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Involvement of lysine 1047 in type I collagen-mediated activation of polymorphonuclear neutrophils

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Oxidative functions of polymorphonuclear neutrophils (PMNs), which play a deciding role in the phagocytosis process, are stimulated by extracellular matrix proteins such as type I collagen. Previous studies have demonstrated the involvement of a DGGRYY sequence located within the α_1 chain C-terminal telopeptide in type I collagen-induced PMN activation, but so far the mechanism has not been completely elucidated. We have recently demonstrated that collagen carbamylation (i.e. post-translational binding of cyanate to lysine ε-NH_2 groups) impairs PMN oxidative functions, suggesting the potential involvement of lysine residues in this process. The present study was devoted to the identification of lysine residues involved in the collagen-induced activation of PMNs. The inhibition of PMN activation by collagen in the presence of 6-amino-hexanoic acid, a structural analogue of lysine residues, confirmed the involvement of specific lysine residues. Modification of lysine residues by carbamylation demonstrated that only one residue, located within the α_1CB6 collagen peptide, was involved in this mechanism. A recombinant α_1CB6 peptide, designed for the substitution of lysine 1047 by glycine, exhibited decreased activity, demonstrating that the lysine residue at position 1047 within the collagen molecule played a significant role in the mechanism of activation. These results help to understand in more detail the collagen-mediated PMN activation mechanism and confirm the prominent involvement of lysine residues in interactions between extracellular matrix proteins and inflammatory cells.

The activation of polymorphonuclear neutrophils (PMNs) constitutes the first step of phagocytosis and is characterized by the release of proteolytic enzymes and reactive oxygen species (ROS) that actively participate in the host defence mechanisms against pathogenic agents [1,2]. Several stimuli may trigger this process, including type I collagen, a major extracellular matrix protein. Previous studies in our laboratory have demonstrated the ability of type I collagen to stimulate ROS production by PMNs through a mechanism involving the binding of an $\alpha_L\beta_2$ integrin [3,4] to a consensus sequence (DGGRYY) located on the C-terminal telopeptide of type I collagen, together with the RGD sequences that promote PMN adhesion and probably the participation of other unidentified sequences [5].

However, in a biological context, this interaction must be considered with respect to the intensity of

Abbreviations

AHA, 6-amino-hexanoic acid; CNBr peptides, peptides derived from collagen cleavage by CNBr; CNBr, cyanogen bromide; GST, glutathione *S*-transferase; IPG, immobilized pH-gradient; pI, isoelectric point; PMN, polymorphonuclear neutrophil; ROS, reactive oxygen species.

protein alterations generated *in vivo* by the so-called 'late post-translational modifications'. These modifications are characterized by the non-enzymatic binding of reactive by-products derived from simple molecules (sugars, lipids, protides) to amino groups of proteins, their subsequent molecular re-arrangement, and their critical effects on protein structural and functional properties [6]. In this regard, we have recently demonstrated that carbamylation alters the ability of type I collagen to activate PMNs [7]. Carbamylation is the post-translational modification of proteins caused by the non-enzymatic binding of isocyanic acid, a reactive urea by-product, to ε-amino-groups of lysine residues. Our previous experiments suggested that one or several lysine residues were involved in collagen-mediated PMN activation. This hypothesis is supported by previous studies that have already underlined the deciding role of lysine residues in type I collagen structures and/or in its interactions with other proteins. For instance, lysine residues contribute to electrostatic interactions required for collagen triple-helix stability [8], but also represent targets for lysyl hydroxylase to form hydroxylysine residues involved in collagen cross-links [9], so that any over-hydroxylation or post-translational modifications of lysine ε-amino-groups are responsible for an alteration of collagen fibrils [10,11] or for an impaired sensitivity towards enzymatic proteolysis [12]. In a more general context, lysine residues are usually described as key residues for protein–protein interactions. For example, they represent preferential targets of histone acetylation [13,14] or govern the interactions of plasmin(ogen) through specific domains named 'lysine-binding sites' [15–17].

This study was designed to identify lysine residues involved in PMN activation induced by type I collagen and used different methodological approaches, such as competition with a lysine structural analogue, modification of lysine side chain by carbamylation and directed mutagenesis. It demonstrated that collagen lysine 1047 is a key residue involved in this process.

