In vitro glyoxidation alters the interactions between collagens and human polymorphonuclear leucocytes

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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-leucyl-phenylalanine (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).

Glyoxidized 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-I collagen with pepsin

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

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(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 10000 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 (λemission 335 nm–400 nm, λexcitation 370 nm–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 2500 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 2500 arbitrary units, respectively).

The superoxide anion (O2−) scavenger activity of glycoxidized collagen I was determined by the SOD-inhibitable reduction of ferricytochrome c. O2− 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/Polynprep 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 5 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 μg 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 × 105 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 O2− production**

O2− release was determined by the SOD-inhibitable reduction of ferricytochrome c [25]. The PMN suspension (107 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. O2− release was induced by adding 0.1 ml of 3 mM 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 O2− 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 μg/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 × 106 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 μg/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 × 105 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 mM PMA, 0.5 μM f-Met-Leu-Phe or 0.3 μM 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 μM), 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-methoxyxysuccinyl-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 enzymatic 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 chemottractant solution: Dulbecco’s solution as control, 5 nM f-Met-Leu-Phe, or glycated or non-glycated type-I collagen (5 or 10 μg/ml). A 0.22-μm-pore-size polystyrene membrane-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% CO₂ 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 glycated collagen I (0–10 μg/ml) and (ii) various mixtures of non-glycated/glycated collagen I (final concentration 10 μg/ml).

**Statistical calculations**

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

**RESULTS**

**Adhesion of PMNs to glycated substrates**

PMN adhesion to various proteins was studied (Figure 1). Adhesion of PMNs to glycated type-I collagen was increased by 37% (*P* < 0.05) when compared with non-glycated 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 glycated 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.

**Activation of PMNs by glycated collagens**

Glycation of type-I collagen inhibited its ability to trigger O₂⁻ production by PMNs, whether adhering to collagen I or in suspension, as evaluated by the intracellular reduction of NBT or SOD-inhibitable reduction of ferricytochrome c reduction, respectively. Activation of PMNs by glycated type-I collagen was decreased by 27–52% (*P* < 0.05) compared with activation by non-glycated collagen, under the experimental conditions used. Fibronectin-induced O₂⁻ production by PMNs was of lower intensity (1.85 ± 1.05 versus 12.85 ± 0.34 nmol of O₂⁻/10⁶ cells with collagen I) and was not altered by non-enzymic glycation (Figure 2). On the other hand, glycoxidized collagen I did not exhibit a significant scavenger activity of O₂⁻ 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 glycated type-I collagen on further activation by other stimuli was determined by pre-incubating PMNs for 30 min at 37 °C with glycated type-I collagen, or non-glycated type-I collagen in the control series, and then stimulating them with 0.5 μM f-Met-Leu-Phe. Pre-incubation with 10 μg/ml glycated collagen I significantly increased f-Met-Leu-Phe-induced O₂⁻ production by PMNs by 45% (*P* < 0.05; Figure 3).

**Figure 1 Increased adhesion of PMNs to glycoxidized substrates**

PMN suspensions (1.5 × 10⁶ cells in 0.1 ml of Dulbecco’s solution) were deposited on 96-well culture plates coated with various non-glycated (stippled bars) or glycated (hatched bars) proteins, each at 50 μg/ml 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, fibronectin; 4, pepsin-treated ALC type-IV collagen; 5, NC1 domain of type-IV collagen; 6, fibronectin. Results are expressed as the means ± S.D. of quadruplicate determinations. Differences from non-glycated proteins were significant at: *P* < 0.05 and **P < 0.01.

