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Age-related expression, enzymatic solubility and modification with advanced glycation end-products of fibrillar collagens in mouse lung

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ABSTRACT

Changes in the expression of fibrillar collagens and post-translational modifications with advanced glycation end-products (AGEs) are often associated with tissue aging. Less is known about age-related changes in mouse lung tissue. Therefore, we studied the expression level and AGE load of fibrillar collagens in lungs from young (≤6 months), adult (15 months) and old (≥25 months) mice. The mRNA expression level was reduced in adult and old mice compared with the young. Old mice also showed a reduced protein level, whereas the adults even had more collagen protein. Fractionating of the fibrillar collagens into enzyme-soluble and insoluble collagens revealed a reduced solubility of collagens in old age. The enzymatic solubility of fibrillar collagens correlated inversely with the AGE load in the insoluble collagen as detected by the AGE-related fluorescence. While the intensity of the AGE-related fluorescence was increased in fibrillar collagens in response to age, the fluorescing AGE variant argpyrimidine was less affected. In summary, aging causes a reduced expression, lower enzymatic solubility and increased AGE load of fibrillar collagens in mouse lung tissue, but not all changes occur gradually with age.

1. Introduction

The extracellular matrix (ECM) provides anchorage and structural support to cells in organs and tissues, and it regulates cellular behavior. Numerous age-associated alterations of organs and tissues are believed to result from changes of the ECM proteins including differences in the turnover (Boyer et al., 1991; Bradley et al., 1974; Mays et al., 1991) and post-translational modifications, such as advanced glycation end-products (AGEs) (Baynes, 2001; Brownlee, 1995; Degroot, 2004).

AGEs are irreversible end-stage products of the non-enzymatic reaction of carbonyl groups of reducing sugars, degradation products of carbohydrates, lipids, or ascorbic acid with the amino groups of peptides, proteins, amino acids, and some phospholipids (Chuyen, 2006). AGEs exist in a large variety of chemical compounds, which are divided into fluorescent AGEs, such as argpyrimidine (ArgPyr), and non-fluorescent AGEs, such as N-ε-carboxymethyllysine (ε-CML) (Chuyen, 2006). Because the chemical structures of few AGEs are currently identified, AGE-related fluorescence (AGE-FL) is mostly analyzed to obtain a representative measure of AGEs (Monnier et al., 1986; Mulder et al., 2006; Verzijl et al., 2000a).

Collagens are the most abundant proteins of the ECM. On the basis of permanent non-enzymatic protein glycations *in vivo* and the low turnover of collagen (Dyer et al., 1991; Tessier, 2010), the AGE load of collagens (glycation, glycoxidation and cross-links) is increased in many aged tissues (Dyer et al., 1993; Miksik and Deyl, 1991; Monnier et al., 1984; Verzijl et al., 2000a). In addition, the age-related AGE load can be influenced by the degree of glucose and lipid metabolism with the subsequent formation of reactive carbonyls (Monnier et al., 1984; Sell et al., 2001), dietary habits (Lingelbach et al., 2000; Sell et al., 2003), the activity of enzymatic detoxification systems (Wu and Monnier, 2003) and oxidative stress (Grimsrud et al., 2008).

AGE modifications of ECM proteins including the collagens result in changes of their physical properties, including fiber stiffness and higher enzyme resistance (Avery and Bailey, 2006; Reddy, 2004). As lung tissue is chronically subjected to mechanical strains, the age-related AGE load of collagens might be one reason for the impaired mechanical properties and other changes in the aging lung (Janssens et al., 1999; Krumpe et al., 1985). The dominant collagen types in total lung tissue are the fibrillar collagens (type I and III) of the interstitium, bronchi and blood vessels, whereas the basement membrane collagen (type IV) is less present (Kelley et al., 1989; Kirk et al., 1984; Reiser

Abbreviations: AGE, advanced glycation end-product; AGE-FL, AGE-related fluorescence; ArgPyr, argpyrimidine; ATPase, adenosine triphosphate synthase; ε-CML, N-ε-carboxymethyllysine; CS, calf serum; CTGF, connective tissue growth factor; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ISF, insoluble fraction; logFC, log fold change; MMP, matrix metalloprotease; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SI, signal intensity; SF, soluble fraction; TIMP, tissue inhibitor of metalloprotease.