Results

Inhibition of collagen-mediated activation of PMNs by 6-amino-hexanoic acid

In a first set of experiments, the potential involvement of lysine residues in PMN activation was evaluated by measuring ROS production by PMNs incubated with type I collagen in the presence of a lysine structural analogue, 6-amino-hexanoic acid (AHA), used as a competitive agent (Fig. 1). AHA inhibited ROS production by PMNs in a dose-dependent manner and the

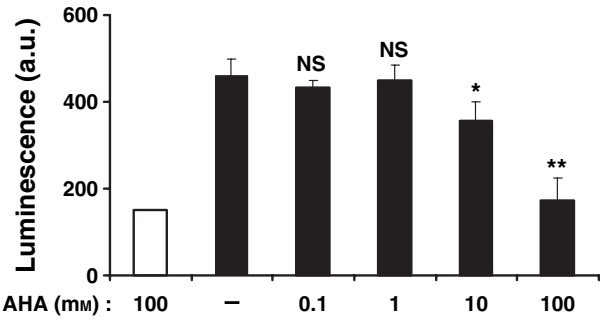

Fig. 1. Role of lysine residues in collagen-induced PMN activation. Approximately 10^6 PMNs, suspended in 1 mL of Dulbecco's solution, were incubated for 15 min at 37 °C with (black bars) or without (white bar) 100 $\mu g \cdot mL^{-1}$ of type I collagen in the presence of various concentrations (0.1–100 mM) of AHA. The production of ROS was measured by chemiluminescence. Results are expressed as means ± standard deviations ($n = 3$). Significant differences versus control series: NS, non-significant, $^*P < 0.05$, $^{**}P < 0.01$. a.u., arbitrary units.

effect was considered to be significant at concentrations of ≥ 10 mM (inhibition of 23% at 10mM concentration, $P < 0.05$). At 100 mM AHA, ROS production was inhibited by 62% ($P < 0.01$), whereas PMN viability was not modified (data not shown). At 100 mM, AHA exhibited no scavenger activity on *in vitro* ROS production by the xanthine oxidase-hypoxanthine system (data not shown). These results suggested the involvement of lysine-containing sequences in the activation mechanism.

Involvement of lysine residues contained within α_1CB6 peptides

In order to localize lysine residues in sequences involved in PMN activation, the activating role of cyanogen bromide (CNBr) peptides (i.e. peptides obtained after collagen cleavage by CNBr) was investigated. CNBr peptides prepared from control, carbamylated (i.e. with modified lysine residues) and pepsinized (i.e. deprived of telopeptides) type I collagen, were separated by electrophoresis (Fig. 2A) and blotted onto a nitrocellulose membrane. Their ability to modulate PMN functions was studied as described in the Experimental procedures. The production of ROS by PMNs was selectively mediated by the interaction with α_1CB6 peptides prepared from control collagen (Fig. 2B). A higher-molecular-weight band, corresponding to partly digested collagen, was also able to activate PMNs. No activation was observed when PMNs interacted with α_1CB6 peptides derived from carbamylated collagen or with CNBr peptides derived from pepsinized collagen (used as a negative control of activation). This effect

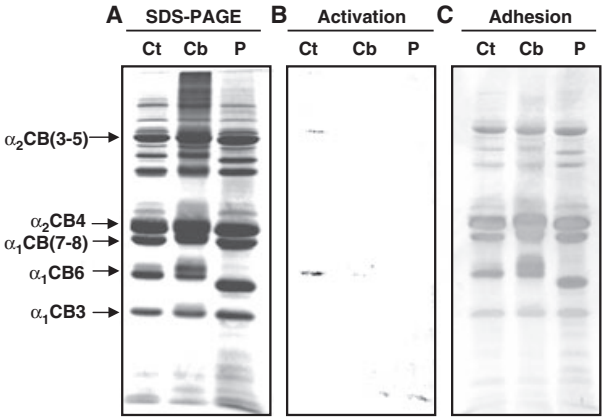

Fig. 2. Influence of α_1CB6 peptide carbamylation on PMN activation. CNBr peptides (50 μg) were separated by electrophoresis through a 12.5% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS and blotted onto a nitrocellulose membrane. Adhesion and activation of PMNs on CNBr peptides were studied according to the protocol described in the 'Experimental procedures'. CNBr peptides of pepsinized collagen, deprived of telopeptides, were used as a negative control of PMN activation. (A) Coomassie Brilliant Blue-stained CNBr peptides separated by electrophoresis. (B) Activation of PMNs by CNBr peptides separated by electrophoresis. (C) Adhesion of PMNs on CNBr peptides separated by electrophoresis. Cb, 6-h-carbamylated collagen CNBr peptides; Ct, control collagen CNBr peptides; P, pepsinized collagen CNBr peptides.

