Clusterin Associates with Altered Elastic Fibers in Human Photoaged Skin and Prevents Elastin from Ultraviolet-Induced Aggregation *in Vitro*

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Clusterin is a secreted glycoprotein with stress-induced expression in various diseased and aged tissues. It shares basic features with small heat shock proteins because it may stabilize proteins in a folding-competent state. Besides its presence in all human body fluids, clusterin associates with altered extracellular matrix proteins, such as β -amyloid in Alzheimer senile plaques in the brain. Because dermal connective tissue alterations occur because of aging and UV radiation, we explored the occurrence of clusterin in young, aged, and sun-exposed human skin. Immunohistochemical analysis showed that clusterin is constantly associated with altered elastic fibers in aged human skin. Elastotic material of sun-damaged skin (solar elastosis), in particular, revealed a strong staining for clusterin. Because of the striking co-localization of clusterin with abnormal elastic material, we investigated the interaction of clusterin with elastin *in vitro*. A chaperone assay was established in which elastin was denatured by UV irradiation in the absence or presence of clusterin. This assay demonstrated that clusterin exerted a chaperone-like activity and effectively inhibited UV-induced aggregation of elastin. The interaction of both proteins was further analyzed by electron microscopy, size exclusion chromatography, and mass spectrometry, in which clusterin was found in a stable complex with elastin

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Clusterin is a well-conserved secreted 75-kDa glycoprotein present in most body fluids and in a variety of tissues.¹ Besides its basic expression, clusterin is highly induced in stressed² and senescent^{3–5} cells. Similar to other heat shock proteins (Hsps), stress-induced transcription of clusterin is mediated by the heat shock transcription factor 1 (HSF1),² resulting in its up-regulation in response to a variety of stress conditions including ischemia, neuronal injury, aging, and oxidative stress.^{2,6} Clusterin associates with itself⁷ via amphipathic helices and may interact with hydrophobic domains of a wide range of other proteins, including β -amyloid, prion proteins, immunoglobulins, apolipoprotein A-I, and complement components.⁴ *In vitro* studies have demonstrated that clusterin binds to exposed hydrophobic domains of partially unfolded proteins and thereby efficiently suppresses stress-induced aggregation.^{8,9} It forms soluble high molecular weight complexes with these proteins and maintains them in a folding-competent state.⁹ Similar to the action of small Hsps (sHsps), clusterin has no refolding capacity, but proteins bound to clusterin can be specifically refolded by ATP-dependent chaperones.^{9,10} In this respect, clusterin is part of the stress response, but in contrast to other Hsps, it has a classical secretion signal peptide and is secreted into the extracellular space, where it binds to target molecules.⁴ Therefore, clusterin belongs to a novel class of extracellular chaperones.¹¹ *In vitro* studies revealed that clusterin prevents aggregation of β -amyloid, associated with Alzheimer's disease,¹² and of prion protein (PrP 106–126), associ-

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ated with bovine spongiform encephalopathy.¹³ These findings implicate a role of clusterin in extracellular stress and diseases with extracellular protein deposition.

We have recently found the association of clusterin with fibrotic areas of cirrhotic human livers.¹⁴ The staining pattern of clusterin resembles the distribution of elastic fibers. This observation led us to investigate the occurrence of clusterin in context with altered elastic fibers in human skin. The connective tissue of human skin is mostly composed of collagen, elastin, and glucosaminoglycans.¹⁵ In aged individuals, two processes lead to alterations of the dermal connective tissue. First, the skin is affected by intrinsic (chronological) aging in the same way as other organs. Intrinsically aged skin is thin and has reduced resistance and elasticity. At the histological level, it is characterized by a reduction of fibroblasts, mast cells, extracellular matrix components, and blood vessels.¹⁶ Elastic fibers, which are produced early in life, undergo progressive degradation.¹⁷ One of the first signs of aging is the disappearance of oxytalan fibers.¹⁸ Moreover, elastic fibers of aged skin are thicker than those of young individuals, although there is no difference in their organization.¹⁹ Second, sun-exposed skin is affected by extrinsic aging (photoaging), initiated by chronic exposure to UV radiation. Clinically, photoaged skin is characterized by a leathery appearance with deep wrinkles and a yellowish coloration. In contrast to intrinsic aging, photodamage leads to qualitative and quantitative alterations of elastin with massive deposition of elastic material, referred to as solar elastosis. The elastic fibers appear thickened, tangled, and amorphous.²⁰ *In vivo* and *in vitro* studies showed that UV radiation activates the elastin promoter, leading to increased elastin production.¹⁶

