***In vitro* glycoxidation alters the interactions between collagens and human polymorphonuclear leucocytes**

Jean-Claude MONBOISSE¹, Laure RITTIE, Hasnae LAMFARRAJ, Roselyne GARNOTEL and Philippe GILLERY

Laboratoire de Biochimie Médicale et Biologie Moléculaire, CNRS UPRESA 6021, IFR-53 Biomolécules, Faculté de Médecine, University of Reims Champagne-Ardenne, 51 Rue Cognacq-Jay, 51095 Reims cedex, France

Glycation and glycoxidation processes, which are increased in diabetes mellitus, are generally considered causative mechanisms of long-term complications. With reference to our previous studies, type-I and -IV collagens could induce differentially the adhesion and stimulation of polymorphonuclear leucocytes (PMNs). As PMNs play a role in sustained diabetic oxidative stress, the present study was designed to determine whether *in vitro* glycoxidation of these macromolecules could alter PMN adhesion, activation and migration. The adhesion of PMNs to *in vitro*-glycoxidized collagens was significantly increased when compared with control collagens: +37 % ($P < 0.05$) and +99 % ($P < 0.01$) for collagens I and IV, respectively. Glycoxidized type-I collagen increased the chemotactic properties of PMNs without significant stimulatory effect on respiratory burst,

whereas pre-incubation of PMNs with glycoxidized type-I collagen induced a priming on subsequent stimulation by *N*-formyl-methionyl-leucyl-phenylalanine. Glycoxidation of type-IV collagen suppressed its inhibitory effect on further PMN stimulation or migration. Collectively, these results indicate that glycoxidation of two major extracellular-matrix collagens considerably alters their ability to modulate PMN migration and production of reactive oxygen species. This imbalance in PMN metabolism may be a major event in the increased oxidative status that characterizes diabetes mellitus.

Key words: chemotaxis, extracellular matrix, non-enzymic glycation, oxygen free radicals, respiratory burst.

INTRODUCTION

Non-enzymic glycation of proteins due to high glucose concentrations has been considered for two decades a major determining factor in the development of long-term degenerative complications of diabetes mellitus [1]. This metabolic reaction between sugar moieties and amino groups of proteins is basically a physiological process that proceeds through several stages involving reactive metabolic intermediates, and finally leads to the formation and tissue accumulation of various products with a long half-life, known as advanced glycation end products or AGEs, that have been largely involved in diabetic complications [2].

Most biochemical alterations induced by non-enzymic glycation involve the generation of reactive oxygen species [3–5] and a subsequent increase in oxidative stress. Common metabolic pathways involving glycation and oxidation are known as glycoxidation [6–8]. Glycation and glycoxidation products induce cellular and extracellular-matrix (ECM) tissue damage, as well as damage to circulating proteins and endothelial structures [9–11]. In connective tissues, collagens are particularly susceptible to glycation. Type-I collagen glycation not only modifies its structural properties [12–14], but also alters its interactions with the surrounding ECM molecules and cells [15,16]. However, other types of collagen are modified by non-enzymic glycation. For example, the overglycation of type-IV collagen, a major component of basement membranes, has been shown to alter its structural and functional properties, particularly its susceptibility to degradation by metalloproteinases [17,18].

In this study, we investigated the effects of collagen-I and -IV glycation on their interactions with polymorphonuclear

leucocytes (PMNs), which are major sources of reactive oxygen species [19]. As we have shown previously that PMNs were activated by contact with type-I collagen, and that collagen IV was able to prevent activation [20,21], we investigated the effects of *in vitro* non-enzymic glycation of collagen I and IV on PMN functions, and demonstrated a significant alteration of the interactions.

EXPERIMENTAL

Reagents

Ferricytochrome *c*, superoxide dismutase (SOD) from bovine erythrocytes, BSA, *N*-formylmethionyl-leucylphenylalanine (f-Met-Leu-Phe), 5-hydroxymethylfurfural (5-HMF), PMA, Nitro Blue Tetrazolium (NBT), xanthine oxidase and hypoxanthine were all purchased from Sigma (St Louis, MO, U.S.A.). The other chemical reagents, of analytical grade, were provided by Prolabo (Paris, France) or Merck (Darmstadt, Germany). Human fibronectin was purchased from Institut J. Boy (Reims, France).

Glycoxidized collagen preparation

Type-I collagen was prepared from rat tail tendon by 0.5 M acetic acid extraction and precipitation with 0.7 M NaCl as described previously [20]. Type-IV collagen was isolated and purified in its native form from bovine anterior lens capsule (ALC) by extraction with 0.5 M acetic acid and anion-exchange chromatography on DEAE-cellulose (Whatman, Maidstone, Kent, U.K.). Limited treatment of type-IV collagen with pepsin

Abbreviations used: AGE, advanced glycation end product; ALC, anterior lens capsule; f-Met-Leu-Phe, *N*-formylmethionyl-leucylphenylalanine; 5-HMF, 5-hydroxymethylfurfural; NBT, Nitro Blue Tetrazolium; PMN, polymorphonuclear leucocyte; SOD, superoxide dismutase; ECM, extracellular matrix; TBA, thiobarbituric acid.

¹ To whom correspondence should be addressed (e-mail jc.monboisse@univ-reims.fr).