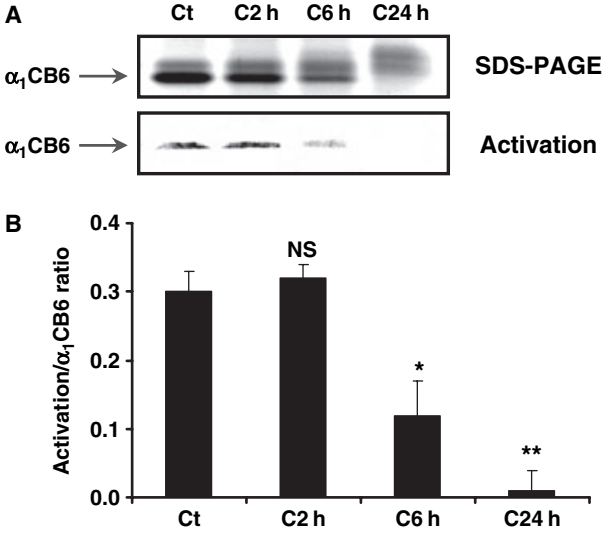

Fig. 3. Influence of carbamylation rate on PMN activation by the α_1CB6 peptide. (A) CNBr peptides (50 μg) were separated by electrophoresis through a 12.5% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS and blotted onto a nitrocellulose membrane. PMN activation on α_1CB6 peptides was studied according to the protocol described in the 'Experimental procedures'. One representative experiment of three independent experiments is shown. (B) Each band was quantified by densitometry (with the results obtained in arbitrary units) and activation of PMNs by α_1CB6 peptides was expressed as a ratio of the intensity of activation to the amount of α_1CB6 peptides deposited. The results are expressed as means ± standard deviations ($n = 3$). Significant differences versus control collagen CNBr peptides: NS, non-significant; $*P < 0.05$, $**P < 0.01$. Ct, control collagen CNBr peptides; C2h, 2-h-carbamylated collagen CNBr peptides; C6h, 6-h-carbamylated collagen CNBr peptides; C24h, 24-h-carbamylated collagen CNBr peptides.

was independent of any impairment of adhesion because neither carbamylation nor pepsin digestion of collagen modified adhesion of PMNs to CNBr peptides, especially to α_1CB6 peptides (Fig. 2C). The inhibition of PMN activation was correlated to the extent of the α_1CB6 peptide carbamylation rate (Fig. 3A). No significant difference was observed between control and 2-h-carbamylated collagen-derived α_1CB6 peptides, whereas a significant decrease of PMN activation was observed with 6- and 24-h-carbamylated collagen-derived α_1CB6 peptides (−60%; $P < 0.05$ and −95% $P < 0.01$, respectively) (Fig. 3B). These results confirmed that lysine residues located within α_1CB6 peptides played a significant role in PMN activation by type I collagen.

Relationship between α_1CB6 peptide lysine carbamylation and PMN activation

Before identifying the lysine residues of α_1CB6 peptides involved in this process among six residues, we first had to determine the number of modified lysine residues at each carbamylation rate, considering the fact that the conditions of collagen carbamylation were expected to generate a mixture of molecules with a heterogeneous rate of lysine modification. Monodimensional electrophoresis was not sufficiently resolvent to permit the separation of such slightly modified peptides, so CNBr peptides were submitted to 2D electrophoresis because the carbamylation of lysine side chains was responsible for a decrease in the isoelectric point (pI) (Fig. 4). As the shift of spots towards a lower pI was directly related to the carbamylation rate of peptides, each new spot corresponded to the modification of a new lysine residue. Separation of control collagen-derived α_1CB6 peptides revealed three spots: two major spots and one minor spot. Preparations obtained from 2-h-carbamylated collagen contained four spots, three of which were identical to those obtained from control collagen, and a new spot of a lower pI that was less visible. In α_1CB6 peptides derived from 6-h-carbamylated collagen, the intensity of the minor spots previously detected in control collagen α_1CB6 peptides increased, indicating the progressive modification of lysine residues. In preparations obtained from 24-h-carbamylated collagen two

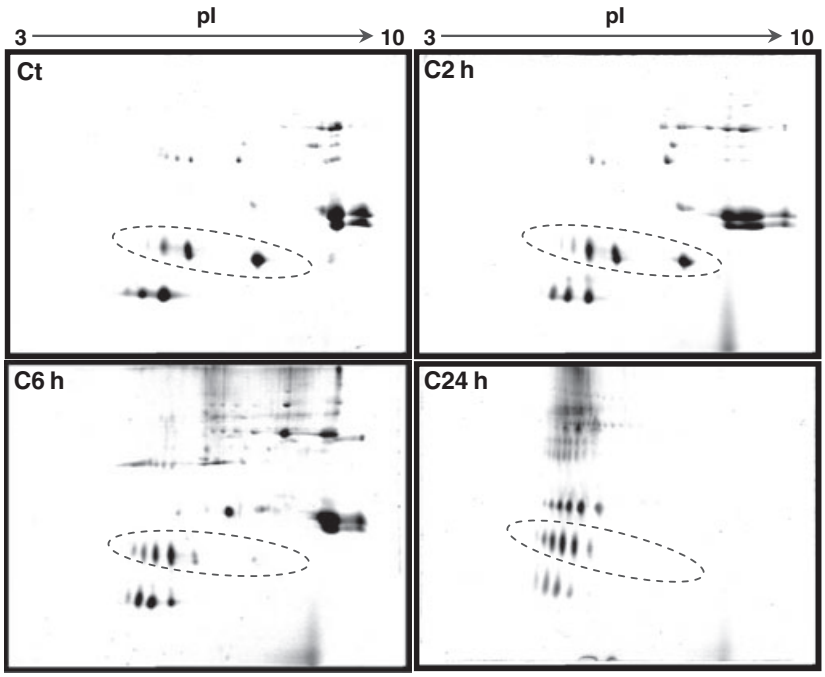

Fig. 4. Separation of carbamylated α_1CB6 peptides by 2D electrophoresis. CNBr peptides (300 μg) were first submitted to IEF (pH 3–10) and then separated by electrophoresis through a 12.5% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS. After electrophoresis, gels were stained with Coomassie Brilliant Blue R250. Spots corresponding to α_1CB6 peptides are enclosed by dotted lines. Ct, control collagen CNBr peptides; C2h, 2-h-carbamylated collagen CNBr peptides; C6h, 6-h-carbamylated collagen CNBr peptides; C24h, 24-h-carbamylated collagen CNBr peptides.