Recent reports show that reactive oxygen species play an important role in photoaging²¹ because UV light leads to free radical formation in skin.^{22,23} Furthermore, reactive oxygen species have been reported to enhance directly elastin expression.²⁴ Therefore, reactive oxygen species may contribute to the overlapping pathogenic mechanisms involved in intrinsic as well as extrinsic aging of the skin.^{25,26} As an evolutionarily conserved defense mechanism, several molecular chaperones such as sHsps, Hsp72, and Hsp90 are produced in the skin, which bind intracellular unfolded proteins and prevent them from irreversible denaturation in stress conditions.²⁷ Whereas chaperones were originally described to act intracellularly, Hsp90 can also be secreted by cells and activate extracellular proteases, thus promoting invasiveness of tumor cells.^{28,29} Recent *in vitro* studies of human fibroblasts showed that expression of clusterin is induced after UVB irradiation,³⁰ UVA irradiation, and oxidative stress.^{6,31} Because clusterin is a stress-induced protein that is able to stabilize proteins in the extracellular space and because we have observed that clusterin co-localizes with elastic fibers in the cirrhotic livers,¹⁴ we explored the association of clusterin with altered elastin in the skin. Therefore, we investigated clusterin in intrinsically and extrinsically aged skin and characterized the interaction of both proteins *in vitro*.

Materials and Methods

Human Tissue

Formaldehyde-fixed and paraffin-embedded tissue samples of human skin were obtained from the Biobank of the Institute of Pathology, Medical University of Graz, Graz, Austria.³² We selected samples of sun-exposed facial skin from 16 aged patients (patient age between 70 to 80 years) and samples from 10 young patients (15 to 30 years of age) as well as skin samples from not sun-exposed regions from 10 aged patients (70 to 80 years of age) and from 10 young patients (15 to 30 years of age) derived from operated breasts.

Immunohistochemistry and Elastin Staining

Serial sections of formaldehyde-fixed, paraffin-embedded tissues (2 μ m thick) were subjected to immunohistochemical and routine (hematoxylin and eosin, H&E) staining. To visualize elastic fibers, sections were stained with the Elastica van Gieson stain using standard histological protocols.³³

For immunohistochemical detection of clusterin, the sections were deparaffinized and rehydrated. Antigen retrieval was performed by microwaving at 150 W for 15 minutes in 0.01 mol/L citrate buffer, pH 6.0. For detection of elastin, the deparaffinized and rehydrated sections were pretreated with protease type XXIV (0.1%; Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) at 37°C for 15 minutes. Endogenous peroxidase was blocked by incubation with 1% H₂O₂ in methanol for 15 minutes. For demonstration of clusterin, sections were incubated with a polyclonal rabbit antibody to clusterin [H-330 (Santa Cruz Biotechnology, Santa Cruz, CA) 1:100 diluted with DAKO diluent (DAKO, Glostrup, Denmark)] for 1 hour at room temperature. Selected cases were stained in addition with a polyclonal goat antibody raised against the C terminus of mouse clusterin [clusterin- β (M18) (Santa Cruz Biotechnology) 1:100 diluted with DAKO diluent]. For elastin immunostaining a mouse anti-human elastin (Novocastra, diluted 1:200 in DAKO antibody diluent; Novocastra Laboratories, Newcastle, UK) was applied at room temperature for 1 hour. After washing with PBS, the sections were incubated with Multi Link biotinylated swine anti-goat-mouse-rabbit immunoglobulins and Strept ABCComplex/HRP (DAKO). Horseradish peroxidase was visualized using AEC as substrate (DAKO). Negative controls were performed by omitting the primary antibody or by using nonimmune rabbit serum.

