Enhanced activation of and increased production of matrix metalloproteinase-9 by human blood monocytes upon adhering to carbamylated collagen

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Abstract Carbamylation refers to chemical modification of protein side chains by cyanate derived e.g. from urea. It alters their structural and functional properties. We have studied the influence of the carbamylation of type I collagen in vitro on its interactions with elutriated human monocytes, and its potential role in atherosclerosis. Adhesion of monocytes onto carbamylated collagen was significantly enhanced compared to native collagen. There was no change in superoxide anion production. On the other hand, there was an increase in the production and the activation of matrix metalloproteinase-9. No effect was found on tissue inhibitor of metalloproteinase-1 production. Thus, the presence of carbamylated collagen may stimulate the remodelling of extracellular matrix mediated by activated monocytes. Such alterations may contribute to enhanced atherosclerosis in renal insufficiency, a pathological condition associated with elevated levels of carbamylation.
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1. Introduction

Many cell functions such as protein synthesis are controlled by direct interactions with extracellular matrix (ECM) components, which trigger various transduction pathways [1]. However, such interactions cannot be considered without reference to the post-translational modifications of proteins, which occur throughout their lifespan in vivo and are characterized by non-enzymatic binding of various low molecular weight molecules to amino groups of proteins. These cumulative modifications not only alter protein structure, but also impair protein interactions with surrounding cells and trigger deleterious reactions. Because of their long biological half-life, ECM proteins, among them type I collagen, represent preferential targets for post-translational reactions. For instance, collagen glycoxidation, due mainly to the binding of glucose,

is involved in pathophysiology of ageing, diabetes mellitus, and other pathologies including atherosclerosis [2]. Atherosclerosis is a complex process involving a variety of events which have still been only partially elucidated [3]. A critical factor in its progression is vessel wall remodelling, conditioned by the local inflammatory status and the balance between degradative and reparative reactions. ECM proteins play a pivotal role in this process, mainly through their interactions with inflammatory cells such as monocytes, which are known to be actively involved in the development of atherosclerosis [4–6].

One recently revealed post-translational modification of proteins is carbamylation [7]. The process occurs physiologically in humans and is based on covalent binding of isocyanic acid, a decomposition product of urea, to proteins. Elevated levels of carbamylated proteins are seen in renal insufficiency, especially in uremic patients [8]. However, the consequences of carbamylation on cell–matrix interactions are still poorly understood.

We have previously shown that type I collagen elicits adhesion and activation of human blood monocytes via interaction with $\alpha x \beta_2$ integrin [9]. Protein carbamylation has been proposed as a causal factor of atherosclerosis, particularly in renal insufficiency [10]. Therefore, it was of interest to study the influence of collagen carbamylation on its interactions with monocytes. We have found in this study that carbamylated collagen selectively regulates metabolic functions of monocytes, favoring their adhesion and matrix metalloproteinase-9 (MMP-9) release, thus increasing the inflammatory potential of monocytes and their ability to remodel ECM.

2. Materials and methods

2.1. Reagents
Chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise mentioned. Reagents for cell cultures were from Life Technologies (Cergy-Pontoise, France).

2.2. Preparation of carbamylated collagen
Acid-soluble type I collagen was prepared from Sprague–Dawley rat tail tendons by 0.5 M acetic acid extraction [9], and carbamylated by incubation with 0.1 M KCNO in 0.15 M phosphate buffer, pH 7.4, for 6 h at 37°C [11]. Control series were prepared in the presence of 0.1 M KCl. After incubation, collagen was extensively dialyzed against water, until no potassium could be detected by flame photometry (Chiron Diagnostics, model 480). Subsequently, collagen was lyophilized and solubilized in 18 mM acetic acid (2 mg/ml). In vitro carbamylation generated four residues of homocitrulline from lysine residues per 1000 amino acid residues (one $\alpha 1$ chain of type I collagen

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contains 1056 residues), as evaluated by anion exchange chromatography (Hitachi 880 analyzer, Roche Diagnostics).