new spots were identified. These results confirmed that α_1CB6 peptides separated by monodimensional electrophoresis exhibited a heterogeneous number of modified lysine residues. For that reason, we then evaluated the activity of peptides exhibiting a known degree of modification (i.e. with a homogeneous carbamylation rate). To that end, α_1CB6 peptides derived from control and 6-h-carbamylated collagen were purified by preparative IEF and their ability to activate PMNs was measured (Fig. 5). Among the three α_1CB6 peptides obtained from control collagen-derived CNBr peptides, only two (with pI values of 6.8 and 7.7) were able to activate PMNs. Among the five α_1CB6 peptides resulting from the separation of 6-h-carbamylated collagen-derived CNBr peptides (with lower pI values, ranging from 5.2 to 6.8), only one peptide was able to activate PMNs, corresponding to the same peptide as that isolated from control collagen-derived CNBr peptides with a pI of 6.8. These results indicated that the modification of only one lysine residue was sufficient to support the loss of ability of α_1CB6 peptides to activate PMNs.

Involvement of lysine 1047 in collagen-mediated PMN activation

The localization of lysine 1047 was determined after verifying the α_1CB6 peptide primary sequence that highlighted the presence of a lysine residue in position 1047, located three amino acids upstream from the consensus activating DGGRYY sequence (Fig. 6A). The importance of this lysine residue in the PMN activation process was studied by the production of a mutated (K1047G) recombinant peptide and the measurement of its ability to activate PMNs (Fig. 6B). The mutated peptide exhibited a significantly decreased ability to stimulate ROS release by PMNs (−70%; $P < 0.01$) when compared with control peptides and taking into account the basal activation state of PMNs.

Discussion

PMNs interact with various types of collagen *in vivo*, especially with type I collagen, the most abundant collagen of interstitial connective tissues. These interactions constitute key mechanisms of the regulation of PMN functions by their extracellular environment and are probably involved in pathophysiological events such as inflammation or infection [3]. Previous studies from our laboratory have shown that type I collagen stimulates the release of ROS by PMNs via a specific DGGRYY sequence located in the C-terminal region of type I collagen α_1 chains [3,5], after binding to $\alpha_L\beta_2$ integrin and subsequent phosphorylation of p^{125}FAK [4,7]. We have recently demonstrated that carbamylation (i.e. binding of cyanate to ε-NH_2 groups of lysine residues) alters the ability of type I collagen to activate PMNs [7]. The *in vivo* relevance of the carbamylation process has been confirmed by various studies that have established a link between protein carbamylation

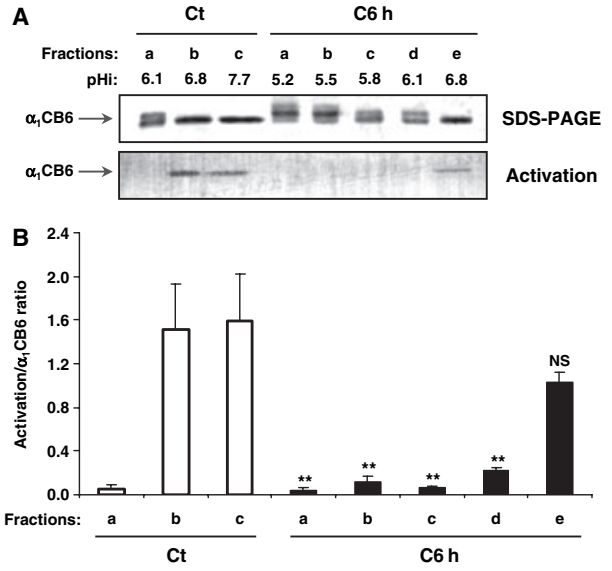

Fig. 5. PMN activation by α_1CB6 peptides separated by IEF. (A) CNBr peptides (50 μg), previously separated by preparative IEF, were submitted to electrophoresis through a 12.5% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS and then blotted onto a nitrocellulose membrane. PMN activation on α_1CB6 peptides was studied according to the protocol described in the 'Experimental procedures'. The results of one representative experiment out of three independent experiments is shown. (B) Each band was quantified by densitometry (with the results obtained in arbitrary units), and activation of PMN by α_1CB6 peptides was expressed as a ratio of the intensity of activation to the amount of α_1CB6 peptides deposited. The results are expressed as means ± standard deviations (n = 3). Significant differences versus control collagen α_1CB6 peptide (fraction c): NS, non significant; $^{**}P < 0.01$. Ct, control collagen CNBr peptides; C6h, 6-h-carbamylated collagen CNBr peptides.