UV-Induced Aggregation of Elastin

Purified soluble elastin from bovine neck ligament³⁴ (2.1 μ mol/L in ddH₂O, E6527; Sigma) was UV-irradiated (CL-1000, 254/302/365-nm lamps, 100 mJ/cm²; UVP, Cambridge, UK) for 1, 2, 5, 10, 20, and 30 minutes at room temperature. Assays were performed in the absence and presence of clusterin (2.1 μ mol/L in ddH₂O; Alexis Biochemicals, Grünberg, Germany). As controls, the elastin

aggregation was also assayed in the presence of bovine serum albumin (5 $\mu\text{mol/L}$) and vitamin C (1 mmol/L). To monitor the kinetics of UV-induced elastin alterations and the possible effect of clusterin, absorption was measured from 240 nm to 600 nm using a UV-visible spectrophotometer (Biochrom 4060; GE Health Care, Freiburg, Germany). The data obtained were analyzed using the Sigma Plot software package (Jandel Scientific, Tallahassee, FL) and the increase of UV signal at 400 nm, which correlates to the light scattering signal of the aggregated elastin, was plotted.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) was performed using a TosohHaas TSK 4000 PW column equilibrated in PBS (premixed PBS; Roche Diagnostics, Mannheim, Germany) at a constant flow rate of 0.75 ml/minute (separation range, 10 to 1500 kDa). Elastin and clusterin were detected by fluorescence at an excitation wavelength of 275 nm and an emission wavelength of 307 nm using a FP 920 fluorescence detector (Jasco, Grossumstadt, Germany). Standard proteins from the molecular weight marker kit (aldolase, ferritin, and bovine serum albumin; Sigma) were used for calibration.

Electrophoresis and Mass Spectrometry

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a SE 250 Mighty Small electrophoresis system (GE Health Care) on 12.5% acrylamide gels at a constant current of 30 mA per gel. Coomassie Blue staining was performed as stated elsewhere.^{35,36} For identification of protein bands with mass spectrometry, spots were excised and digested following the protocol of Schäfer and colleagues.³⁷ Sample preparation for MALDI-MS was performed using ZipTips (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For MALDI-MS and MALDI-MS/MS an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) was used. Data were analyzed using the BioTools (Bruker) and Mascot (Matrix Science, London, UK) software packages.

Electron Microscopy

To determine morphological changes, purified elastin and clusterin as well as mixtures of both proteins were obtained before or after UV treatment (100 mJ/cm^2 , 30 minutes). The samples were adsorbed onto carbon-coated grids, glow-discharged in air before the application of 5 μl of protein solution. Excess protein solution was blotted off after 2 minutes. The adsorbed material was negatively stained for 30 seconds using 5 μl of 1.5% (w/v) ammonium molybdate (pH 5.5). Electron micrographs were recorded at a nominal magnification of $\times 50,000$ using a JEOL JEM 2010 electron microscope (JEOL Ltd., Tokyo, Japan) operated at 120 kV. Well-separated particles were selected and extracted into boxes using boxer from the EMAN package.³⁸ The image processing procedures were performed using the IMAGIC suite.³⁹ Approximately 50 views were selected of each sample and iteratively aligned to their average to bring them all to a

common center. The centered images were analyzed by multivariate statistical analysis to obtain class averages.⁴⁰

Animals

Specific pathogen-free female C3H/HeNcr (MTV⁻) mice were purchased from the National Cancer Institute, Frederick Cancer Research Facility, Animal Production Area, Frederick, MD. The animals were kept under alternating 12 hours light and dark cycles and controlled temperature and humidity in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, in accordance with current regulations of the United States Department of Health and Human Services. Water and food were provided *ad libitum*. All animal procedures were approved by the University of Texas M.D. Anderson Cancer Center Institutional Animal Care and Use Committee. Mice were 8 to 10 weeks old at the start of the experiments and age-matched within each experiment.

