Vimentin Is the Specific Target in Skin Glycation

Structural Prerequisites, Functional Consequences, and Role in Skin Aging

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Until now, the glycation reaction was considered to be a non-specific reaction between reducing sugars and amino groups of random proteins. We were able to identify the intermediate filament vimentin as the major target for the AGE modification N-alpha-(carboxymethyl)lysine (CML) in primary human fibroblasts. This glycation of vimentin is neither based on a slow turnover of this protein nor on an extremely high intracellular expression level, but remarkably it is based on structural properties of this protein. Glycation of vimentin was predominantly detected at lysine residues located at the linker regions using nanoLC-ESI-MS/MS. This modification results in a rigorous redistribution of vimentin into a perinuclear aggregate, which is accompanied by the loss of contractile capacity of human skin fibroblasts. CML-induced rearrangement of vimentin was identified as an aggresome. This is the first evidence that CML-vimentin represents a damaged protein inside the aggresome, linking the glycation reaction directly to aggresome formation. Strikingly, we were able to prove that the accumulation of modified vimentin can be found in skin fibroblasts of elderly donors in vivo, bringing AGE modifications in human tissues such as skin into strong relationship with loss of organ contractile functions.

The formation of advanced glycation end products (AGEs)2 is the result of the reaction between reducing sugars and amino groups of proteins based on the Maillard reaction. This reaction is termed glycation or non-enzymatic glycosylation (1). AGEs constitute a heterogeneous group of structures, whereas N-alpha-(carboxymethyl)lysine (CML) adducts are the most prevalent AGEs present in vivo (1, 2). CML along with other AGEs accumulate during normal aging (3–8) leading to stiffening and a lack of elasticity in connective tissues and vessel walls (9). This accumulation is enhanced in diabetic patients (9) as well as in human lung cancer tissues (10), here attributed to a high glycolytic rate of tumors in general. Furthermore, AGEs are considered to be markers of various additional diseases, such as atherosclerosis, renal failure, and Alzheimer disease (3, 8, 11). In the current view, the longevity of proteins is the major catalyst for the glycation reaction because of the extended time for glycation. In skin, AGE deposits have been observed in long-lived proteins namely fibronectin, laminin, collagen (12), and elastin (1). Additionally actinic elastosis tissues contain AGE accumulations based on the glycation of elastic fibers that play a significant role in photoaging (1). AGEs were initially thought to arise mainly from the reaction between extracellular proteins and glucose. However, recent studies report that the intracellular AGE formation from glucose-derived dicarbonyl precursors is much higher compared with the extracellular formation (13, 14). These dicarbonyl precursors consist mainly of glyoxal, methylglyoxal, and 3-deoxyglucosone (15), which are substrates for reductases (16). Glyoxal and methylglyoxal are detoxified by the glyoxalase system (17). Intracellular AGEs induce oxidative stress, activate NF-kB and heme oxygenase, produce lipid peroxidation products, and cross-link proteins (18). As yet, only a few examples are known, in which AGEs were degraded intracellularly (19, 20); others report that cells store AGEs in lysosomes or phagosomes (21) because of the persistent character of the glycated proteins.

In this report, we identified the intermediate filament protein vimentin as the major target for CML modification in human dermal fibroblasts. Furthermore pentosidine, pyrraline and CEL glycate the protein vimentin. Intermediate filaments are one of the major structural components of the cytoskeleton in addition to actin microfilaments and microtubules. All cytoplasmic intermediate-filament proteins have a common secondary structure, consisting of a central alpha-helical rod domain of about 310 amino acids that is flanked by non-alpha-helical domains of variable size (22). Vimentin is required for many vital cell functions like cell motility, chemotactic migration, and wound healing (23, 24). There is strong evidence that the biological impact of the identified vimentin modification is related to the loss of contractility of the fibroblasts caused by the structural breakdown of the intermediate filament system finally accelerating the process of aging.
**Vimentin Glycation in Skin Fibroblasts**