(Worthington, Freehold, NJ, U.S.A.) to remove the NC1 domain or with purified bacterial collagenase (CLSPA grade, Worthington, provided by Serlabo) to generate the NC1 domain was performed as described previously [21,22]. Type-I and -IV collagens were sterilized with 70% (v/v) ethanol for 2 h, rinsed extensively with sterile distilled water and dissolved at 1 mg/ml in sterile 18 mM acetic acid.

Sterile solutions of type-I or -IV collagen, BSA or human fibronectin were incubated under sterile conditions for 7 weeks at 37 °C in the absence or in the presence of 0.25 M glucose in 50 mM phosphate buffer, pH 7.4, without chelating agents, dialysed extensively against distilled water and lyophilized.

The intensity of glycoxidation of type-I or -IV collagen was evaluated by the thiobarbituric acid (TBA) method for protein-bound sugars, expressed as nmol of 5-HMF/mg of collagen, and by fluorescence measurement of AGEs. Type-I or -IV collagen (1 mg) was digested by bacterial collagenase (1 unit/ml) in 2 ml of Tris buffer [50 mM Tris/HCl (pH 7.6)/0.15 M NaCl/4 mM CaCl₂] for 24 h at 37 °C and then centrifuged at 10 000 *g* for 30 min. The TBA method was performed as described previously [23,24], and the fluorescence of the supernatant was measured with a Perkin-Elmer LS50B spectrofluorimeter (Beaconsfield, Bucks., U.K.) at two different ranges of wavelengths ($\lambda_{\text{excitation}}$ 335 nm– $\lambda_{\text{emission}}$ 385 nm; $\lambda_{\text{excitation}}$ 370 nm– $\lambda_{\text{emission}}$ 440 nm), versus a control corresponding to the same Tris buffer containing the collagenase [23,24]. Glycated type-I collagen showed a 8.3-fold increase in the fluorescence at the different wavelengths (2500 versus 300 and 2900 versus 300 arbitrary units, respectively), as well as a 3.1-times increase in the content of protein-bound sugars, evaluated by TBA assay (123.5 versus 40.1 nmol of 5-HMF/ μ g of collagen). Likewise, type-IV collagen fluorescence increased 7.3 times (1980 versus 270 and 1840 versus 240 arbitrary units, respectively), BSA fluorescence 15.7 times (3010 versus 260 and 2800 versus 178 arbitrary units, respectively) and fibronectin 8.8 times (2300 versus 260 and 2150 versus 220 arbitrary units, respectively).

The superoxide anion (O_2^-) scavenger activity of glycoxidized collagen I was determined by the SOD-inhibitable reduction of ferricytochrome *c*. O_2^- ions were produced by the xanthine oxidase/hypoxanthine system as described previously [25].

Preparation of PMNs

Human blood was obtained from healthy subjects among the laboratory staff after receipt of informed consent, and PMNs were isolated by a single-step centrifugation procedure through a metrizoate/Polyprep gradient (Nycomed, Oslo, Norway) at 240 *g* for 35 min at room temperature. The PMN-rich layer was removed and washed once in Dulbecco's solution, pH 7.4 (0.137 M NaCl/2.7 mM KCl/10 mM glucose/30 mM Hepes/1.3 mM CaCl₂/0.5 mM MgCl₂) and then centrifuged at 600 *g* for 15 min at room temperature. The contaminating erythrocytes were eliminated by hypotonic lysis in 15 mM ammonium chloride. PMNs were suspended at 10⁷/ml in Dulbecco's solution, pH 7.4, at 4 °C until use [26]. The percentage of PMNs in the cell preparation exceeded 95%, and cell viability, as determined by Trypan Blue exclusion test, was greater than 98%.

Measurement of PMN adhesion

Polystyrene 96-well plates (Nunc, Copenhagen, Denmark) were coated with 50 μ g of each protein or collagen solubilized at 0.5 mg/ml in a 18 mM acetic acid solution. The protein-coated plates were washed three times with 0.15 M NaCl prior to the

addition of 1.5×10^5 cells in 0.1 ml of Dulbecco's solution. After a 30-min incubation period, the non-adherent cells were discarded. The adherent cells were fixed with 0.1 ml of a 1.1% (v/v) glutaraldehyde solution for 15 min, washed extensively with distilled water, and stained by adding 0.1 ml of Crystal Violet solution (1 g/l in 0.2 M Hepes buffer, pH 6.0). The adhesion of PMNs was evaluated by measuring absorbance at 560 nm [27].

Measurement of O_2^- production

O_2^- release was determined by the SOD-inhibitable reduction of ferricytochrome *c* [25]. The PMN suspension (10⁶ cells in 0.1 ml) was added to a glass test tube containing 0.1 ml of 1 mM ferricytochrome *c* solution and 0.85 ml of Dulbecco's solution. O_2^- release was induced by adding 0.1 ml of 3 μ M collagen-I solution (glycated or not) or 0.1 ml of human fibronectin solution at 1 mg/ml (glycated or not). The increase in absorbance at 550 nm was followed spectrophotometrically as an index of the amount of liberated superoxide ion. Production of O_2^- was also evaluated by the intracellular reduction of NBT by PMNs adherent to 96-well culture plates coated with type-I collagen or human fibronectin (100 μ g/well). NBT reduction was determined by absorbance measurement at 560 nm as described previously [27].