**Figure 2 Decreased activation of PMNs by glycoxidized type-I collagen**

(A) O₂⁻ 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 μg/well). PMNs were incubated as described in Figure 1 for 30 min at 37 °C in the presence of 1.67 × 10⁻⁴ M NBT, and NBT reduction was measured at 560 nm. (B) O₂⁻ 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-glycated (stippled bars) or glycated (hatched bars) proteins: 1, BSA; 2, type-I collagen; 3, fibronectin. Results represent the means ± S.D. of quadruplicate determinations. Differences from non-glycated proteins were significant at *P < 0.05 and ***P < 0.001.
Table 1 Absence of $\text{O}_2^−$ scavenger activity of glycoxidized collagen I

$\text{O}_2^−$ scavenger activity of collagen I was determined by the SOD-inhibitable reduction of ferricytochrome c using the xanthine oxidase/hypoxanthine system to produce $\text{O}_2^−$ ions. Ferricytochrome c reduction was spectrophotometrically evaluated at $\lambda = 550$ nm. A millimolar absorption coefficient of 15.5 mM$−1$·cm$−1$ was used to calculate $\text{O}_2^−$ ion production. Results are expressed as the means ± S.D. of quadruplicate determinations. NS, not significant, when compared with 0 µg/ml collagen I.

<table>
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<tr>
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<th>0</th>
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<th>100</th>
<th>250</th>
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<tr>
<td>Non-glycated collagen I</td>
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<td>30.6 ± 3.8</td>
<td>26.7 ± 2.5 (NS)</td>
<td>28.3 ± 5.0 (NS)</td>
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<td>Glycated collagen I</td>
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<td>32.5 ± 3.4 (NS)</td>
<td>29.0 ± 2.7 (NS)</td>
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</table>

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 µM f-Met-Leu-Phe. $\text{O}_2^−$ 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 µg/ml); 3, preincubation with type-I collagen (10 µg/ml). Results represent the means ± S.D. of quadruplicate determinations. Differences from non-glycated type-I collagen were significant at *P < 0.05.

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 $\text{O}_2^−$ 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 $\text{O}_2^−$ 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 chemotaxic 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 µg/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.

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 × 10⁵ 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 µg/ml); and 3, type-I collagen (10 µg/ml). PMN migration was assessed by nuclear staining with Crystal Violet measured at 560 nm. Results represent the means ± S.D. of quadruplicate determinations. Differences from non-glycated forms of 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 µg/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 ± S.D. of quadruplicate determinations. NS, not significant, when compared with the control without glycoxidized collagen in the upper and lower compartments.

(a)

<table>
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<th>Lower µg/ml</th>
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<th>10 µg/ml</th>
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<td>0</td>
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<td>0.699 ± 0.119 (NS)</td>
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</tr>
<tr>
<td>5</td>
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<td>0.655 ± 0.031 (NS)</td>
<td>0.668 ± 0.035 (NS)</td>
</tr>
<tr>
<td>10</td>
<td>0.809 ± 0.089 (P &lt; 0.01)</td>
<td>0.746 ± 0.048 (NS)</td>
<td>0.582 ± 0.091 (NS)</td>
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</table>

(b)

<table>
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<tr>
<th>Lower (control/glycoxidized)</th>
<th>Upper (control/glycoxidized) 10/0 µg/ml</th>
<th>5/5 µg/ml</th>
<th>0/10 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/0 µg/ml</td>
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<td>0.619 ± 0.062 (NS)</td>
<td>0.592 ± 0.043 (NS)</td>
</tr>
<tr>
<td>5/5 µg/ml</td>
<td>0.746 ± 0.027 (NS)</td>
<td>0.644 ± 0.048 (NS)</td>
<td>0.606 ± 0.061 (NS)</td>
</tr>
<tr>
<td>0/10 µg/ml</td>
<td>0.828 ± 0.023 (P &lt; 0.01)</td>
<td>0.759 ± 0.048 (NS)</td>
<td>0.617 ± 0.033 (NS)</td>
</tr>
</tbody>
</table>

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

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 µg/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 µg/ml); and 3, glycated type-I collagen (5 µg/ml). Results represent the means ± 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, glycated collagen-I matrices impair the proteolytic properties of fibroblasts [29]. This study was designed to focus on the specific interactions between glycated 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 z1(1) 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-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 z3(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 α1(I) and α2(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 α1(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 α1(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-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, controlled by both culture conditions 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 hyperglycemic 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.

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**REFERENCES**


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