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human facial skin biopsies were isolated from healthy donors. Primary human dermal cells were enzymatically prepared using a standardized dispase (Roche Applied Science, Mannheim, Germany) digestion technique. The dermal fraction was cultured at 37 °C and 7% CO₂ (in air) in 6-well plates containing Dulbecco’s modified Eagle’s medium (Invitrogen, Eggenstein, Germany) supplemented with 10% fetal calf serum (Invitrogen) and penicillin/streptomycin (50 μg/ml, Invitrogen). After 5–6 weeks of incubation, confluent fibroblasts were seeded into the appropriate flasks. If desired for the experiment fibroblasts were incubated for 7 days with 200 μM glyoxal (18, 20) supplemented to the medium described above. Keratinocytes of the epidermal fraction were cultured in KGM-2 (Cambrex, Apen, Germany). Additional vimentin- and vimentin⁺ fibroblasts, derived from murine embryos, were kindly provided by Dr. Robert Evans (University of Colorado Health Sciences Center, Denver, CO). Vimentin⁻ and vimentin⁺ fibroblasts were grown with additional 10 μg/ml gentamicin (GIBCO, Auckland, New Zealand). Fibroblasts expressing vimentin-GFP (25) were a nice gift from Dr. Ronald Liem (Columbia University). The vimentin-GFP cells were grown with additional 300 μg/ml geneticin (GIBCO).

**Immunoblotting Analysis**—Proteins from cell extracts (20 μg per lane) were separated by gel electrophoresis under denaturing conditions on 10% SDS-PAGE gels. Immunoblotting was performed following standard procedures using chemiluminescence methods. Protein bands, blotted on nitrocellulose or polyvinylidene difluoride sheets, were visualized with the Lumilight Plus Western blot Detection kit (Roche Applied Sciences). Imaging was performed using LUMI-Imager (Boehringer, Mannheim, Germany). Signals were quantified using LumiAnalyt (Roche Applied Sciences). The CML (26) antibody used was clone 6D12. CEL (27), pentosidine (28), and pyrraline (29) were purchased from TransGenic (TransGenic Inc., Kumamoto, Japan, 1:200 dilution). Antibodies detecting vimentin (sc-7558 dilution 1:200, sc-32322 dilution 1:100,000) and actin (sc-1615 dilution 1:500) were purchased from Santa Cruz Biotechnology. Antibody detecting GAPDH (clone 5G4, Kumamoto, Japan, 1:200 dilution). Secondary antibodies labeled with peroxidase were purchased from Sigma Aldrich (A6782 dilution 1:1,000, A8919 dilution 1:20,000). Antibodies were detected using enhanced chemiluminescence-labeled secondary antibodies (AlexaFluor 488 chicken anti-mouse IgG, AlexaFluor 594 donkey anti-goat IgG, AlexaFluor 546 donkey anti-goat IgG, Molecular Probes, Eugene, OR) at 1:1,000 to visualize the target protein and DAPI (Molecular Probes). After extensive washing with PBS fluorescence images were recorded on a fluorescence microscope (Olympus, Hamburg, Germany) with an attached closed-circuit display camera. Confocal fluorescence images were recorded on LSM 510 Meta (Carl Zeiss, Jena, Germany).

**Immunoprecipitation and Fractionation Studies**—50 μl of a vimentin agarose conjugate (sc-7558AC, Santa Cruz Biotechnology) was added to 500 μl of 2× immunoprecipitation buffer (300 mM NaCl, 20 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 0.4 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 2% Triton X-100) and 400 μl of ddH₂O. 100 μl of lysate was added containing 100–500 μg of protein. This solution was incubated at 4 degrees for 1 h followed by centrifugation. The pellet was washed several times with 1× immunoprecipitation buffer, resuspended and boiled for 5 min in 30 μl 2× Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris-HCl, pH 6.8). Every centrifugation was performed at 4 degrees and 13,000 rpm. The fractionation was done with the ProteoExtract® Subcellular Proteome Extraction Kit (Merck, Darmstadt, Germany).