In order to measure the inhibitory effect of type-IV collagen on PMN functions, a PMN suspension (1.2 ml containing 6×10^6 cells) in Dulbecco's solution was incubated for 30 min at 37 °C with native or pepsin-treated ALC type-IV collagen or with the NC1 domain (glycated or not; all at 50 μ g/ml) or with Dulbecco's solution as control. After the first incubation period, PMNs were collected by centrifugation at 600 *g* for 5 min, washed twice with Dulbecco's solution, and resuspended in 1.2 ml of Dulbecco's solution. Aliquots of 0.1 ml of this suspension (corresponding to 5×10^5 cells) were added to glass tubes containing 0.85 ml of Dulbecco's solution, 0.1 ml of 1 mM ferricytochrome *c* solution and the stimulating agent (8 nM PMA, 0.5 μ M f-Met-Leu-Phe or 0.3 μ M collagen I, final concentrations). After a 15-min incubation period at 37 °C, ferricytochrome *c* reduction was measured spectrophotometrically at 550 nm.

Measurement of granule secretion

Preincubation of PMNs with type-IV collagen was performed as described previously. PMN (10⁶ cells in 0.1-ml aliquots) were added to glass tubes containing 0.8 ml of Dulbecco's solution and 0.1 ml of f-Met-Leu-Phe solution (final concentration 0.5 μ M), and incubated for 30 min at 37 °C. After the incubation period, cells were removed by centrifugation at 800 *g* for 5 min. Granule exocytosis was determined in the supernatant by elastase activity assay, using *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide as substrate and lactate dehydrogenase activity, used as a control of absence of PMN lysis, as described previously [20]. The enzymic activities released into the medium were expressed as percentages of the respective total enzymic activity of the PMN preparation.

PMN migration assays

PMN migration assays were performed using modified Boyden chambers (Blindwell, Nuclepore, provided by Corning-Costar, Brumath, France). The bottom compartment was filled with the chemoattractant solution: Dulbecco's solution as control, 5 nM f-Met-Leu-Phe, or glycated or non-glycated type-I collagen (5 or 10 μ g/ml). A 0.22- μ m-pore-size polyvinylpyrrolidone-free poly-

carbonate membrane was then placed over the wells and covered with a second membrane (polyvinylpyrrolidone-free polycarbonate, 3 μm porosity) according to the method described in [28]. PMN suspensions (5×10^5 cells, or 10^6 cells for checkerboard analyses, in 0.1 ml of Dulbecco's solution) were deposited into the upper compartment. Migration was measured at 37 °C in a humidified atmosphere of 95 % air/5 % CO_2 for 3 h. At the end of the incubation period, the cells remaining at the upper surface of the 3- μm -pore-size membrane were removed with a cotton swab. The membranes were fixed with methanol for 15 min and stained with Crystal Violet. The number of migrating cells was determined by measuring the absorbance at 560 nm. Checkerboard analyses were performed using (i) various concentrations of glycosylated collagen I (0–10 $\mu\text{g}/\text{ml}$) and (ii) various mixtures of non-glycosylated/glycosylated collagen I (final concentration 10 $\mu\text{g}/\text{ml}$).

Statistical calculations

All experiments were performed in quadruplicate and the results expressed as means $\pm$ S.D.. Significance of the differences was calculated using the Student's *t* test.

RESULTS

Adhesion of PMNs to glycosylated substrates

PMN adhesion to various proteins was studied (Figure 1). Adhesion of PMNs to glycosylated type-I collagen was increased by 37 % ($P < 0.05$) when compared with non-glycosylated control. Adhesion of PMNs to type-IV collagen was also increased to a higher extent by glycation. Adhesion was increased by 99 %, 74 % and 71 % ($P < 0.05$), respectively, when using glycosylated forms of ALC type-IV collagen, pepsin-treated type-IV collagen and the NC1 domain of type-IV collagen. Adhesion of PMNs to human fibronectin was also increased by glycation (+51 %), while glycation did not affect the adhesion of PMN to BSA, which was actually of low intensity in basal conditions.

Figure 1 Increased adhesion of PMNs to glycosylated substrates

PMN suspensions (1.5×10^5 cells in 0.1 ml of Dulbecco's solution) were deposited on 96-well culture plates coated with various non-glycosylated (stippled bars) or glycosylated (hatched bars) proteins, each at 50 $\mu\text{g}/\text{well}$ and incubated for 30 min at 37 °C. The number of adherent PMNs was measured by nuclear staining with Crystal Violet at 560 nm. 1, BSA; 2, type-I collagen; 3, ALC type-IV collagen; 4, pepsin-treated ALC type-IV collagen; 5, NC1 domain from ALC type-IV collagen; 6, fibronectin. Results are expressed as the means $\pm$ S.D. of quadruplicate determinations. Differences from non-glycosylated proteins were significant at: * $P < 0.05$ and ** $P < 0.01$.