2.3. Cell incubations

Monocytes were prepared by elutriation from whole blood obtained with informed consent from healthy volunteers [9]. The purity of the preparations (percentage of CD14-positive cells) and cell viability (trypan blue exclusion test) were respectively higher than 95% and 98%.

Ninety-six-well plates were coated with either untreated or carbamylated collagen, 25 μg per well, washed three times with Dulbecco's solution and saturated for 2 h at 37°C with 100 μl of 2% (w/v) bovine serum albumin, then washed three more times before cell addition. The amount of control or carbamylated collagen bound to plastic wells was verified by 4-hydroxyproline assay [12]: 2.25 ± 0.22 μg of 4-hydroxyproline in control collagen-coated wells vs. 2.18 ± 0.25 μg in carbamylated collagen-coated wells.

2.4. Measurement of cell adhesion and superoxide anion (O_2^-) production

Cell adhesion was measured at 37°C by staining cell nuclei with crystal violet. 1.5×10^5 cells were incubated from 0 to 6 h, and the number of adherent cells was calculated from a standard calibration curve [9].

O_2^- production was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome c, using monocytes in suspension, as previously described [13]. Briefly, 1.5×10^6 monocytes were suspended in 850 μl of Dulbecco's solution supplemented with 100 μl of 1 mM ferricytochrome c solution. O_2^- production was evaluated after addition of 100 μl of a 2 mg/ml solution of collagen I in 18 mM acetic acid. The increase in absorbance at 550 nm was evaluated spectrophotometrically, test tubes supplemented with 50 μl of a 1000 U/ml superoxide dismutase solution being used as blanks to assess the specificity of the reaction.

2.5. Evaluation of MMP-9 and TIMP-1 production

MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) amounts were quantitatively assayed in 100 μl culture medium by two-site enzyme-linked immunosorbent assay (ELISA) sandwich kits (Biotrak RPN2614 and Biotrak RPN2611 respectively, Amersham Pharmacia Biotech, Buckinghamshire, UK). These methods evaluated total MMP-9 (pro-MMP-9 and pro-MMP-9–TIMP-1 complexes) and total TIMP-1 (free and MMP-complexed TIMP-1).

MMP-9 activity was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) zymography, which allowed the determination of latent and activated forms, as previously described [14]. Culture medium (20 μg proteins) was subjected to electrophoresis under non-reducing conditions in a SDS–9% polyacrylamide gel containing 1 mg/ml gelatin. Gels were incubated overnight at 37°C in 50 mM Tris–HCl, 150 mM NaCl, 10 mM CaCl$_2$ buffer, pH 7.4, then stained with Coomassie brilliant blue. Unstained areas corresponding to gelatinolytic activities were quantified by image analysis (Vilbert-Lourmat, Marne La Vallée, France).

TIMP identification was assessed by gelatin reverse zymography. Culture medium (1 μg proteins) was subjected to electrophoresis in SDS–15% polyacrylamide gels containing 1 mg/ml gelatin and 20 ng/ml pro-MMP-2. Reverse zymography revealed inhibitory activity, which appeared as blue zones against a clear background, demonstrating inhibition of gelatin lysis in the gels.

3. Results

3.1. Effect of collagen carbamylation on monocyte adhesion and O_2^- production

Monocytes seeded on untreated acid-soluble collagen-coated plates gradually adhered to the substratum, reaching a plateau after 2 h incubation. A significantly higher number of monocytes adhered to carbamylated collagen. Kinetic analyses also showed that adhesion occurred faster (+44% at 30 min, $P < 0.001$) and reached a higher plateau level (+52% after 3 h, $P < 0.001$) (Fig. 1A).