**nanoLC-ESI-MS/MS**—Protein identification using nanoLC-ESI-MS/MS was performed by the Proteome Factory (Proteome Factory AG, Berlin, Germany). The MS system consisted of an Agilent 1100 nanoLC system (Agilent, Boeblingen, Germany), PicoTip emitter (New Objective) and an Esquire 3000 plus ion trap MS (Bruker, Bremen, Germany). Protein spots were in-gel-digested and applied to nanoLC-ESI-MS/MS. After trapping and desalting the peptides on enrichment column (Zorbas SB C18, 0.3 × 5 mm, Agilent) using 3% acetonitril/0.1% formic acid solution for 5 min, peptides were separated on Zorbas 300 SB C18, 75 μm × 150 mm column (Agilent) using an acetonitrile/0.1% formic acid gradient from 5–40% acetonitrile within 40 min. MS spectra were automatically taken by Esquire 3000 plus according to the manufacturer’s instrument settings for nanoLC-ESI-MS/MS analyses. Proteins were identified using MS/MS ion search with the Mascot search engine 1.9.0 (Matrix Science, London, England) and nr protein data base (National Center for Biotechnology Information, Bethesda) for gels using standard electrophoresis buffer (250 mM Tris-HCL, 1.9 mM glycine, 1% SDS). Preparative gels for mass spectrometric analysis were stained with 0.2% Coomassie Blue R-250.

**Cell-Monolayer Immunohistochemistry**—For immunostaining, cells were fixed in 3% paraformaldehyde for 30 min at room temperature, washed with PBS and permeabilized with 0.5% Triton X-100 for 5 min, after which the cells were blocked with 3% BSA for 30 min. Cells were then incubated with primary antibody in 1% BSA for 1 h. Antibodies detecting vimentin (sc-7558 dilution 1:100, sc-32322 dilution 1:200), actin (sc-1615 dilution 1:100), and CFTR (sc-8910 dilution 1:100) were purchased from Santa Cruz Biotechnology. The antibody detecting CML (26) (6D12, dilution 1:50) was purchased from TransGenic Inc. (Kumamoto, Japan). After this incubation, the cells were washed with 0.05% Nonidet P-40 and PBS. Cells were then incubated for 1 h with 1% BSA solution containing fluorescence-labeled secondary antibodies (AlexaFluor 488 chicken anti-mouse IgG, AlexaFluor 594 donkey anti-goat IgG, AlexaFluor 546 donkey anti-goat IgG, Molecular Probes, Eugene, OR) at 1:1,000 to visualize the target protein and DAPI (Molecular Probes). After extensive washing with PBS fluorescence images were recorded on a fluorescence microscope (Olympus, Hamburg, Germany) with an attached closed-circuit display camera. Confocal fluorescence images were recorded on LSM 510 Meta (Carl Zeiss, Jena, Germany).

**Subcellular Proteome**

**Proteome Factory AG, Berlin, Germany.**
vimentin (Homo sapiens). The ion charge in search parameters for ions from ESI-MS/MS data acquisition were set to “1+, 2+ or 3+” according to instruments and methods common charge state distribution. CML-modified peptide candidates were identified by the CML-characteristic mass shift of 58.01 $m/z$ with respect to the mass of unmodified peptides containing target residues for carboxymethylation. Duplicate MS/MS spectra were reduced by active precursor exclusion after two spectra for 1.5 min. Candidates were verified by MS/MS data search according to the scoring algorithm implemented by Mascot 1.9.0 for the characteristic fragment ions (parameters: vimentin (Homo sapiens), gi 21040384, max. target sequence 100, expect threshold 10, Matrix BLOSUM62, variable modification carboxymethyl (K), peptide mass tolerance ± 0.1%, fragment mass tolerance ± 0.5 Da). Mascot search was performed using the mgf MS/MS datafile created. Only modified peptides with a score of at least 3 were considered for further analysis to exclude false positives. Software used for MS/MS-data acquisition and analysis was HP Chemstation for Agilent LC 1100 A 09.03 with Bruker Daltonics Esquire Control 1.0 for general nanoLC and ESI-MS/MS control. Bruker DataAnalysis V3.2 was used for deconvolution, deisotoping, and mgf-file creation.