UV Treatment of Animals

The dorsal skin of mice was shaved with electric clippers 1 day before the start of UVB treatment. UVB radiation was provided by a bank of six FS40 sunlamps (National Biological, Beachwood, OH), which have a peak at 313 nm and deliver $\sim 65\%$ of their total energy within the UVB (280 to 320 nm) wavelength range; their UVB irradiance was monitored, as previously described.⁴¹ During all UV irradiations, the mice were housed five per standard cage, individually separated with Plexiglas dividers, on a shelf 20 cm below the light bulbs under a wire cage top. Mice were either exposed to a single dose of 20 kJ/m^2 UVB or to multiple doses of 15 kJ/m^2 UVB (corresponding to $\sim 10\times$ the minimal inflammatory dose) three times per week for a total of 16 weeks (control mice, $n = 5$; single exposure, $n = 5$; 16 weeks exposure, $n = 10$). Mice were sacrificed before and at 24 or 48 hours after the (last) UV exposure. Approximately 1×1 cm pieces of dorsal skin were excised or entire mouse ears were fixed immediately in 4% phosphate-buffered formaldehyde, paraffin-embedded, and sectioned at 5 μm for H&E, Elastica van Gieson, or immunohistochemical staining.

Results

Clusterin Co-Localizes with Altered Elastic Material in UV-Damaged and Aged Skin, but Not with Regular Elastic Fibers

To explore the association of clusterin with elastin, we performed histological studies in young and aged skin. To distinguish the effect of intrinsic and extrinsic aging, skin was obtained from sun-exposed as well as from sun-protected skin. Co-localization of elastic material with clusterin was demonstrated by immunostaining of consecutive sections with antibodies to clusterin and elastin or by Elastica van Gieson staining (Figure 1). All 16 cases

Figure 1. Co-localization of clusterin with elastin in UV-damaged and aged skin. Consecutive sections of skin samples of UV-exposed skin from aged (**A** and **B**, **C** and **D**, **E** and **F**) and young (**G** and **H**) patients as well as non-UV-exposed samples from aged (**I** and **J**) and young (**K** and **L**) skin are shown. Elastin was detected by immunohistochemistry (IHC Ela) or by Elastica van Gieson staining (EvG), and the presence of clusterin was demonstrated by immunohistochemistry (IHC Clu). Clusterin co-localizes with amorphous elastic material in UV-exposed aged skin (solar elastosis; **asterisk** in **A**, **B**, **E**, and **F**) as well as with thickened elastic fibers (**arrowheads** in **C** and **D**). **H**: In young UV-exposed skin, clusterin was absent. **G**: **Arrows** indicate thin oxytalan fibers, which are negative for clusterin. **I** and **J**: In aged non-UV-exposed skin, clusterin is associated with thickened elastic fibers (**arrowheads**). **K**: Young non-UV-exposed skin elastic fibers (**arrows**) were negative for clusterin. Scale bars = 50 μ m.

of sun-aged facial skin showed the typical histological appearance with massive accumulation of elastic material in the papillary and reticular dermis (solar elastosis). Immunohistochemical analysis of parallel sections for clusterin in all cases revealed strong staining and a co-localization with elastic material (Figure 1, A–F). Staining patterns were well comparable using two different antibodies against clusterin. The clusterin immunoreaction correlated with the extent of elastin damage, whereas thickened elastic fibers and amorphous elastic material were strongly positive for clusterin (Figure 1, B, D, and F). However, the staining reaction with clusterin was slightly stronger in the superficial than in the deeper layers of the dermis. In contrast to the aged skin, young facial skin had a well-organized elastic fiber network, which was negative for clusterin in all 10 samples investigated (Figure 1, G and H).

Intrinsically aged skin of breast samples showed no deposition of amorphous elastic material, as observed in sun-aged skin. Nevertheless, clusterin was present in all 10 cases of aged breast skin. Eight cases showed a strong positive reaction for clusterin in association with thickened elastic fibers (Figure 1, I and J), whereas in two cases only weak clusterin staining was seen. Young breast skin samples had fine elastic fibers, which were negative for clusterin in all 10 cases investigated (Figure 1, K and L). The differences in the presence of clusterin in young and aged skin as well as the pronounced accumulation of clusterin in association with amorphous elastic material in aged UV-damaged skin indicates that clusterin selectively binds to altered elastin.