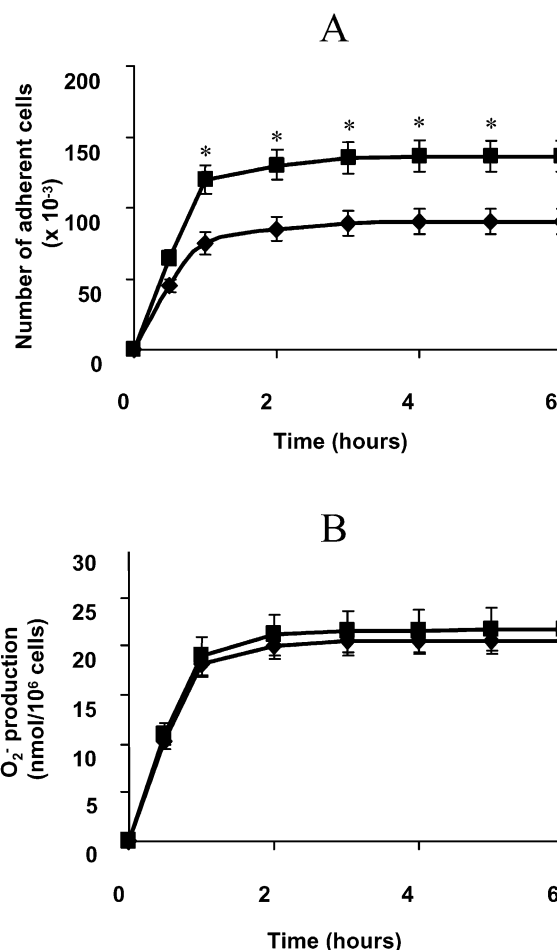By contrast, no change was seen in O_2^- amount produced

Fig. 1. Effect of collagen carbamylation on monocyte adhesion (A) and O_2^- production (B). A: Monocytes were seeded in wells coated with untreated (♦) or carbamylated (■) type I collagen. The number of adherent cells was determined by measurement of absorbance at 560 nm using the crystal violet method and a standard calibration curve. Results are the means of four experiments ± S.E.M. Significant difference (Student's t-test) between the two series: *$P < 0.001$. B: Monocytes were incubated in suspension with untreated (♦) or carbamylated (■) type I collagen, and O_2^- production measured by the superoxide dismutase-inhibitable reduction of ferricytochrome c. Results are the means of four experiments ± S.E.M. No significant difference was found between the two series.

when the cells adhered to carbamylated collagen as compared to untreated collagen (Fig. 1B).

3.2. Effect of collagen carbamylation on MMP-9 production and activation by monocytes

The contact of monocytes with carbamylated collagen strongly enhanced both production and activation of MMP-9 when compared to untreated collagen (Fig. 2A). This effect occurred in a time-dependent manner. In the presence of untreated collagen, total MMP-9 gradually increased in the culture medium, reaching about three times the 6-h level after 24 h incubation. A highly accelerated MMP-9 production was seen on carbamylated collagen: increases of 46% vs. control after 6 h ($P < 0.001$), and 70% ($P < 0.001$) after 24 h (Fig. 2B). After 24 h, MMP-9 concentrations, as measured by ELISA in culture medium, were 10.8 ± 3.7 ng/ml in control series (KCl-

incubated collagen) vs. 18.8 ± 4.1 ng/ml in monocytes cultivated on carbamylated collagen ($P < 0.001$).

The activation of pro-MMP-9 to active MMP-9 was also increased upon carbamylated collagen. After 6 h, only the 84-kDa active form could be found in culture medium of monocytes adhering to untreated collagen, at very low levels ($< 1.5\%$ of total MMP-9), whereas the two activated forms (84 and 78 kDa) were present in the culture medium of monocytes incubated with carbamylated collagen. The activation of MMP-9 induced by carbamylated collagen increased progressively with time, the percentage of active forms being 4.1%, 8.0%, 12.2% at 6 h, 12 h and 24 h respectively, vs. 1.4%, 2.5%,

Fig. 3. Effect of collagen carbamylation on TIMP-1 and TIMP-2 production by monocytes. Monocytes were incubated with untreated (1), KCl-incubated (2) or carbamylated (3) collagen for up to 24 h. Conditioned media were analyzed by gelatin reverse zymography (1 μg protein per lane).