**FACS Analysis**— Fibroblasts were incubated for 7 days with 200 $\mu$m glyoxal (18, 20). The trypsinized cells were centrifuged at 13,000 rpm and washed twice with PBS. The resulting pellet was resuspended in 3% PFA and incubated for 30 min at room temperature. After 2 additional washing steps the cells were permeabilized with 0.5% Triton X-100 (Sigma) in PBS, washed again, and blocked with 3% BSA (Roth, Karlsruhe, Germany) in PBS for 30 min at room temperature. FITC-labeled antibody detecting CML (26) (Transgenic Inc., Kumamoto, Japan, 1:100 dilution) was added in 1% BSA and incubated for 1 h at room temperature. After three washing steps with PBS, the fluorescence was measured using FACSCanto (BD Biosciences, San Jose) and analyzed by the software BD FACSDiva 4.0.

**Contraction Capacity Studies**—100 mg of Type I collagen (Sigma) was dissolved at 4 °C in 33.3 ml of 0.1% sterile acetic acid. 0.9 ml of 10× Hank’s Buffer Salt Solution (Biochrom, Berlin, Germany) was added to 4.5 ml of this solution of collagen. 1.9 ml of 0.1% acetic acid was supplemented. NaOH was added dropwise to neutralize the solution. 1.5 $\times$ 10⁵ fibroblasts were resuspended in 0.1 ml of fetal calf serum and added to the solution for each ml of the gel. Casy Model TT (Scharfe System, Reutlingen, Germany) was used to count the fibroblasts and to control their viability. 2 ml of the final collagen solution containing the fibroblasts were applied to 6-well plates and incubated at 37 °C and 7% CO₂ (in air) for 1 h. The gels were covered with 2 ml of Dulbecco’s modified Eagle’s medium and incubated for another 10 h at 37 °C and 7% CO₂ (in air). To examine the contraction capacity, gels were detached from the side of the wells and further incubated. Diameters were measured at 0 h and 24 h. The methodical approach of collagen lattices is based on Bell et al. (30) and was described by Delvoye et al. (31) for measuring contraction in fibroblasts.

**Paraffin Section Immunohistochemistry**— Briefly, facial skin biopsies from healthy human donors were incubated for 2 h in 4% paraformaldehyde followed by incubation in water for 3 h. The biopsies were dehydrated with ethanol and isopropyl alcohol followed by the histoprocessing at 80 °C and the embedding in paraffin (32). 5-$\mu$m sections were prepared, and the paraffin was released using xylol. After stepwise rehydration sections were blocked with 1% BSA, washed with PBS and incubated for 1 h with the primary antibody followed by additional washes with PBS. Afterward the incubation for 1 h with the secondary antibody was performed. Past extensive washing the imaging was started on a fluorescence microscope (Olympus, Hamburg, Germany).

**Electron Microscopy**—Cells were embedded as described earlier (33) according to Ito and Karovsky (34) and analyzed on a Zeiss EM 109 electron microscope (Zeiss).

**Statistical Analysis**—Statistical data were analyzed using Statistica 7.1 (StatSoft, Tulsa, OK). Normality was tested performing the Lilliefors-test. Statistical significance was determined using the two-tailed Student’s unpaired t test.

**RESULTS**

**Characterization of Major AGE Targets in Human Skin Cells**— Lysates of primary human fibroblasts and keratinocytes isolated from healthy donors were analyzed by SDS-PAGE, and immunoblotting for CML epitopes was performed. Fibroblast lysates exhibited one prominent CML signal in the lanes of each human donor. This single band was not detected in the protein pool of the keratinocytes (Fig. 1a). This result was confirmed using two-dimensional electrophoresis (Fig. 1b, top) leading to one distinct spot positive for CML. The CML signal was analyzed using nanoLC-ESI-MS/MS and identified as vimentin with a sequence coverage of 47%. Because of that, immunoblotting for vimentin (Fig. 1b, bottom) was performed resulting in a strong positional match of the two signals representing vimentin and CML. This positional match was found not only for CML, but also for CEL (27), pyrraline (29), and pentosidine (28). To test whether the vimentin signal and the CML signal are also overlapping in the cell, we performed immunofluorescence studies. Analysis by immunofluorescence microscopy (Fig. 1c) showed a strong colocalization of the two fluorescence signals. This colocalization is especially given in the perinuclear region and to lesser extent in the distal areas of the cytosol, where non-modified vimentin is located. In further fractionation studies we were able to demonstrate that the fibroblast cytoskeletal fraction contains vimentin and actin (Fig. 1d, CF). In this fraction only vimentin exhibited a detectable CML signal (Fig. 1d, middle lane).