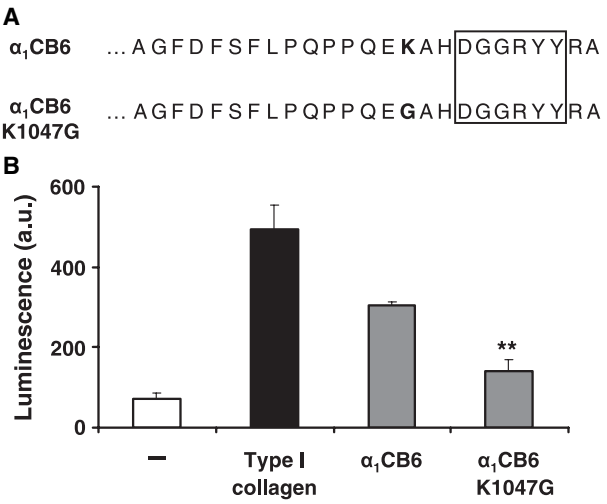

Fig. 6. Influence of the K1047G mutation on PMN activation mediated by α_1CB6 peptides. (A) Representation of the amino acids primary sequence surrounding mutation site in recombinant α_1CB6 peptide. (B) Approximately 10^6 PMNs suspended in 1 mL of Dulbecco's solution were incubated for 15 min at 37 °C with 50 μg·mL^{-1} of control or mutated recombinant α_1CB6 peptides (grey bars) and the production of ROS by PMNs was analysed by chemiluminescence (see the Experimental procedures). Incubation of PMNs with 100 μg·mL^{-1} of type I collagen (black bar) was used as a positive control of PMN activation, whereas incubation of PMNs without effector was used as a negative control (white bar). Results are expressed as means ± standard deviation (n = 3). Significant differences versus control α_1CB6 peptides: $^{**}P < 0.01$. a.u., arbitrary units.

and characteristic complications of several diseases such as chronic renal failure or atherosclerosis [18,19], together with other post-translational modification of proteins such as glycoxidation [20].

As these results suggested the participation of collagen lysine residues in PMN activation, the present study was devoted to identification of the residues involved in this process. To that end, three evaluations were carried out: (a) the competitive effect of AHA on collagen-induced PMN activation, (b) the effect of the carbamylation of lysine side chains on collagen-induced PMN activation and (c) the effect of a recombinant peptide mutated on lysine 1047 on collagen-induced PMN activation.

AHA, a lysine structural analogue, was first shown to be a competitive agent of the interaction between collagen and PMNs because it induced a dose-dependent inhibition of ROS production, indicating the impairment of the interaction. This inhibitory effect was independent of any direct scavenger effect of AHA on ROS and was observed at 10 mM, which is a somewhat lower active concentration than that already reported in the literature (for example 200 mM for the inhibition of apo(a) lysine-binding sites [21]).

We then used carbamylated collagen to determine to what extent specific modifications of lysine residues could induce a loss of effect. Carbamylation has already been used to determine the role of specific amino acids in protein–protein interactions. For instance, selective carbamylation of the α-amino group of the tissue inhibitor of metalloproteinases-2 NH_2-terminal cysteine has been used to demonstrate the key role of this amino group in the inhibitory effect of tissue inhibitor of metalloproteinases-2 towards matrilysin and gelatinase-A [22]. The results presented in Figs 2 and 3 confirmed the specific ability of α_1CB6 peptides to stimulate ROS production by PMNs, as previously demonstrated [5], and showed that this peptide progressively lost its stimulating effect with an increasing carbamylation rate. These experiments indicated that the involvement of the lysine ε-NH_2 group(s) could be explained by the increased probability of the six lysine residues located in α_1CB6

peptides to be carbamylated, as illustrated by 2D electrophoresis patterns. This technique allowed us to demonstrate relative heterogeneity in the carbamylation rate of collagen CNBr peptides obtained from the incubation of collagen with cyanate and to establish a correlation between the number of spots detected and the number of modified lysine residues, as previously demonstrated by Qin *et al.* for alpha-crystallins [23]. In this respect, we evaluated the activity of the different α_1CB6 peptides separated by preparative IEF. IEF separation of control collagen-derived α_1CB6 peptides revealed three different peptides, of which only two exhibited a stimulatory effect on PMNs. These three spots corresponded to collagen molecules with different basal carbamylation rates because the new spots generated by carbamylation experiments exhibited the same pI value as the minor spot derived from control collagen. Experiments performed with 6-h-carbamylated collagen-derived α_1CB6 peptides revealed that none of the peptides identified as carbamylated peptides was able to activate PMNs. These results supported the hypothesis that only one lysine residue among the six contained within the α_1CB6 peptide was crucial in the PMN activation process and represented a preferential target of carbamylation.