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and Last, 1980). A small number of studies already demonstrated an increased AGE load of lung collagens in monkeys and rats (Bellmunt et al., 1995a,b; Reiser et al., 1987).

For a better understanding of age-related processes *in vivo*, experimental mouse models are frequently used in the aging research (Kirkland and Peterson, 2009). However, no study has extensively analyzed the expressions at the mRNA and protein levels, enzyme-mediated solubility and AGE modifications of lung collagens together in one mouse model. Therefore, our study aimed at the analysis of these parameters in mouse lung collagens and its changes in the process of aging.

2. Material and methods

2.1. Animals

We studied lung tissues of C57BL/6 mice when they were young ($n=21$; 11 female, 10 male; 5–6 months; 20–33 g), adult ($n=11$; 5 female, 6 male; 15 months; 25–38 g) or old ($n=17$; 11 female, 6 male; 25–34 months; 26–34 g). For lung removal, mice were killed by cervical dislocation. After washing the lung tissue in 0.9% sodium chloride solution, one part of the tissue was fixed in phosphate-buffered formaldehyde and embedded in paraffin, and another part was frozen in liquid nitrogen. This animal procedure was approved by the local Animal Care and Use Committee Halle (Saale).

2.2. mRNA expression analysis

Total RNA was isolated from frozen lung tissue with TRIZOL® reagent (Invitrogen; Karlsruhe, Germany) and cleaned up using the RNeasy Mini Kit (Qiagen; Hilden, Germany). For gene chip studies biotin-labeled cRNA was synthesized from total RNA by use of the WT Expression kit (Applied Biosystems/Ambion; Darmstadt, Germany) and subjected to the GeneChip hybridization procedure using Mouse Array 2.0 and GeneChip Fluidics station 450 from Affymetrix® (Santa Clara, CA). Gene chips were scanned using the Affymetrix GeneChip Scanner 7G with a GeneChip Command Console 3.1 software. Quality assessment, GCRMA mode-based background adjustment and calculation of the data were performed with the Robin software (Lohse et al., 2010).

For real-time polymerase chain reaction (PCR) cDNA was synthesized from total RNA by reverse transcription, and then amplified by cDNA-specific primer pairs (Biomers; Ulm, Germany) and GoTaq qPCR mix (Promega; Mannheim, Germany). We used primers for PCR amplification of mouse COLIα2 (sense: 5′-CCA GCG AAG AAC TCA TAC AGC-3′; antisense: 5′-AAT GTC CAG AGG TGC AAT GTC-3′), COLIIIα1 (TGG TAT GAA AGG ACA CAG AGG C; TCC AAC TTC ACC CTT AGC ACC), COLIVα1 (GGG AGC ATG AAG GGA CAG AA; AGG CAC ACC TTT TTC TCC AG), MMP-2 (CCC TCA AGA AGA TGC AGA AGT T; CCA TCA AAC GGG TAT CCA TC), MMP-9 (AGA CGA CAT AGA CGG CAT CC; ATT GTC TGC CGG ACT CAA AG), TIMP-1 (AGA CCA CCT TAT ACC AGC GTT AT; GAT AGA TAA ACA GGG AAA CAC TG), TIMP-2 (CTG GAC GTT GGA GGA AAG AA; TCT CTT GAT GCA GGC GAA G), TIMP-3 (CCA CGT GCA GTA CAT TCAC AC; CCA GGT GGT AGC GGT AAT TG), CTGF (ACC GAC TGG AAG ACA CAT TTG G; CAG GCT TGG CGA TTT TAG GTG), and ATPase 5A1 (TAT GCG GGT GTA CGG GGT TAT CTT; TGA GCT TTG CGT CTG ACT GTT CTG) as reference gene. Real-time PCR was performed using the iCycler iQ™ system (Bio-Rad; Hercules, CA). The mRNA levels were calculated according to the mathematical model for the relative quantification in real-time PCR (Pfaffl, 2001).