Strikingly the protein actin did not reveal a detectable CML signal in the fractionation studies, even though it was present in high amounts in the cytoskeletal fraction according to the silver-staining pattern. This clearly indicates a preferential CML modification of vimentin. To validate further, that the signal for colocalization of CML and vimentin is not due to an accidental colocalization with another cytoskeletal protein, we performed immunoprecipitation studies. As demonstrated in Fig. 1d (IP) the precipitated vimentin showed a strong CML signal. The current view of the non-enzymatic glycation reactions favors the protein half-life as a major factor contributing to the formation of CML and other derivatives. In other words, a slow turnover of a protein seems to be the most prominent reason for a high glycation rate of proteins (5, 8). Therefore, we decided to
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Primary human fibroblasts and keratinocytes of three different donors were isolated, cultured, and lysed as described under “Experimental Procedures.” Cell lysates were separated by SDS-PAGE and analyzed using an anti-CML antibody (18, 26). a, lanes represent the analysis of three donors using keratinocyte (K) or fibroblast (F) lysates. The right lane shows the silver staining pattern of a fibroblast lysate of donor C. Fibroblast lysates were analyzed by 2DE followed by immunoblotting. b, position of the CML (upper panel) signal with the position of the signal for vimentin (lower panel) was compared. The colocalization of vimentin and CML was examined using immunofluorescence staining (c) as described under “Experimental Procedures.” d, lysates of fibroblasts were fractionated as described above. The cytoskeletal fraction (CF) was separated by SDS-PAGE and either silver-stained (stain, left lane) or immunoblotted with an α-CML antibody (α-CML). Additionally, an immunoprecipitation of vimentin was performed (IP). The bands of the CF were identified as actin (A) and vimentin (V) using the corresponding antibodies.

Identification of vimentin as major target for CML modification in primary human fibroblasts. Primary human fibroblasts and keratinocytes of three different donors were isolated, cultured, and lysed as described under “Experimental Procedures.” Cell lysates were separated by SDS-PAGE and analyzed using an anti-CML antibody (18, 26). a, lanes represent the analysis of three donors using keratinocyte (K) or fibroblast (F) lysates. The right lane shows the silver staining pattern of a fibroblast lysate of donor C. Fibroblast lysates were analyzed by 2DE followed by immunoblotting. b, position of the CML (upper panel) signal with the position of the signal for vimentin (lower panel) was compared. The colocalization of vimentin and CML was examined using immunofluorescence staining (c) as described under “Experimental Procedures.” d, lysates of fibroblasts were fractionated as described above. The cytoskeletal fraction (CF) was separated by SDS-PAGE and either silver-stained (stain, left lane) or immunoblotted with an α-CML antibody (α-CML). Additionally, an immunoprecipitation of vimentin was performed (IP). The bands of the CF were identified as actin (A) and vimentin (V) using the corresponding antibodies.

Identification of CML Modification Sites Using Nano LC-ESI-MS/MS—To test our hypothesis that structural properties of vimentin are among the most important factors for the CML modification, we decided to study the exact locations of the modification sites of vimentin by nanoLC-ESI-MS/MS. Thus, we isolated vimentin directly from fibroblasts by cell fractionation and analyzed the molecule for modified lysines. This approach enables us to detect modifications generated intracellularly to identify the real in vivo occurring modification sites. This is an advantage over most of the presently available studies based on in vitro modified proteins (36). Fig. 3a summarizes the results of several nanoLC-ESI-MS/MS analyses. Lysines found to be CML-modified in one of the nanoLC-ESI-MS/MS experiments are marked in red color. Most remarkably, all lysines located in the linker regions of the vimentin molecule (37) could be detected with a CM modification. In the folded protein domains the degree of modification was less prominent while there only 16% of the potential modification sites (3 of 19 lysines) were detected with this modification. Fig. 3c shows representative data of a single run of nanoLC-ESI-MS/MS. In this figure, two peptides of the linker regions L1 and L12 are shown detected with carboxymethylated lysines. According to Strelkov et al. (37) the linker regions are exposed to the surrounding area and seem to be, therefore, susceptible for CML formation (Fig. 3b).
In our studies presented here we were able to demonstrate that vimentin in fibroblasts is the preferential intracellular protein CML-modified in human skin. This high degree of modification seems to be based on structural properties of the vimentin molecule and not on half-life or intracellular localization. This consequently raises the question: if vimentin moieties are really selectively susceptible to CML modifications, do vimentin-deficient cell clones exhibit reduced CML modification levels?