Clusterin Suppresses UV-Induced Aggregation of Elastin *in Vitro*

Because of the striking co-localization of clusterin with abnormal elastic material in human UV-damaged skin, we investigated the interaction of clusterin with elastin *in vitro* and explored if clusterin may act as a chaperone for elastin and prevent UV-induced denaturation of elastin. For this goal, we established an *in vitro* UV irradiation assay in which elastin was treated with UV light for up to 30 minutes in the absence or presence of clusterin. The kinetics of UV-induced alterations of elastin was measured for up to 30 minutes by recording the increase in light scattering. UV irradiation resulted in increased light scattering, which was directly proportional to the exposure time (Figure 2). These results provide indirect evidence that elastin formed aggregates under these conditions (see also Figure 4D). Although bovine serum albumin or vitamin C showed no significant influence on the aggregation of elastin, addition of clusterin efficiently prevented the UV-induced increase of light scattering, indicating that clusterin is able to suppress aggregation of elastin (Figure 2).

To explore further the role of clusterin and its interaction with elastin, electron microscopy and SEC analyses were performed. Electron micrographs show that clusterin forms globular oligomeric complexes of variable size ranging from 10 to 18 nm in diameter (Figure 3A).

Figure 2. Effect of clusterin on UV-induced elastin aggregation. Elastin was treated *in vitro* in the absence (●) or presence (○) of clusterin with UV light, and absorption was measured after 0, 2, 5, 10, 20, and 30 minutes. Changes in light scattering were measured at 400 nm. The maximal absorption was normalized to 1. The increase in absorption reflects the aggregation of elastin, which is prevented in the presence of clusterin. Clusterin alone (▲) is not affected by UV irradiation. As control, elastin aggregation was monitored in presence of bovine serum albumin (5 μ mol/L, ▼) and vitamin C (1 mmol/L, ▽).

The variability in particle diameters indicates that clusterin forms dynamic complexes compatible with 4 to 24 subunits. This observation correlates with results on polydisperse sHsps, such as α B-crystallin and Hsp27, which also form variable homo-oligomers of 4 to 50 subunits.^{42–44} Furthermore, these findings are in good agreement with previously observed high molecular weight complexes, from which active clusterin dimers dissociate to achieve their chaperone activity.^{8,9} Interestingly, after application of clusterin to SEC at low concentration, a 120-kDa peak was eluted, corresponding to clusterin dimers and indicating a concentration-dependent self-assembly mechanism (Figure 3B). Thus, clusterin shows high structural and functional similarity to other sHsps.

In a next step we investigated the interaction of clusterin with native and UV-treated elastin. The negative-stained electron micrographs of native elastin showed regular, globular particles with diameters of 7.5 to 10 nm (Figure 4A). When elastin was treated with UV light for 30 minutes, large amorphous protein aggregates were detected (Figure 4B), which is in accordance with the increase in light scattering (Figure 2). UV-induced formation of elastin aggregates was efficiently prevented by addition of clusterin (Figure 4C). Notably, as demonstrated by electron microscopy (Figure 4C, inset), the clusterin-elastin complexes were smaller than the clusterin homo-oligomers (Figure 3A), indicating that a dissociated clusterin species binds to the substrate molecule, similar to the mechanism previously proposed for sHsps.^{10,44–46}

The UV irradiation-dependent interaction of clusterin with elastin was further investigated by SEC, SDS-PAGE, and mass spectrometry. When elastin alone was exposed to UV for 30 minutes and separated by SEC, a peak in the void volume of the column, representing the elastin aggregates was detected (Figure 4D, dotted line). SDS-PAGE analysis of the respective elastin aggregates demonstrated that they were insoluble in reducing SDS-

Figure 3. Clusterin forms homo-oligomers. **A:** Electron micrographs of clusterin after negative staining [1.5% (w/v) ammonium molybdate]. The protein occurs in different size classes as demonstrated by the differently sized class averages of the molecules (lower image row). **B:** SEC analysis of clusterin. At low concentrations, clusterin elutes as a dimer corresponding to a molecular mass of ~120 kDa (Clu dimer).

