5, GA 10 mg/ml (furosine 96 nmol/ml); lane 6, GA 10 mg/ml + 0.01 M L-NAME; lane 7, GA 10 mg/ml + 1 μg/ml cycloheximide; lane 8, 0.01 M sodium nitroprusside.

Fig. 5. Immunoperoxidase staining for p53. Immunoperoxidase staining for p53 in t End.1 ECs incubated with Amadori adducts comprised of furosine at 96 nmol/ml in supplemented medium contained in 10 mg/ml of the GA probe (A), and with 10 mg/ml HSA (B). The positivity was inhibited by co-incubation with 1 μg/ml cycloheximide, 0.01 M L-NAME and 0.01 M AMG (C and D). Unaffected by 0.01 M L-NAME, 0.01 M AMG and 1 μM/ml CHX (data not shown).

Discussion

Early glycation products of serum albumin represent the largest fraction of circulating glycated proteins in vivo [4]. In the present study, we found that these products enhanced apoptosis in both murine and human EC lines, whereas native albumin had no effect. We incubated ECs with Amadori adducts of...
GA, containing furosine that ranged from 0.48 to 96 nmol/ml, in order to mimic conditions in human diabetic patients who have serum furosine levels of 76 ± 0.02 nmol/ml [14]. We also found that apoptosis was mediated by overexpression of iNOS mRNA accompanied by enhanced NOS activity in ECs, whereas cNOS mRNA expression was unaltered.

Several observations indicate that induction of iNOS by GA may have been the cause of apoptosis. For example, the frequency of apoptosis, the increased NOS activity and the heightened expression of iNOS mRNA were all interrelated. In addition, each was a monotonic function of GA concentration and all showed similar dose responses.

Both competitive (L-NAME) and covalent non-competitive (AMG) inhibitors of NOS eliminated GA-induced apoptosis, which contrasted with maximal percentages of TUNEL-positive cells and clear fragmentation of DNA in gel electrophoresis that were stimulated by the NO donor SNP. Even though AMG may additionally act as an anti-oxidant and inhibitor of AGE formation, iNOS was also inhibited in GA-treated ECs by the specific competitive NOS inhibitor, L-NAME, which caused a similar inhibition of apoptosis. The time course of our findings also supported a causal role for NO since apoptosis reached a peak between 8 and 12 h. Transcription and translation of iNOS require 6–7 h [8], whereas the apoptotic phenomenon requires 1–2 h [7,9,10]. Apoptosis clearly represents an active biological function of cells, since CHX, a protein synthesis inhibitor, completely abolished the phenomenon. However, studies with CHX that prevent de novo protein synthesis do not allow identification of specific cellular proteins that are critical elements for entry into the apoptotic pathway.

In agreement with our results, Chen et al. [15] recently found enhanced apoptosis in ECs incubated with glycated collagen I (containing 30 nmol fructosamine/mg collagen, indicating a degree of glycation similar to our GA probe). However, they failed to detect increased NO concentrations, even though increased eNOS expression was observed. NO is an unstable gas that promptly reacts with thiol residues to form nitrotyrosine-modified proteins. Therefore, the lack of increased NO during increased nitrotyrosine-modified protein formation in the study by Chen et al. [15] may have been due to the rapid reaction of NO with protein thiol groups leading to low NO levels even during enhanced synthesis. In their study, Chen et al. [15] suggested that the coincident increase in nitrotyrosine-modified proteins indicated a diminished time for NO to form peroxinitrite. Indeed, when we measured enzymatic NOS activity, which is a sensitive test of production activity, we found increased NOS activity in ECs incubated with GA. Hence, the findings from Chen et al. [15] and from the present study both indicate that GA exposure leads to increased apoptosis in EC.

It was reported recently that GA induces increases in intracellular calcium which may in turn activate the apoptotic pathway. In support of this, iNOS enhancement may follow increases in intracellular calcium induced by GA [16]. Since apoptosis was inhibited by the NOS inhibitors L-NAME and AMG, iNOS may be the final effector of cell death.

NO appears to induce apoptosis by promoting expression of the tumour suppressor protein, p53 [17,18]. The unpaired electron of NO modifies the intracellular redox state [19] to result in stimulation of p53 expression that, in turn, modulates the expression of the WAF1/CIP1 gene [20]. This latter gene regulates the synthesis of the protein p21, the inhibitor of
cyclin E. The final effect is an inhibition of the DNA polymerase $\epsilon$ isoform, which is the most active enzyme involved in DNA replication [10]. In our study, incubation of ECs with the Amadori adduct furosine (GA) resulted in nuclear expression of p53 protein that was blunted by co-incubation with either of two iNOS activity inhibitors (L-NAME or AMG) or by the protein synthesis inhibitor CHX. These effects of L-NAME and AMG on p53 synthesis lend further support to a causal role for NO in the apoptotic response.

The low basal rates of apoptotic cells in the current study were probably due, in part, to the use of immortalized cells. However, we have observed similar low basal apoptotic rates in mesangial, tubular and endothelial cells [13]. Nevertheless, the theoretical resistance of immortalized ECs to undergo apoptosis further reinforces our results since we detected a clear pro-apoptotic effect of GA in these cell lines. This pro-apoptotic effect may even be amplified in non-immortalized cells.

It is unlikely that Amadori adducts are uniquely responsible for the vascular complications of diabetes or that they evoke vascular injury exclusively via iNOS-elicted apoptosis. However, the present data support and point to an important role for Amadori adducts in the development of endothelial apoptosis.

In both the microvasculature and larger vessels, the loss of the endothelial layer following apoptosis is likely to promote contact of the plasma with the extracellular matrix and vascular smooth muscle cells, thereby initiating a cascade of events similar to those occurring during atherogenesis. Moreover, different mechanisms of response to distinct stimulators may predominate at various time points.

In conclusion, our findings suggest that albumin modified by Amadori glucose adducts has potent cytotoxic effects in ECs and that NO is critically involved in this process.

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Conflict of interest statement. None declared.

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