Characterization of CML Levels in Vimentin Knock-out Fibroblasts—To test whether vimentin deletion is leading to lower CML modifications we used a murine fibroblast cell line and a vimentin (−/−) subclone of these cells and compared levels of CML modifications (both cell clones were a kind gift from Dr. Robert Evans, University of Colorado Health Sciences Center, Denver, CO). Both, FACS analysis (Fig. 4, a and b) and immunoblot analysis (Fig. 4b, right), displayed a strong reduction in mean CML signal in vimentin-deficient fibroblasts compared with vimentin-positive fibroblasts. The mean CML signal in the knock-out fibroblasts is 46% less in value compared with mean signal of vimentin positive fibroblasts. Dot blots showed similar results indicating a reduction of 48% in CML level between vimentin-positive and vimentin-negative fibroblasts. These data confirm the high importance of vimentin as major target for CML modification in human skin.

Eckes et al. (23) reported earlier on a reduced contractile capacity of vimentin-deficient fibroblasts compared with vimentin-positive fibroblasts, accompanied by a diminished stiffness of the knock-out fibroblasts. Because the CML modification takes place in the vimentin linker region, which is important for the formation of filamentous structures (37–39), it seems to be reasonable to test the functional
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a. Vimentin-Knockout and CML

![Graph showing Vimentin-Knockout and CML]

b. Quantification of CML-Signal

![Graph showing Quantification of CML-Signal]

c. Glyoxal-Treatment and Contraction

![Graph showing Glyoxal-Treatment and Contraction]

d. 3D collagen lattices

![Graph showing 3D collagen lattices]

FIGURE 4. Murine vimentin-knock-out fibroblasts exhibit reduced levels of CML modifications. Vimentin (+/−) and vimentin (−/−) fibroblasts were cultured, harvested, and analyzed by FACS or dot-blot analysis for CML content. For FACS analysis a fluorescence-labeled antibody detecting CML was used (a). Quantitative analysis of the FACS data is shown in b (left columns). Right columns in panel b represent the quantitative analysis of dot blots detecting the CML level of these two populations. The signal intensity for vimentin (+/−) was set as 100%. Each column represents the mean of five independent measurements. The data are mean ± S.D. with: **, p < 0.01.

effects of CML-vimentin modification on the contractile function of fibroblasts.

Influence of Glyoxal-induced CML on the Contractile Capacity of Fibroblasts—To test the effect of CML modifications on vimentin function we treated cells with glyoxal to induce CML-vimentin modifications in fibroblasts. Primary human fibroblasts were incubated with 200 μM glyoxal for 7 days to enhance CML formation (18, 20, 40). This incubation resulted in a massive increase of CML as measured by FACS (Fig. 5, a and b) and immunoblot analysis (Fig. 5b). Immunoprecipitation studies (Fig. 5b, right) also exhibit a strong enhancement of CML modification in vimentin after glyoxal treatment. The higher rate of CML-vimentin formation is leading to a predominant modification of vimentin in the linker region of the molecule (data not shown).

Using this approach we were able to compare fibroblasts with a higher CML-vimentin modification to normal fibroblasts for contractile capacities. The contractile capacity was determined using three-dimensional free-floating collagen lattices (30, 31). Any increase in CML-vimentin leads to a significantly (p = 0.00056) diminished contractile capacity of the fibroblasts (Fig. 5, c and d) when seeded into a free-floating three-dimensional collagen gel (30, 31). The difference in this capacity is displayed by a collagen gel that is less contracted (Fig. 5d, right) compared with the contracted gel containing the untreated control fibroblasts (Fig. 5d, left).