3.8% in control conditions. The difference was highly significant ($P < 0.001$) at all times (Fig. 2C).

3.3. Effect of collagen carbamylation on TIMP-1 production by monocytes

Contact of monocytes with carbamylated collagen was not associated with the production of the major physiological inhibitor of MMP-9, TIMP-1, when compared to untreated collagen (Fig. 3). After 24 h incubation, TIMP-1 concentrations, as measured by ELISA in culture medium, were 820 ± 83 ng/ml in control series vs. 851 ± 78 ng/ml in monocytes cultivated on carbamylated collagen (+3.8%, NS). No significant change of TIMP-2 production was noticed by reverse zymography.

4. Discussion

Carbamylation results from the non-enzymatic binding of urea-derived cyanate to amino groups of proteins, especially to lysine residues, generating homocitrulline [7,15]. This reaction is increased in vivo by renal insufficiency [8,15]. Studies have shown that carbamylation of plasma proteins and hemoglobin was increased in patients with renal insufficiency. However, there is very little literature on the carbamylation of tissue proteins. The presence of homocitrulline in kidneys of patients with renal insufficiency has been shown in one study [16], but no quantitative results have been reported. Considering the long lifespan of ECM proteins and with reference to the accumulation of glycoxidation end-products in various diseases [2], we may expect a much higher rate of carbamylation in tissue proteins than in circulating proteins. The concentration of potassium cyanate used in our study to carbamylate collagen is compatible with the ones used by others in in vitro studies on the effects of serum carbamylated proteins on cultured mesangial cells [17], or on the effects of cyanate on isolated polymorphonuclear neutrophils [15]. In our experimental procedures, four homocitrulline residues were formed per 1000 amino acid residues, which is a lower rate than that obtained by Shaykh et al. (10 per 1000) for carbamylated serum proteins [17].

Atherosclerosis, a major complication of renal insufficiency, is not fully explained by conventional risk factors. Previous studies have suggested that the carbamylation of low density lipoproteins could enhance their oxidation, and thus promote atherogenesis [10]. More recently, carbamylation has been shown to alter structural and/or functional properties of ECM proteins: in glomerular mesangial cells for instance, carbamylated proteins stimulate collagen deposition [17]. Such findings point out the potential role of protein carbamylation in atherosclerosis related to renal insufficiency.

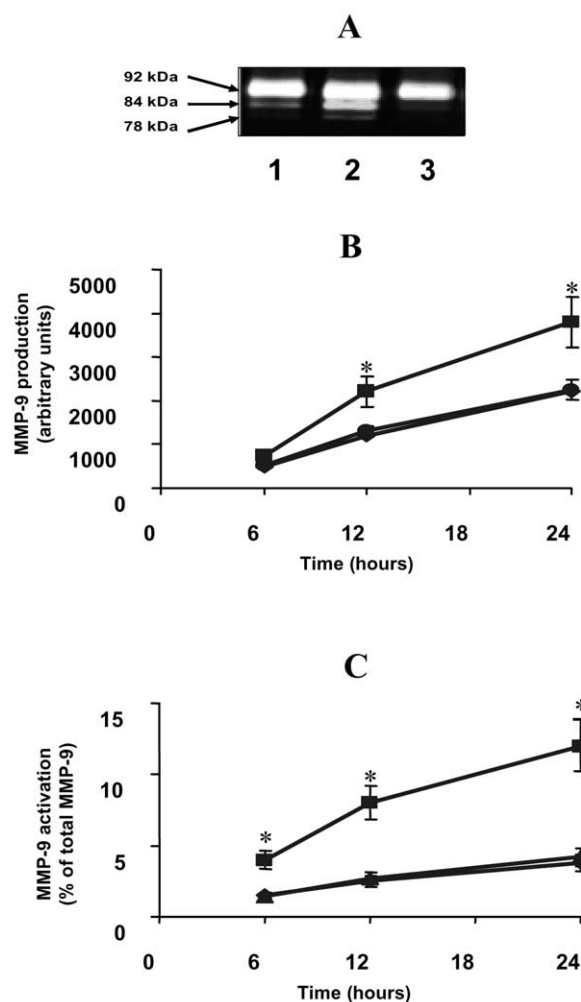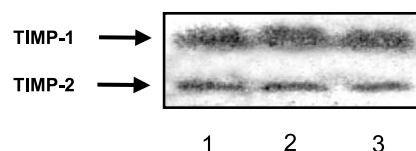We have therefore studied how the carbamylation of colla-