Figure 2 Decreased activation of PMNs by glycosylated type-I collagen

(A) O_2^- production was measured by the intracellular reduction of NBT by PMNs adherent to 96-well culture plates coated with type-I collagen or fibronectin (100 $\mu\text{g}/\text{well}$). PMNs were incubated as described in Figure 1 for 30 min at 37 °C in the presence of 1.67×10^{-5} M NBT, and NBT reduction was measured at 560 nm. (B) O_2^- production was also measured in PMN suspensions by the SOD-inhibitable reduction of ferricytochrome *c* at 550 nm, as described in the Experimental section. In both series, PMNs were incubated with non-glycosylated (stippled bars) or glycosylated (hatched bars) proteins. 1, BSA; 2, type-I collagen; 3, fibronectin. Results represent the means $\pm$ S.D. of quadruplicate determinations. Differences from non-glycosylated proteins were significant at * $P < 0.05$ and *** $P < 0.001$.

Activation of PMNs by glycosylated collagens

Glycation of type-I collagen inhibited its ability to trigger O_2^- production by PMNs, whether adhering to collagen I or in suspension, as evaluated by the intracellular reduction of NBT or SOD-inhibitable ferricytochrome *c* reduction, respectively. Activation of PMNs by glycosylated type-I collagen was decreased by 27–52 % ($P < 0.05$) compared with activation by non-glycosylated collagen, under the experimental conditions used. Fibronectin-induced O_2^- production by PMNs was of lower intensity (1.85 ± 1.05 versus 12.85 ± 0.34 nmol of $\text{O}_2^-/10^6$ cells with collagen I) and was not altered by non-enzymic glycation (Figure 2). On the other hand, glycosylated collagen I did not exhibit a significant scavenger activity of O_2^- ions produced by the xanthine oxidase/hypoxanthine system, as determined by the SOD-inhibitable reduction of ferricytochrome *c* (Table 1).

The effect of contact of PMNs with glycosylated type-I collagen on further activation by other stimuli was determined by preincubating PMNs for 30 min at 37 °C with glycosylated type-I collagen, or non-glycosylated type-I collagen in the control series, and then stimulating them with 0.5 μM f-Met-Leu-Phe. Preincubation with 10 $\mu\text{g}/\text{ml}$ glycosylated collagen I significantly increased f-Met-Leu-Phe-induced O_2^- production by PMNs by 45 % ($P < 0.05$; Figure 3).

Table 1 Absence of O_2^- scavenger activity of glycoxidized collagen I

O_2^- scavenger activity of collagen I was determined by the SOD-inhibitable reduction of ferricytochrome *c* using the xanthine oxidase/hypoxanthine system to produce O_2^- ions. Ferricytochrome *c* reduction was spectrophotometrically evaluated at $\lambda = 550$ nm. A millimolar absorption coefficient of $15.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ was used to calculate O_2^- ion production. Results are expressed as the means $\pm$ S.D. of quadruplicate determinations. NS, not significant, when compared with 0 $\mu\text{g/ml}$ collagen I.

	O_2^- (nmol/min)			
	Collagen I concentration ($\mu\text{g/ml}$) ... 0	50	100	250
Non-glycated collagen I	30.6 ± 3.8	26.7 ± 2.5 (NS)	28.3 ± 5.0 (NS)	30.6 ± 4.5 (NS)
Glycated collagen I	30.6 ± 3.8	25.8 ± 3.0 (NS)	32.5 ± 3.4 (NS)	29.0 ± 2.7 (NS)

Figure 3 Glycoxidized type-I collagen primes PMN stimulation by f-Met-Leu-Phe

PMN suspensions were pre-incubated for 30 min at 37°C with non-glycated (stippled bars) or glycated (hatched bars) type-I collagen and then stimulated with $0.5 \mu\text{M}$ f-Met-Leu-Phe. O_2^- production was assessed by the SOD-inhibitable reduction of ferricytochrome *c* measured at 550 nm. 1, Control, pre-incubation with Dulbecco's solution; 2, pre-incubation with type-I collagen ($5 \mu\text{g/ml}$); 3, preincubation with type-I collagen ($10 \mu\text{g/ml}$). Results represent the means $\pm$ S.D. of quadruplicate determinations. Differences from non-glycated type-I collagen were significant at $*P < 0.05$.

Figure 4 Glycoxidation of ALC type-IV collagen suppresses its inhibitory effect on PMN stimulation

PMN suspensions were preincubated for 30 min at 37°C with non-glycated (stippled bars) or glycated (hatched bars) forms of ALC type-IV collagen ($50 \mu\text{g/ml}$) and then stimulated with $0.5 \mu\text{M}$ f-Met-Leu-Phe. O_2^- production was assessed by the SOD-inhibitable reduction of ferricytochrome *c* measured at 550 nm. 1, Dulbecco's solution (control); 2, native ALC type-IV collagen; 3, pepsin-treated type-IV collagen; 4, NC1 domain from ALC type-IV collagen. Results represent the means $\pm$ S.D. of quadruplicate determinations. Differences from non-glycated forms of ALC type-IV collagen were significant at $**P < 0.01$ and $***P < 0.001$.

Since we demonstrated previously that type-IV collagen exerts a preventative effect on PMN activation [21], we investigated the influence of type-IV collagen glycation on the action. PMNs were pre-incubated for 30 min at 37°C with glycated type-IV collagen, or glycated NC1 domain of type-IV collagen, and further stimulated by type-I collagen, f-Met-Leu-Phe or PMA. Glycation of type-IV collagen or the NC1 domain suppressed the preventative effect on PMN activation by f-Met-Leu-Phe (Figure 4). The amount of O_2^- obtained was not significantly different from the amount obtained under control conditions (pre-incubation with Dulbecco's solution), whereas non-glycated type-IV collagen and NC1 domain still exerted a preventative effect (-44 and -62% , respectively, $P < 0.001$). We obtained similar results on O_2^- production when PMNs were further stimulated with type-I collagen or PMA and by measurement of granule exocytosis (results not shown).