2.3. Collagen analysis

Paraffin sections of mouse lung tissue were stained for ECM according to the protocol of the Elastic stain kit (Sigma; Deisenhofen, Germany). The van Gieson's picrofuchsin staining intensity for collagen was evaluated by use of the Axiovert microscope equipped with Spot Camera (Carl Zeiss; Jena, Germany) and Metamorph 4.6.5 software (Visitron Systems; Puchheim, Germany). Moreover, we evaluated the tissue background stained with eosin–hematoxylin solution according to Ehrlich (Fluka; Buchs, Switzerland). The total pixel area of the collagen staining per a defined area (121,839 μm^2) was calculated for four defined areas of one image and then averaged. Finally, ten images per section were evaluated, averaged and normalized per background staining.

Fibrillar collagens were isolated from frozen lung tissue according to the protocol of Meng et al. (2001). The isolation procedure is summarized in Fig. 1. Briefly, 60 mg of lung tissue was washed in 50 mM Tris buffer with 1 M sodium chloride (pH 7.2), homogenized with pestles, and then incubated in 1 ml 0.5 M acetic acid solution containing 1.8 mg pepsin from porcine gastric mucosa (≥250 units/mg; Sigma, P-7000) for 18 h at room temperature. After centrifugation, fibrillar collagens were precipitated from the supernatant by adding one volume of 1.4 M sodium chloride and then dissolved in phosphate buffer saline (PBS) to get the first soluble fraction (SF1). The non-precipitated part of the supernatant containing collagen type IV and other proteins (Maurel et al., 1990) was stored in some cases. The pepsin-insoluble pellet was washed in PBS and then incubated in 1 ml of a 20 mM Tris buffer with 1 mM calcium chloride (pH 7.5) containing 0.1 mg proteinase K (Applichem, Darmstadt, Germany) and 0.1 mg *Clostridium histolyticum* collagenase type I (≥125 units/mg; Sigma, C-0130) for 18 h under slight agitation at 37 °C. After centrifugation, the supernatant was removed to get the second soluble fraction (SF2), and the enzyme activity was inhibited by adding 40 mM disodium–EDTA. The final pellet, called insoluble fraction (ISF), was made accessible for further analysis by short-term sonication with subsequent incubation in 6 M hydrochloric acid for 12 h at room temperature. The amount of collagen in each fraction was measured per standard series of trans-4-hydroxyproline (Sigma-Aldrich; Steinheim, Germany) according to the protocol of the 4-hydroxyproline assay (Lin and Kuan, 2010).

2.4. AGE measurement

Samples of the three isolation fractions were adjusted to the same collagen concentration in the respective fraction buffer. The AGE-FL of the collagen samples minus background fluorescence (AGE-FL of the dilution buffer) was measured in a black microplate at 370/10 nm excitation and 440/10 nm emission by using the FLUOstar OPTIMA

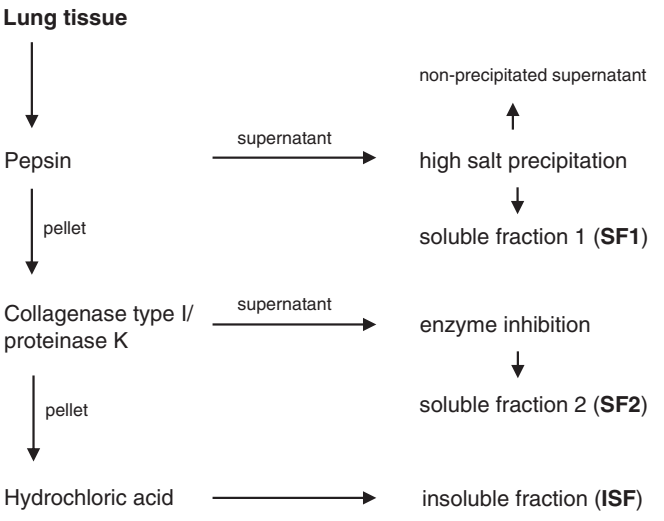

Fig. 1. Schematic presentation of the procedure for isolating fibrillar collagens from mouse lung.