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Degradation of oxidized and glycoxidized collagen: Role of collagen cross-linking

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ABSTRACT

Skin aging is a multifactorial process leading to structural and physiological changes. Protein modifications are known to play here an important role. Since collagen is the most abundant protein in the extracellular matrix of the skin and due to its slow turnover rates it is a frequent target of modifications by reactive compounds. Using skin biopsies of young and old mice we demonstrated that advanced glycation end products (AGEs), such as argpyrimidine and pentosidine, accumulate in aged skin, whereas protein carbonylation is unchanged. To investigate whether this discrepancy in accumulation is the result of an increased formation or due to reduced degradation we used modified collagen type I in *in vitro* experiments and tested for proteolytic susceptibility. We were able to show that collagenase is able to degrade oxidized and AGE-modified collagen. However, if collagen is cross-linked heavily, collagenase is unable to degrade the modified collagen. Cross-linking of collagen is preferentially taking place in collagen fibers treated with glycoxidizing agents.

In summary, the low presence of oxidized collagen in aged skin seems to be the result of a sufficient degradation by collagenases, whereas the reason of the accumulation of AGE-modified collagen is at least partially an insufficient degradation.

Introduction

Skin aging is studied for some time for health as well as cosmetic reasons [1]. Skin aging is due to both intrinsic and extrinsic factors causing a unique combination of intrinsic skin aging and photoaging [2]. In general, aging is a complex process with a decline of cellular function because of an accumulation of damaged cellular components during lifetime [3]. In skin one characteristic functional age-associated decline is a reduction of its elasticity and an increase in skin stiffness [4]. This decline is due to changes in the dermal and subcutical layers of the skin, which are composed of connective tissue and fat. The loss of subcutical fat and most importantly the changes of the extracellular matrix in the dermal layer are the major players in this process [5,6]. An important mechanism, which is described in this context, are posttranslational modifications of collagen, which is the most abundant protein in the extracellular matrix of the skin. Such posttranslational age-related changes are unavoidable and for unknown reasons the fibroblasts, responsible for maintaining the collagen homeostasis, are not able to keep up with this task during aging.

Important posttranslational modifications of collagen are oxidation and the formation of advanced glycation end products (AGEs)[1], which both might occur due to intrinsing or extrinsing factors [7,8]. AGE formation is the result of a series of complex reactions starting with the reaction of a reducing sugar with the amino group of proteins. In this so-called Maillard reaction the amino group and the carbonyl group form a Schiff base and further on Amadori products [9]. After Amadori rearrangement further modifications take place resulting finally in the formation of AGEs. As already mentioned, beside the formation of AGEs an enhanced accumulation of oxidatively modified proteins is considered to be a hallmark of the aging process. Proteins of organisms in an aerobic environment are continuously exposed to oxygen free radicals and other reactive species [10,11]. Since oxidized proteins are associated with failure of their biological functions, proteolytic degradation of modified proteins is necessary to maintain cellular homeostasis. However, certain pathological conditions, aging, increasing oxidant flux or impaired degradation systems cause the accumulation of damaged proteins [3]. The AGE formation, the oxidation of intracellular proteins during aging as well as the fate of these modified proteins in cells are investigated to some extent [12–15]. However, almost nothing is known about the degradation of posttranslationally modified collagen,

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[1] *Abbreviations used:* AGE, advanced glycation end product; MMP, matrix metalloproteinase.

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why it is accumulating and whether the degradation of such modified collagen is selective. The major enzymes involved in skin collagen turnover are the zinc-dependent matrix metalloproteinases (MMPs) [16]. Therefore, we tested in the here presented study which protein modifications accumulate in aged skin and whether modified collagen can be degraded preferentially by collagenase.

Materials and methods

Reagents

Rat tail collagen type I was purchased from Life Technologies (Darmstadt, Germany). All other chemicals were from Sigma–Aldrich (Deisenhofen, Germany) or Carl Roth AG (Karlsruhe, Germany) unless otherwise indicated.

Animals

C57BL6/J mice, obtained from Charles River Laboratories (Sulzfeld, Germany), were kept under controlled humidity (55 ± 10%), temperature (22–24 °C) and on a 12 h light/dark schedule. Food (Sniff Spezialdiäten GmbH, Soest, Germany) and water were provided *ad libitum*. Skin biopsies were isolated from the back of the animals after dislocation of the cervical spine.