Chemotactic effect of glycated collagens on PMNs

The influence of glycation on the ability of collagens to exert a chemoattracting effect on PMNs was tested in Boyden chambers for 3 h (Figure 5). PMN migration was elicited by type-I collagen at 5 or $10 \mu\text{g/ml}$ to an extent similar to that induced by 5 nM f-Met-Leu-Phe. PMN migration was significantly increased in the presence of glycated compared with non-glycated type-I collagen

Figure 5 Increased chemotactic effect of glycoxidized type-I collagen

Chemotactic effect of non-glycated (stippled bars) or glycated (hatched bars) type-I collagen was measured in modified Boyden chambers. Suspensions of PMNs (5×10^5 cells in 0.1 ml of Dulbecco's solution) were deposited in the upper compartment of the chambers and migration was elicited by: 1, 5 nM f-Met-Leu-Phe; 2, type-I collagen ($5 \mu\text{g/ml}$); and 3, type-I collagen ($10 \mu\text{g/ml}$). PMN migration was assessed by nuclear staining with Crystal Violet measured at 560 nm. Results represent the means $\pm$ S.D. of quadruplicate determinations. Differences from non-glycated type-I collagen were significant at $*P < 0.05$ and $**P < 0.01$.

Table 2 Glycoxidation increases chemotactic properties of type-I collagen towards PMN

Chemotactic properties of glycoxidized collagen I were determined by checkerboard analyses with (a) glycoxidized collagen I alone (as shown by upper and lower, with concentrations shown in $\mu\text{g/ml}$) or (b) a mixture of control and glycoxidized collagen I (upper and lower). PMN migration was determined as described for Figure 5, using PMN suspensions of 10^6 cells in 0.1 ml of Dulbecco's solution. Results are expressed as absorbance values at 560 nm and correspond to the means $\pm$ S.D. of quadruplicate determinations. NS, not significant, when compared with the control without glycoxidized collagen in the upper and lower compartments.

(a)				
Lower	Upper ...	A_{560}		
		0 $\mu\text{g/ml}$	5 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$
0 $\mu\text{g/ml}$		0.689 $\pm$ 0.082	0.699 $\pm$ 0.119 (NS)	0.693 $\pm$ 0.108 (NS)
5 $\mu\text{g/ml}$		0.814 $\pm$ 0.059 (NS)	0.655 $\pm$ 0.031 (NS)	0.666 $\pm$ 0.035 (NS)
10 $\mu\text{g/ml}$		0.809 $\pm$ 0.089 ($P < 0.01$)	0.746 $\pm$ 0.048 (NS)	0.582 $\pm$ 0.091 (NS)
(b)				
Lower (control/glycoxidized)	Upper (control/glycoxidized) ...	A_{560}		
		10/0 $\mu\text{g/ml}$	5/5 $\mu\text{g/ml}$	0/10 $\mu\text{g/ml}$
10/0 $\mu\text{g/ml}$		0.661 $\pm$ 0.049	0.619 $\pm$ 0.062 (NS)	0.592 $\pm$ 0.043 (NS)
5/5 $\mu\text{g/ml}$		0.746 $\pm$ 0.027 (NS)	0.644 $\pm$ 0.048 (NS)	0.606 $\pm$ 0.061 (NS)
0/10 $\mu\text{g/ml}$		0.828 $\pm$ 0.023 ($P < 0.01$)	0.759 $\pm$ 0.048 (NS)	0.617 $\pm$ 0.033 (NS)

Figure 6 Glycoxidation of ALC type-IV collagen suppresses its inhibitory effect on PMN chemotactism

PMN suspensions were preincubated for 30 min at 37 °C with Dulbecco's solution (white bars), or with non-glycated (stippled bars) or glycated (hatched bars) ALC type-IV collagen (50 $\mu\text{g/ml}$). PMN migration was then measured in modified Boyden chambers as described in Figure 5. Migration was elicited by: 1, 5 nM f-Met-Leu-Phe; 2, non-glycated type-I collagen (5 $\mu\text{g/ml}$); and 3, glycated type-I collagen (5 $\mu\text{g/ml}$). Results represent the means $\pm$ S.D. of quadruplicate determinations. Differences from non-glycated ALC type-IV collagen were significant at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

(Table 2). Checkerboard analyses showed that glycoxidized collagen I did not induce random PMN migration.

The capacity of type-IV collagen to inhibit the chemotactic effects of type-I collagen was tested using the same model. Preincubation of PMNs with non-glycated type-IV collagen induced a significant decrease in the non-glycated collagen-I-induced migration (-22% , $P < 0.001$) and in the glycated collagen-I-induced migration (-38% , $P < 0.01$). By contrast, glycation of type-IV collagen suppressed its inhibitory effect on collagen-I-induced PMN migration. Similar results were obtained

when PMN migration was elicited with 5 nM f-Met-Leu-Phe (Figure 6).