To localize this residue, we analyzed primary sequences of a collagen α_1 chain of various species. This study revealed the presence of a conserved lysine residue at position 1047, located three amino acids upstream from the active DGGRYY sequence. This residue was not identified as a target for hydroxylation by lysine hydroxylase (i.e. it was not a component of the GXK consensus sequence recognized by the enzyme) and could subsequently be assumed to be free from modifications related to collagen cross-linking. As the use of short synthetic peptides was not convenient because such peptides could only exert a competitive effect in the presence of collagen [5], we produced a recombinant mutated peptide. In our approach it was necessary to use the whole α_1CB6 peptide (including RGD sequences) to obtain PMN activation. We chose to replace lysine 1047 with a glycine residue to evaluate simultaneously the influence of the ε-NH_2 group charge and the steric hindrance of the side chain. The residue deprived of a side chain did not disturb the particular structure of the collagen α chain. We found that this mutation significantly decreased the ability of the recombinant peptide to activate PMNs. The inhibition of the stimulatory effect was major, resulting in a 70% decrease in PMN activation compared with the control peptide. However, it was not complete. We thus can hypothesize that this lysine residue strengthens the interaction between PMNs and the DGGRYY sequence and that the interaction is less efficient when the residue is modified.

Two hypotheses may explain the role of lysine 1047: either this amino acid participates in the stabilization of the DGGRYY sequence conformation or it interacts directly with a PMN receptor ($\alpha_L\beta_2$ integrin), as does the DGGRYY sequence [4]. The first hypothesis was supported by our previous studies demonstrating that collagen carbamylation led to a partial loss of its triple helical structure [7], but not by the competitive effect of AHA, as shown here. We can therefore assume that lysine 1047 acts as an anchoring point on the type I collagen molecule for $\alpha_L\beta_2$ integrin, even though we cannot exclude that the substitution of lysine by glycine in the recombinant peptide can induce subtle modifications of DGGRYY sequence conformation. Until recently, no data were available in the literature that reported a direct interaction between collagen lysine residues and β_2 integrins. However, such a mechanism has already been described for disintegrin-specific sequences containing lysine residues. Ivaska *et al.* have demonstrated that the three-amino acid sequence RKK (contained within the cyclic peptide CTRKKHDNAQC derived from jararhagin disintegrin) is essential for binding to the I domain of α_2 integrins [24]. Similarly, members of the 'lysine–threonine–serine (KTS)–disintegrin' family contain the consensus KTS sequence, rather than RGD, in their integrin- binding loop [25,26]. In addition, glycation of collagen lysine side chains is responsible for an impaired interaction of type I collagen with β_1 integrins [27,28]. Thus, we hypothesized that lysine 1047 might play a similar role in the interaction of collagen with PMN $\alpha_L\beta_2$ integrin. These experiments show, for the first time, the specific role of lysine 1047 in the activation of PMNs by type I collagen, even though the interaction experiments were performed using peptides instead of the whole type I collagen molecule. This experimental design does not fully reproduce physiological conditions, but it is well known that cell interactions may be modulated not only by whole proteins but also by macromolecule-derived peptides (matrikines) that are cleaved from extracellular matrix proteins *in vivo* and exert specific effects [29].

In conclusion, our results confirm the paramount importance of lysine residues in protein–protein or protein–cell interactions and suggest that any side chain modification of these residues, which are exposed *in vivo* to post-translational modifications (e.g. glycation or carbamylation), may have important consequences in human pathophysiology. In this regard, our results are in line with recent studies using other experimental approaches [19,30], which indicate carbamylation as a

major post-genomic mechanism of the 'post-translational pathophysiology' of atherosclerosis and renal failure [7,19,31–33]. This concept should be further considered for the design of new therapeutic strategies.

Experimental procedures

Materials

All chemicals were obtained from Sigma (St Louis, MO, USA), unless stated otherwise.

Preparation of collagen

Acid-soluble type I collagen was prepared from Sprague–Dawley rat tail tendons by acetic acid extraction, as previously described [34]. Pepsin-digested type I collagen was obtained after digestion of collagen with 0.1% (m/v) pepsin in 100 mM acetic acid for 18 h at 4 °C. In some experiments, collagen was carbamylated by incubation with 100 mM KCNO in a 150 mM phosphate buffer, pH 7.4, for 2, 6 or 24 h at 37 °C, leading to the transformation of 2, 6 and 11 lysine residues, respectively, into homocitrulline residues per collagen α chain [7]. After incubation, collagen was extensively dialyzed against distilled water until no potassium could be detected by flame photometry (model 480; Chiron Healthcare SAS, Suresnes, France). Subsequently, collagen was lyophilized and solubilized at 2 $mg \cdot mL^{-1}$ in 18 mM acetic acid. Collagen preparations were verified to be endotoxin free (< 0.05 endotoxin units$\cdot mL^{-1}$) using the limulus amebocyte lysate kinetic-QCL kit (Cambrex BioSciences, Emerainville, France).