PAGE loading dye because bands in the high molecular mass range were observed (Figure 4D, inset), indicating that the degradation products became cross-linked by the UV light during prolonged exposure. The protein composition of the high-molecular weight material was confirmed as elastin aggregates by MS-fingerprint analysis. In addition to the large aggregates, another set of peaks with apparent molecular masses less than 60 kDa was observed by SEC (Figure 4D). SDS-PAGE analysis of these peaks showed several bands in the lower molecular mass range (Figure 4D, inset) that were identified as elastin degradation products by MS-fingerprint analysis. The amount of soluble degradation products decreased with the exposure time to UV light, whereas the amount of aggregates increased (not shown). Thus, elastin seems to degrade during the exposure to UV light, and the

degradation products form amorphous, fibrous aggregates consisting of degraded elastin molecules. Interestingly, clusterin was not altered by the exposure to UV light (data not shown). To test if clusterin suppresses the aggregation of elastin by forming stable complexes, we performed SEC analysis of elastin irradiated with UV for 30 minutes in the presence of clusterin. Under these conditions peaks corresponding to apparent molecular masses of 500 and 120 kDa, as well as a broad peak ranging from 60 to 10 kDa were detected (Figure 4E). The peaks were subjected to SDS-PAGE and the resulting bands were analyzed by mass spectrometry to identify the corresponding proteins. Degraded elastin was found in the broad, low molecular weight peak (Figure 4E, inset). The 120-kDa peak represented clusterin dimers (Figure 4E, inset), which is in line with the observation that UV treatment did not affect the elution profile of clusterin alone (data not shown). In the 500-kDa peak, however, both clusterin and elastin were identified (Figure 4E, inset). The presence of both proteins in the same peak indicates that clusterin forms stable complexes with elastin.

Clusterin Does Not Accumulate in Mouse Skin after 16 Weeks of UV Irradiation

To explore further the functional relevance of clusterin in photoaged skin, we analyzed mice after a single exposure to UV and after repeated UV treatments for 16 weeks. Multiple UV exposures for 16 weeks provoked a moderate epidermal hyperplasia and a sparse mixed cellular infiltrate in the dermis. The elastic fiber network was not altered after a single UV exposure, whereas there was slight increase of elastic fibers in the dermis of the mice after 16 weeks of UV treatment. Immunohistochemical staining was performed with two different antibodies to human and mouse clusterin and revealed no accumulation of clusterin in short-term or in long-term irradiated mice (data not shown). In this context it is noteworthy that chronic UV irradiation of mice leads to an increase in elastin expression and to increased density of elastic fibers but, at least under the experimental conditions and duration of UV irradiation protocol applied in this study, not to accumulation of elastotic material as observed in human photoaged skin.

Discussion

The results of the immunohistochemical analyses demonstrate that clusterin constantly appears in aged skin in association with altered elastic material. Clusterin deposition is found in intrinsically as well as extrinsically aged (UV-damaged) skin, and its occurrence is particularly striking in solar elastosis. Clusterin immunostaining in intrinsically aged skin was less pronounced, in line with its minor extent of elastosis. In contrast, clusterin did not co-localize with the network of thin elastic fibers in skin samples from young patients. Thus, the presence of clusterin was selectively linked to the occurrence of damaged elastic material.

Figure 4. Interaction of clusterin with elastin. Electron micrographs of negatively stained (1.5% w/v ammonium molybdate) native elastin (**A**) and elastin UV-irradiated in the absence (**B**) or presence (**C**) of clusterin. Note that clusterin prevents elastin aggregation (compare **B** and **C**). In addition, new particles representing a clusterin-elastin complex arise (**C**, inset: multivariate statistical analysis of clusterin-elastin complex). Samples shown in **A–C** were further analyzed by SEC, SDS-PAGE, and mass spectrometry (**D** and **E**). **D**: SEC analysis of native elastin (interrupted solid line) and elastin after 30 minutes of UV irradiation (dotted line). Peaks eluted were analyzed for protein composition by SDS-PAGE (**D**, insets), and major protein bands (arrowheads) were characterized by mass spectrometry (Ela nat, native elastin; Ela deg, degraded elastin; Ela agg, aggregated elastin). **E**: SEC analysis of elastin after treatment with UV for 30 minutes in presence of clusterin (2.1 $\mu\text{mol/L}$). Peaks eluted were analyzed for protein composition by SDS-PAGE (**E**, insets), and major protein bands (arrowheads) were identified by mass spectrometry (Clu, clusterin; Ela, elastin; Clu/Ela complex, clusterin/elastin complex; Ela deg, degraded elastin).