DISCUSSION

Non-enzymic glycation of proteins is increased in the presence of high glucose concentrations and is involved in pathological processes such as diabetes mellitus [1,2], particularly in the triggering of oxidative processes (glycoxidation), which are deleterious to many biological molecules [3,8]. These post-translational modifications have been shown to take place at various sites in both type-I and -IV collagens, inducing structural and functional alterations of these molecules [12,17]. For example, resistance of type-IV collagen to metalloproteinase degradation is increased by non-enzymic glycation [18]. Collagens not only play a structural role in ECMs, but also exert metabolic activity as modulators of various cellular functions. For example, glycosylated collagen-I matrices impair the proteolytic properties of fibroblasts [29]. This study was designed to focus on the specific interactions between glycosylated collagens and PMNs.

We have demonstrated previously that type-I collagen elicits PMN adhesion and respiratory burst [26,27]. Two short peptide sequences of the $\alpha 1(\text{I})$ collagen chain are responsible for PMN activation: an Arg-Gly-Asp sequence located in the triple-helix domain (positions 915–917) and the Asp-Gly-Gly-Arg-Tyr-Tyr sequence (1034–1039) in the C-terminal telopeptide [26]. Conversely, type-IV collagen was able to prevent PMN activation through a specific sequence corresponding to residues 185–203 of the $\alpha 3(\text{IV})$ chain [21]. This balanced effect of type-I and -IV collagens is supposed to be altered when matrix degradation occurs (e.g. in case of inflammation or during wound repair) or in the case of structural modifications of collagens. With regard to these results, it was relevant to study the influence of *in vitro* non-enzymic glycation on the interactions between collagens and PMNs.

By *in vitro* incubation with 0.25 M glucose, glycoxidized collagen I was obtained that exhibited an AGE fluorescence level about 7 times higher than control collagen I. The extent of glycation is higher than that found in diabetic skin collagen I, which was reported to be twice as high as in control skin collagen [30,31]. However, large differences in AGE content have been found in various diabetic connective tissues, for example up to 5 or 6 times in aortic connective tissue [30,31]. In addition, high glucose concentrations up to 0.5 M have been used for glycation experiments *in vitro* by other authors [32,33].

These results demonstrate that *in vitro* glycoxidation of ECM proteins increases PMN adhesion. Similar results have been reported for human monocytes activated with endotoxin, whereas freshly isolated monocytes exhibited a decreased adhesion to glycated collagen substrates [34]. The increased adhesion of PMNs was observed on type-I collagen and fibronectin, which belong to the most abundant components of connective tissues, as well as on type-IV collagen, the major collagenic component of basement membranes. Results seem to indicate that PMN adhesion to ECM proteins is generally increased by glycoxidation. In contrast with adhesion, the ability of type-I collagen to activate PMNs is strongly inhibited by glycoxidation. The differential effect of collagen-I glycoxidation on adhesion and activation of PMNs might be explained by structural features. PMN adhesion occurs on to various domains of both $\alpha 1(I)$ and $\alpha 2(I)$ chains [27], and it may be hypothesized that glycoxidation-induced modifications create new potential adhesion sites. In contrast, only two short sequences are responsible for PMN activation, and the binding of glucose (or glucose derivatives) to lysine residues close to these sequences may induce conformational changes impairing PMN activation. A preferential site of glycation has been shown to correspond to lysine 924 of the $\alpha 1(I)$ chain [12], close to the Arg-Gly-Asp sequence necessary for PMN activation. The second peptide sequence responsible for activation is located in the C-terminal telopeptide of the $\alpha 1(I)$ chain, which also comprises one lysine residue (position 1031) in its sequence. Its glycation may induce cross-linking of collagen I molecules and masking of the Asp-Gly-Gly-Arg-Tyr-Tyr sequence. We have demonstrated previously that type-I collagen could slightly increase the dismutation rate of O_2^- ions in pulse-radiolysis experiments [35]. Nevertheless, the poor scavenger activity, exhibited by both control and glycoxidized collagen I, on O_2^- ions produced by the xanthine oxidase/hypoxanthine system, does not explain the major impairment of glycoxidized collagen I in stimulating O_2^- production. This was confirmed by the absence of a significant degranulating effect. Previous studies have shown that O_2^- production by monocytes or PMNs was not directly modified by glycation of collagen I, but the studies have used glycated type-I collagen only as an adhesion substratum, activation being elicited by zymosan particles [19]. However, even if glycoxidation suppressed the ability of type-I collagen to stimulate O_2^- production by PMNs, we have shown in this study that glycoxidized type-I collagen is able to prime PMNs significantly to a further stimulation with chemotactic peptides such as f-Met-Leu-Phe and thus to enhance their oxidative responses. The same response is expected for other inflammatory mediators, such as complement factors or leukotriene B₄. The findings are consistent with other studies, suggesting for example that PMNs from diabetic patients exhibit an altered reactive oxygen metabolism in relation to hyperglycation processes [34].

Results also indicate clearly that *in vitro* glycoxidation of type-I collagen increases its chemotactic properties for PMNs, and corroborate previous data showing that fibronectin modified by advanced glycation increased trans-endothelial migration of PMNs [36]. Moreover, high glucose concentration promotes

PMN adhesion to the endothelium, increasing extravascular migration of these cells [37]. This finding might indicate that ECM glycation or glycoxidation induces an increased chemotactic recruitment of PMNs. However, as discussed above, the ability of PMNs to be locally stimulated is strongly altered.