Preparation of collagen CNBr peptides

Collagen-derived CNBr peptides were prepared as described by Epstein *et al.* [35]. Briefly, collagen solubilized at 10 $mg \cdot mL^{-1}$ in 70% (v/v) formic acid was incubated under N_2 for 4 h at 30 °C in the presence of an excess of CNBr. CNBr peptides were then lyophilized and dissolved in distilled water.

2D electrophoresis

Collagen CNBr peptides were first submitted to IEF using the 'Protein IEF cell' system (BioRad, Marnes-la-Coquette, France). Briefly, immobilized pH-gradient (IPG) strips (BioRad) were rehydrated with 250 μL of rehydratation buffer (8 M urea, 4% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 0.2% (v/v) Bio-Lytes® (pH 3–10; BioRad), 200 mM dithiothreitol) containing 300 μg of CNBr peptides. Active rehydration of IPG strips was performed under 50 V for 10 h at room temperature. After rehydration, IEF was performed in three steps: conditioning (250 V, 15 min, 20 °C); linear voltage increase to 4000 V; and final focusing (20 000 $V \cdot h^{-1}$ for 5 h). After the IEF step, IPG strips were washed for 15 min in an equilibration buffer [375 mM Tris, 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, pH 8.8] containing 130 mM dithiothreitol and then washed for 20 min in the same buffer containing 135 mM iodoacetamide in place of dithiothreitol. CNBr peptides obtained by electrofocusing were further separated by SDS-PAGE containing 12.5% (w/v) polyacrylamide and the gels were stained with Coomassie Brillant Blue R250.

Preparative IEF

Preparative IEF was carried out using a ROTOFOR® system (BioRad), made up of a 55-mL focusing chamber cooled in its centre by a ceramic tube and divided into 20 compartments surrounded by anode and cathode compartments, filled respectively with 100 mM H_3PO_4 and 100 mM NaOH solutions. The pH gradient was established using Bio-Lytes (BioRad) ampholytes (pH 4–8). The focusing chamber was filled with 45 mL of distilled water, 2 mL of glycerol, 1 mL of ampholytes and 4 mL of a 10 $mg \cdot mL^{-1}$ CNBr peptide solution. Focusing was performed at constant power (12 W) under gentle stirring (1 r.p.m.) for 6 h at room temperature. Fractions corresponding to each compartment of the focusing chamber were then collected by aspiration and their respective pH values were measured. The resolution of separation was improved by a second IEF experiment carried out directly with selected fractions (containing peptides of interest) in order to refine the pH gradient.

Preparation of PMNs

PMNs were isolated from whole blood obtained by venepuncture of healthy subjects, after obtaining informed consent, using a one-step centrifugation procedure (600 ***g***, 30 min, 20 °C) through a Ficoll gradient (Polymorphprep®; Axis-Shield, Oslo, Norway). PMNs were washed in Dulbecco's solution (137 mM NaCl, 2.7 mM KCl, 30 mM HEPES, 10 mM glucose, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) and then centrifuged (1000 ***g***, 5 min, 20 °C). Contaminating erythrocytes were removed by hypotonic lysis using a solution of 15 mM NH_4Cl. Isolated PMNs were counted on a Neubauer hemocytometer and viability was checked using the Trypan Blue exclusion test. Purity and viability of preparations were, respectively, > 95% and > 98%.

Evaluation of ROS production by PMNs

ROS production was evaluated using a chemiluminescence test. Briefly, 10^6 PMNs were incubated in 1 mL of Dulbecco's solution, together with 100 μg of denatured (30 min at 60 °C) type I collagen or 50 μg of purified recombinant

peptides, for 15 min at 37 °C in the presence of 28 μM luminol [36]. Luminescence, expressed in arbitrary units, was directly measured in supernatants using a luminometer (Lumac 3M Biocounter M2010A, Schaesberg, the Netherlands).

Evaluation of PMN adhesion and activation in contact with CNBr peptides separated by electrophoresis

The ability of collagen CNBr peptides, separated by electrophoresis, to modulate PMN functions was assessed using a previously described technique [37]. Briefly, 50 μg of CNBr peptides were submitted to SDS-PAGE containing 12.5% (w/v) polyacrylamide and blotted onto a 9×8 cm nitrocellulose membrane (VWR International, Fontenay sous Bois, France). Membranes were saturated with Dulbecco's solution containing 5% (w/v) BSA for 1 h at room temperature and then rinsed three times with fresh Dulbecco's solution before performing adhesion and activation experiments.

For adhesion experiments, 2×10^7 PMNs in Dulbecco's solution (10 mL) were incubated on the saturated membrane (previously transferred into a specific plastic dish) for 30 min at 37 °C. After incubation, the membrane was washed twice with Dulbecco's solution in order to remove non-adherent cells. The membrane was incubated for 1 h at 37 °C in Dulbecco's solution containing a mixture of mouse antibodies (Monosan, Uden, the Netherlands) raised against PMN surface proteins (CD11a, CD11b and CD11c, at a concentration of 1 $\mu g \cdot mL^{-1}$) and washed three times with Dulbecco's solution. Detection of antibodies bound to PMNs fixed to CNBr peptides was performed using a peroxidase-conjugated secondary antibody and a solution of 4-chloro-1-naphtol.