Analysis of mouse skin

UV-light protected skin of young (<5 months) and old mice (>16 months) was homogenized with lysisbuffer (50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P40, 0.1% SDS, 0.5% Natriumdeoxycholat) using a POLYTRON® PT2100 homogenizer (Kinematica GmbH, Eschbach, Germany). Forty microgram protein of tissue lysates were separated by SDS–PAGE using 10% acrylamide gels. For the determination of protein carbonyls, separated proteins were transferred onto nitrocellulose membrane and treated as described by Catalgol et al. [17]. In brief, the membrane was washed with PBS/methanol (80/20, v/v), 2 N HCl and incubated with 10 mM DNPH solution. After three washing steps with 2 N HCl and five washing steps with 50% methanol, the membrane was blocked with Odyssey® blocking buffer (Li-Cor Biosciences, Bad Homburg, Germany) for 1 h. Immunodetection was performed using rabbit polyclonal anti-DNP antibody (D9656, Sigma–Aldrich). Detection of the membranes was carried out using a secondary fluorescent–conjugated antibody and the Odyssey infrared detection system from Li-Cor Biosciences (Bad Homburg, Germany). For the detection of argpyrimidine, mouse monoclonal anti-MG antibody (AGE06B, Biologo, Kronshagen, Germany) was used.

Preparation of soluble, oxidized and glycoxidized collagen

Rat tail collagen type I was dissolved in 0.02 M acetic acid to 0.5 mg/ml and incubated with 20, 100, 200, 300 and 400 µmol hydrogen peroxide (H_2O_2)/mg protein and 0.2, 1, 2, 3, 4 µmol copper sulfate ($CuSO_4$)/mg protein for 0.5 h at 37 °C. To test time-dependent protein oxidation 0.5 mg/ml collagen solution was incubated with 200 µmol H_2O_2/mg protein and 2 µmol $CuSO_4$/mg protein for 0.5, 1, 1.5 and 2 h. The reaction was stopped by the addition of 1000 U/ml catalase. Glycoxidized collagen was prepared by incubating rat tail collagen type I with glyoxal and methylglyoxal (40, 80, 160, 320 µmol/mg protein) for 1 week at 37 °C and 5% CO_2 followed by dialysis against 0.02 M acetic acid overnight.

Preparation of oxidized and glycoxidized collagen lattices

To allow polymerization, 1 N NaOH was added to 3 mg/ml rat tail collagen type I and collagen solution (2.7 mg/ml) was incubated for 2 h at 37 °C. Collagen gels were treated with 74 µmol H_2O_2/mg protein and 0.74 µmol $CuSO_4$/mg protein as well as various concentrations of glyoxal (0.2, 0.4, 1, 2, 4 µmol/mg protein) and methylglyoxal (0.04, 0.2, 0.4, 1, 2 µmol/mg protein). After 2 h incubation at 37 °C modified collagen lattices were washed twice with phosphate-buffered saline (PBS).

Collagen analyses

For analysis of oxidized collagen 10 µg of protein samples were separated by SDS–PAGE using 6% acrylamide gels which were stained with Coomassie blue solution (45% methanol, 10% acetic acid, 0.2% Coomassie brilliant blue G250) for 0.5 h. Coomassie-stained gels were washed with 10% acetic acid until protein bands were clearly visible. Formation of protein carbonyls was investigated by immunoblot as described above and by ELISA according to Buss et al. [18] with modifications by Sitte et al. [14]. The degree of collagen modification after glyoxal- and methylglyoxal treatment was determined by AGE fluorescence measured at 360 nm excitation and 460 nm emission using a microplate reader (Synergy 2, BioTek, Bad Friedrichshall, Germany). Cross-linking of collagen was characterized by SDS–PAGE. In addition, methylglyoxal-modified collagen was detected for argpyrimidine by immunoblot as described above.

Collagen degradation

Degradation of soluble collagen was performed using collagenase of *Clostridium histolyticum* (⩾125 Collagen Digestion Units (CDU)/mg solid; C5138, Sigma–Aldrich). The final concentration of collagenase was 450 CDU/mg collagen. The reaction was stopped after 0.25, 0.5, 3 or 24 h collagenase incubation with 5 mM ethylenediaminetetraacetate (EDTA) to investigate time-dependant degradation of modified collagen. As a control, the reaction was directly stopped after adding collagenase. To analyze degraded collagen equal sample amounts were separated by SDS–PAGE and stained with Coomassie blue solution to quantify protein expression of collagen chains. Degradation of polymerized collagen was induced by exposing collagen lattices to bacterial collagenase in PBS with Ca^{2+} (30 CDU/mg collagen). By weighing collagen gels before and after the treatment, digestion of polymerized oxidized collagen was measured. The control was incubated with buffer alone. Furthermore, free NH_2 was detected via fluorescamine according to Reinheckel et al. [19]. Fluorescence intensity was measured using a microplate reader at 360 nm excitation and 460 nm emission.

Solubility of modified collagen lattices

250 µl of 2.7 mg/ml collagen solution was polymerized and modified with 74 µmol H_2O_2/mg protein and 0.74 mM $CuSO_4$/mg protein, 0.4 µmol glyoxal/mg protein and 2 µmol methylglyoxal/mg protein. Collagen lattices were incubated in 500 µl of 0.02 M acetic acid overnight at 4 °C and protein concentration was measured after modification as well as after incubation in 0.02 M acetic acid to determine protein recovery rate. Protein concentration was determined by Lowry protein assay (Bio-Rad, Munich, Germany) using rat tail collagen type I as a standard.