Fig. 2. Effect of collagen carbamylation on MMP-9 production and activation by monocytes. Monocytes were incubated with untreated (1, ♦), carbamylated (2, ■) or KCl-incubated (3, ●) collagen for up to 24 h. Conditioned media were analyzed by gelatin zymography (20 μg protein per well). MMP-9 bands were evaluated by densitometric evaluation. Results are the means of four determinations ± S.E.M. Significant difference (Student's t-test) between control and KCl-incubated collagens vs. carbamylated collagen: *$P < 0.001$. A: Gelatin zymography of conditioned media after 24 h incubation. Latent MMP-9 (MW = 92 kDa) and active forms of MMP-9 (MW = 84 kDa and 78 kDa) are indicated by arrows. B: Kinetics of MMP-9 production. Every point indicates the production of total MMP-9 (latent+active forms). C: Kinetics of MMP-9 activation. Every point represents the ratio activated MMP-9 × 100/total MMP-9.

gen, a long-lived tissue protein, altered its interactions with monocytes. Monocytes play an essential role in the initiation and progression of atherosclerosis through their pro-inflammatory properties that contribute to the destabilization of atheroma plaque, leading to superficial erosions or rupture of the plaque [18]. As a matter of fact, monocytes synthesize various components that influence ECM plaque remodelling, including reactive oxygen species and MMPs [6,19]. Circulating monocytes are attracted by numerous cytokines or chemokines to the atherogenic sites of vessel walls, undergo transendothelial migration, interact with type I collagen through CD11c/CD18 integrin [9], and differentiate to macrophages that contribute to the constitution of the plaque.

We have shown here that carbamylation altered the ability of type I collagen to modulate monocyte functions. Firstly, the number of monocytes adherent to carbamylated collagen was much higher than the number of monocytes adherent to untreated collagen, which suggests that monocytes interact more closely with the ECM, for example in blood vessels, when carbamylation is increased. Secondly, we found that the increased binding of monocytes was accompanied by a strong stimulation of active MMP-9 production, corresponding to an increased ability of monocytes to degrade surrounding ECM. Overexpression of MMP-9 increases the risk of rupture of the fibrous cap of the atherosclerotic plaque, and represents a key event in triggering thrombotic events like vascular cerebral accidents or acute coronary syndromes. In addition, MMPs significantly modulate cellular functions, and knockout of various MMP genes in mice has clearly demonstrated their link with the atherosclerosis process, as reviewed elsewhere [20]. In our experiments, the increased MMP-9 secretion and activation were not correlated with an altered production of its major inhibitor, TIMP-1, whereas no effect on oxygen free radical production was noticed, suggesting that other inflammatory properties of monocytes were not stimulated. The mechanism of this interaction is currently under investigation. Other studies have reported that various ECM components (e.g. laminin peptides) could modulate monocyte functions [21], thus explaining some of their roles in atherogenesis [18]. Our findings suggest an increased adhesion and a sustained activity of monocytes in carbamylated tissues, and provide novel insights into the pathophysiology of atherosclerosis, particularly in renal insufficiency.

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