Additionally, glycation of basement-membrane components, such as type-IV collagen, is an important factor in the regulation of PMN functions. These experiments indicate that glycoxidation of type-IV collagen or the NC1 domain suppresses the inhibitory effect on PMN activation, also leading to a potentialization of PMN responses to various stimuli (probably different from natural activators such as specific sequences of type-I collagen) and to an increase of their activation status. Moreover, glycated type-IV collagen no longer inhibits PMN migration elicited by chemotactic molecules such as bacterial peptides or collagen I, in contrast with native type-IV collagen, which prevents the migration of PMNs to extracellular tissues. These results suggest that glycoxidized type-IV and -I collagens act synergistically to increase extravascular migration and inappropriate activation of PMNs.

Collectively, the results of these experiments demonstrate that *in vitro* glycation of type-I and -IV collagens alters the interactions with PMNs, which leads to a large increase in PMN function, inducing enhanced oxidative tissue damage of tissues. These interactions may explain the participation of PMNs in the pathogenesis of various long-term complications of diabetes mellitus.

This work was supported by grants from the University of Reims Champagne-Ardenne and from the CNRS (UPRESA 6021). We thank S. Etienne and E. Deschamps for typing the manuscript, and S. Gobert for improving the English style.

REFERENCES

- 1 Brownlee, M., Vlassara, H. and Cerami, A. (1984) Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann. Intern. Med.* **101**, 527–537
- 2 Vlassara, H. (1997) Recent progress in advanced glycation end products and diabetic complications. *Diabetes* **46**, S19–S25
- 3 Gillery, P., Monboisse, J. C., Maquart, F. X. and Borel, J. P. (1988) Glycation of proteins as a source of superoxide. *Diabetes Metab.* **14**, 25–30
- 4 Jiang, Z. Y., Woollard, A. C. S. and Wolff, S. P. (1990) Hydrogen peroxide production during experimental protein glycation. *FEBS Lett.* **268**, 69–71
- 5 Wolff, S. P. and Dean, R. T. (1987) Glucose autooxidation and protein modification. The potential role of 'autooxidative glycosylation' in diabetes. *Biochem. J.* **245**, 243–250
- 6 Wells-Knecht, M. C., Thorpe, S. R. and Baynes, J. W. (1995) Pathways of formation of glycoxidation products during glycation of collagen. *Biochemistry* **34**, 15134–15141
- 7 Traverso, N., Menini, S., Cottalasso, D., Odetti, P., Marinari, U. M. and Pronzato, M. A. (1997) Mutual interaction between glycation and oxidation during non-enzymatic protein modification. *Biochim. Biophys. Acta* **1336**, 409–418
- 8 Baynes, J. W. and Thorpe, S. R. (1999) Role of oxidative stress in diabetes. A new perspective on an old paradigm. *Diabetes* **48**, 1–9
- 9 Taniguchi, N., Kaneto, H., Asahi, M., Takahashi, M., Wenyi, C., Higashiyama, S., Fujii, J., Suzuki, K. and Kayanoki, Y. (1996) Involvement of glycation and oxidative stress in diabetic macroangiopathy. *Diabetes* **45**, S81–S83
- 10 Lyons, T. J. and Jenkins, A. J. (1997) Glycation, oxidation, and lipoxidation in the development of the complications of diabetes: a carbonyl stress hypothesis. *Diabetes Rev.* **5**, 365–391
- 11 Sakata, N., Miyamoto, K., Meng, J., Tachikawa, Y., Imanaga, Y., Takebayashi, S. and Furukawa, T. (1998) Oxidative damage of vascular smooth muscle cells by the glycated protein-cupric ion system. *Atherosclerosis* **136**, 263–274
- 12 Reiser, K. M., Amigable, M. A. and Last, J. A. (1992) Nonenzymatic glycation of type I collagen. The effects of aging on preferential glycation sites. *J. Biol. Chem.* **267**, 24207–24216
- 13 Tian, S. F., Toda, S., Higashino, H. and Matsumura, S. (1996) Glycation decreases the stability of the triple-helical strands of fibrous collagen against proteolytic degradation by pepsin in a specific temperature range. *J. Biochem. (Tokyo)* **120**, 1153–1162