For activation experiments, 2×10^7 cells suspended in Dulbecco's solution (10 mL) containing 167 μM nitro blue tetrazolium were incubated for 30 min at 37 °C onto the saturated membrane, previously transferred into a specific plastic dish. The CNBr peptides that induced PMN activation appeared as blue-stained bands and were quantified by densitometry (Vilbert-Lourmat, Marne La Vallée, France).

Directed mutagenesis

Total RNA extracted from dermal fibroblasts was submitted to RT-PCR to obtain the corresponding cDNA. Directed mutagenesis was carried out by performing successive PCR steps [i.e. after each PCR, the specificity of the PCR amplification was verified by electrophoresis on a 1% (w/v) agarose gel and the corresponding amplicons were purified from agarose gels by using the Midi Gebaflex Tube® system (Fermentas, Souffelweyershein, France)]. The purified products were then used as matrices for the PCR described below. PCR primers were designed using the GenBank sequence number NG007400 [*COL1A1* gene: collagen, type I, alpha 1 (*Homo sapiens*) – Gene ID: 1277 – locus Z74615]. Primer sequences used in consecutive PCR reactions (denoted a–d) were as follows (note that the position of primers in the whole nucleotide sequence are indicated in square brackets): (a) forward: 5′-TGG TCA GAG AGG AGA GAG A-3′ [position 3011 to position 3029] and reverse: 5′-TGT CCT TGG GGT TCT TGC T-3′ [position 4062 to position 4080]; (b) forward: 5′-AAA CAA GGT CCC TCT GGA GCA AGT GGT GAA CGT-3′ [position 3069 to position 3101] and reverse: 5′-TAG TAG CGG CCA CCA TCG TGA GCC CCC TCT TGA-3′ [primer containing the mutation site - position 3734 to position 3766]; (c) forward: 5′-TCG TGA ATT CAC CTG GAT TGG CTG GA-3′ [position 3127 to position 3140] and reverse: 5′-ATC AGC CCG GTA GTA GCG GCC ACC AT-3′ [position 3751 to position 3776]; (d) forward: 5′-TCG TGA ATT CAC CTG GAT TGG CTG GA-3′ [position 3127 to position 3140] and reverse: 5′-ACT AAG CGG CCG CTA TCA GCC CGG TA-3′ [position 3765 to position 3776]; '(c) forward' and '(d)' primers contained restriction sites used for plasmid construction (*Eco*RI and *Not*I). A control cDNA was obtained in the same conditions by using a reverse primer that did not contain the mutation site during the second PCR step, as follows: (b) forward: 5′-AAA CAA GGT CCC TCT GGA GCA AGT GGT GAA CGT-3′ [position 3069 to position 3101] and reverse: 5′-TAG TAG CGG CCA CCA TCG TGA GCC TTC TCT TGA-3′ [position 3734 to position 3766]. After these different amplification steps, cDNA was digested by *Eco*RI and *Not*I restriction enzymes and then inserted into the pGEX-4T3 plasmid (GE HealthCare, Orsay, France). Sequences of control and mutated cDNA were verified by sequencing (data not shown; Genome Express, Meylan, France) before starting the production of recombinant peptides.

Production and purification of recombinant α_1CB6 peptides

After transformation with a pGEX-4T3 plasmid containing cDNA and clone selection, JM109DE3 bacteria were grown, overnight at 37 °C with agitation, in 100 mL of Luria–Bertani medium supplemented with 100 $\mu g \cdot mL^{-1}$ of ampicillin (used to select transformed bacteria). Protein production by bacteria was then enhanced by stimulation with 400 mM isopropyl-β-D-galactopyranoside for 4 h at 37 °C. Bacteria were collected by centrifugation (900 ***g***, 15 min, 4 °C), and suspended in 50 mM Tris, 1 mM EDTA (pH 8.0) buffer, before sonication. After centrifugation (10 000 ***g***, 5 min, 4 °C) the lysate was recovered for protein purification.

As the pGEX-4T3 plasmid allows the production of a glutathione *S*-transferase (GST) fusion protein, the lysate

was incubated overnight at 4 °C in the presence of 1 mL of glutathione sepharose-4B resin (GE HealthCare). After washing the resin with buffer comprising 50 mM Tris and 1 mM EDTA (pH 8.0), the GST fusion protein was eluted by 30 mM reduced glutathione and then incubated overnight at room temperature with thrombin (20 $U \cdot mg^{-1}$ of fusion protein) in order to release the α_1CB6 peptide from the GST. The digestion product was incubated again with glutathione-sepharose-4B resin, and the α_1CB6 peptide, eluted separately from the GST protein, was recovered and dialyzed for 3 days against distilled water before being lyophilized.

Statistical analysis

All experiments requiring statistical analysis were performed in triplicate and the results are expressed as means ± standard deviations. Significance of differences was calculated using the Student's *t*-test.

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