The selectivity of clusterin for denatured proteins was further underlined by the results of the experiments with chronically UV-irradiated C3H mice. After repeated UV exposures for 16 weeks, mice showed an increase of elastic fibers in the skin but no deposition of elastotic material and no reactivity for clusterin. The absence of elastotic material in mice exposed to UV for 16 weeks is consistent with previous studies in which characteristic features of photoaging, including pronounced focal elastotic deposits, required up to 30 weeks of UV irradiation.^{47,48} However, even after this prolonged UV exposure the histological appearance of photoaging seems to differ between mice and humans in that diffuse and massive dermal deposition of elastotic material is observed in sun-aged human skin whereas rather focal deposits are seen in mice after chronic experimental UV irradiation.⁴⁷ These findings emphasize that in addition to UV-induced overexpression of elastin, elastin misfolding may be a distinct biochemical feature of human solar elastosis.

Clusterin is systemically present in human serum, and production of clusterin might also be locally induced by

stress conditions. Therefore, clusterin that accumulates in UV-damaged skin could be derived from serum as well as produced locally. Because in UV-irradiated mice no clusterin-positive cells or deposition of clusterin was found in the skin, enhanced local production of clusterin is unlikely after physiologically relevant UV exposure.

The constant association of clusterin with damaged elastic material in human skin prompted us to investigate the interaction of clusterin and elastin in more detail. We found that elastin aggregation induced by UV irradiation *in vitro* was efficiently inhibited by addition of clusterin. This finding is in agreement with the promiscuous nature of clusterin to bind a broad variety of unfolded proteins, which are mostly located in the extracellular space.⁴ In electron micrographs, fewer aggregates were seen when clusterin was added to the elastin sample. Notably, the globular complexes of clusterin alone were bigger than the elastin-clusterin complexes. This suggests that clusterin alone forms large oligomers⁹ and that active clusterin dissociates from these complexes and binds to denatured proteins thereby preventing their aggregation similarly as described for other sHsps.¹⁰

Although our *in vitro* studies demonstrated that clusterin may protect elastin from UV-induced aggregation, its *in vivo* significance is presently unclear. Studies in clusterin-deficient mice demonstrated that clusterin may enhance or reduce disease manifestation depending on the disease model. For instance, clusterin enhanced neurotoxicity of amyloid- β in an Alzheimer's disease mouse model.⁴⁹ In contrast, clusterin reduced cardiac inflammation and improved cardiac function in a myosin-induced autoimmune myocarditis mouse model.⁵⁰ Unfortunately, neither acute nor chronic UV exposure of mice led to deposition of solar elastotic material and the respective association of clusterin as seen in the human situation so that further studies in clusterin knockout mice are not reasonable.

Theoretically, clusterin may exert a protective function *in vivo* by stabilizing elastin and delivering it to folding-competent chaperones, such as Hsp90, which has been recently described to occur in the extracellular space.^{28,29} Furthermore, clusterin-bound elastin can be transported via megalin gp330 receptor-mediated endocytosis⁴¹ into cells and degraded intracellularly. However, when rescue systems are overloaded, damaged proteins accumulate, and heat shock proteins are then found in the protein aggregates,^{51,52} a situation that could be the case in photoaged skin. Although clusterin is able to inhibit elastin aggregation *in vitro*, it obviously cannot prevent the formation of solar elastosis. Nevertheless, it is likely that clusterin has an influence on stress-induced damage of connective tissue and may serve as a biomarker for the presence of misfolded proteins.

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