- 14 Odetti, P., Aragno, I., Garibaldi, S., Valentini, S., Pronzato, M. A. and Rolandi, R. (1996) Role of advanced glycation end products in aging collagen. A scanning force microscope study. *Gerontology* **44**, 187–191
- 15 Katayama, Y., Celic, S., Nagata, N., Martin, J. T. and Findlay, D. M. (1997) Nonenzymatic glycation of type I collagen modifies interaction with UMR 201–10B preosteoblastic cells. *Bone* **21**, 237–242
- 16 Paul, R. G. and Bailey, A. J. (1996) Glycation of collagen: the basis of its central role in the late complications of ageing in diabetes. *Int. J. Biochem. Cell. Biol.* **28**, 1297–1310
- 17 Raabe, H. M., Molsen, H., Mlinaric, S. M., Acil, Y., Sinnecker, G. H., Notbohm, H., Kruse, K. and Muller, P. K. (1996) Biochemical alterations in collagen IV induced by *in vitro* glycation. *Biochem. J.* **319**, 699–704
- 18 Mott, J. D., Khalifah, R. G., Nagase, H., Shield, C. F., Hudson, J. K. and Hudson, B. G. (1997) Nonenzymatic glycation of type IV collagen and matrix metalloproteinase susceptibility. *Kidney Int.* **52**, 1302–1312
- 19 Scatena, R., Nocca, G., De Sole, P., Bottoni, P. and Giardina, B. (1998) Impaired reactive oxygen metabolism of phagocytic leukocytes in NIDDM patients. A role for nonenzymatic glycosylation of collagen. *J. Biolumin. Chemilumin.* **13**, 273–278
- 20 Monboisse, J. C., Bellon, G., Dufer, J., Randoux, A. and Borel, J. P. (1987) Collagen activates superoxide anion production by human polymorphonuclear neutrophils. *Biochem. J.* **246**, 599–603
- 21 Monboisse, J. C., Garnotel, R., Bellon, G., Ohno, N., Perreau, C., Borel, J. P. and Kefalides, N. A. (1994) The $\alpha 3$ chain of type IV collagen prevents activation of human polymorphonuclear leukocytes. *J. Biol. Chem.* **269**, 25475–25482
- 22 Monboisse, J. C., Bellon, G., Perreau, C., Garnotel, R. and Borel, J. P. (1991) Bovine lens capsule basement membrane collagen exerts a negative priming on polymorphonuclear neutrophils. *FEBS Lett.* **294**, 129–132
- 23 Furth, A. J. (1988) Methods for assaying nonenzymatic glycosylation. *Anal. Biochem.* **175**, 347–360
- 24 Odetti, P. R., Borgoglio, A. and Rolandi, R. (1992) Age-related increase of collagen fluorescence in human subcutaneous tissue. *Metabolism* **41**, 655–658
- 25 Monboisse, J. C., Braquet, P., Randoux, A. and Borel, J. P. (1983) Non-enzymatic degradation of acid-soluble calf skin collagen by superoxide ion: protective effect of flavonoids. *Biochem. Pharmacol.* **32**, 53–58
- 26 Monboisse, J. C., Bellon, G., Randoux, A., Dufer, J. and Borel, J. P. (1990) Activation of human neutrophils by type I collagen. *Biochem. J.* **270**, 459–462
- 27 Monboisse, J. C., Garnotel, R., Randoux, A. and Borel, J. P. (1991) Adhesion of human neutrophils to and activation by type I collagen involving a $\beta 2$ integrin. *J. Leukocyte Biol.* **50**, 373–380
- 28 Bignold, L. P. (1988) Measurement of chemotaxis of polymorphonuclear leukocytes *in vitro*. *J. Immunol. Methods* **108**, 1–18
- 29 Rittié, L., Berton, A., Monboisse, J. C., Hornebeck, W. and Gillerly, P. (1999) Decreased contraction of glycated collagen lattices coincides with impaired matrix metalloproteinase production. *Biochem. Biophys. Res. Commun.* **264**, 488–492
- 30 Dyer, D. G., Dunn, J. A., Thorpe, S. R., Bailie, K. E., Lyons, T. J., McCance, D. R. and Baynes, J. W. (1993) Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J. Clin. Invest.* **91**, 2463–2469
- 31 Vishwanath, V., Frank, K. E., Elmets, C. A., Dauchot, P. J. and Monnier, V. M. (1986) Glycation of skin collagen in type I diabetes mellitus. *Diabetes* **35**, 916–921
- 32 Bobbink, I. W. G., de Boer, H. C., Tekelenburg, W. L. H., Banga, J. D. and de Groot, P. G. (1997) Effect of extracellular matrix glycation on endothelial cell adhesion and spreading. *Diabetes* **46**, 87–93
- 33 Gilcrease, M. Z. and Hoover, R. L. (1992) Human monocyte interactions with non-enzymatically glycated collagen. *Diabetologia* **35**, 160–164
- 34 Kedziora-Kornatowska, K. Z., Luciak, M., Blaszczyk, J. and Pawlak, W. (1998) Effect of aminoguanidine on the generation of superoxide anion and nitric oxide by peripheral blood granulocytes of rats with streptozotocin-induced diabetes. *Clin. Chim. Acta* **278**, 45–53
- 35 Monboisse, J. C., Gardès-Albert, M., Randoux, A., Borel, J. P. and Ferradini, C. (1988) Collagen degradation by superoxide anion in pulse and gamma radiolysis. *Biochim. Biophys. Acta* **965**, 29–35
- 36 Sengoelge, G., Födinger, M., Skoupy, S., Ferrara, I., Zangerle, C., Rogy, M., Horl, W. H., Sunder-Plassmann, G. and Menzel, J. (1998) Endothelial cell adhesion molecule and PMNL response to inflammatory stimuli and AGE-modified fibronectin. *Kidney Int.* **54**, 1637–1651
- 37 Morigi, M., Angioletti, S., Imberti, B., Donadelli, R., Micheletti, G., Figliuzzi, M., Remuzzi, A., Zoja, C. and Remuzzi, G. (1998) Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF- κ B-dependent fashion. *J. Clin. Invest.* **101**, 1905–1915

Received 11 February 2000/12 June 2000; accepted 11 July 2000