We were able to show that vimentin is preferentially modified by CML and modified vimentin is losing its functionality, but on the other hand the modified protein is not degraded as shown by turnover studies. This, in consequence, is leading to the question: what happens to the CML-modified protein? One of the possibilities is the sequestration of such proteins into aggregates as shown before (41, 42).

Influence of Glyoxal on the Cellular Distribution of Vimentin—To determine whether the loss of contractile capacity is the result of a CML-induced redistribution of vimentin, we used immunofluorescence microscopic analysis of fibroblasts with and without glyoxal treatment. Fig. 6a demonstrates a strong redistribution of vimentin because of glyoxal treatment, resulting in a perinuclear aggregate of vimentin. About 70% of the human fibroblasts showed a comparable aggregate formation following incubation with 200 μM glyoxal (18, 20, 40) for 2 days. About 90% of the fibroblasts were affected after incubation for 7 days. Fig. 6c displays the time-elapsed rearrangement of the intermediate filament vimentin during glyoxal treatment using fibroblasts expressing vimentin-GFP (25) (the vimentin-GFP expressing fibroblasts are a kind gift from Dr. Ronald Liem, Columbia University). Rearrangement takes place during the first 16 h of glyoxal treatment. This destruction of the vimentin filament and the localization of vimentin into an aggregate serves to explain the observed loss of contractile capacity of fibroblasts, because vimentin is significantly involved in the process of contraction (23). The distribution of actin seems to be unaffected, reflecting vimentin as the major CML target (Fig. 5a). Confocal images of this aggregate (Fig. 6b) show a dense vimentin structure inside the aggregate. Interestingly, incubation of fibroblasts with methylglyoxal results in the same redistribution of vimentin as observed by incubation with glyoxal. Methylglyoxal is generated through the glycolysis pathway and plays an important role in the formation of methylglyoxal-derived AGEs such as CEL (43, 44). This finding shows the high relevance of glycation of vimentin.

Formation of a vimentin aggregate because of glyoxal treatment raises the question whether the aggregate itself contains CML-modified vimentin. Fig. 7a displays the accumulation of CML and vimentin, clearly showing that the majority of the CML signal is located in the perinuclear area and preferentially in the formed vimentin aggregate.
The formation of such a vimentin aggregate near the cell nucleus led us to the hypothesis that CML-vimentin is accumulating in aggresomes (42). To study the formation of typical aggresomal structures we tested for vimentin and CFTR (41, 42) distribution in glyoxal-treated fibroblasts (Fig. 7b) because CFTR is a common marker for aggresomes (41, 42). Strikingly the incubation of human fibroblasts with glyoxal resulted in the redistribution of CFTR into the aggregate. Thus the observed vimentin aggregation does contain the aggresome-marker CFTR and can consequently be named aggresome (41, 42, 45). This is the first evidence that CML-vimentin represents the damaged protein inside the aggresome, linking the glycation reaction directly to aggresome formation. Strikingly we were able to show aggregated fibrils which most likely represent aggresomal vimentin (Fig. 7c) as shown by Kopito et al. before (41, 42) using transmission electron microscopy. At low magnification some cells exhibit large areas of electron lucid density (stars) containing only a few membrane compartments reminiscent to endoplasmic reticulum or lysosomal structures as well as glycogen granules.

**Vimentin Is Forming Aggresomes in Vivo**—We have already analyzed the localization of vimentin and CML in cultured non-glyoxal-treated fibroblasts (Fig. 1c). Interestingly, there were no aggresomes detectable in these cells. This might be because of a lower level of vimentin glycation compared with glyoxal-treated fibroblasts as shown in Fig. 5b. Additionally these fibroblasts divide much faster in the cell culture system compared with the in vivo situation leading to shorter time for glycation of the single cell. Therefore, the question about the in vivo relevance of the CML-vimentin aggresomes is important to answer.

As an experimental consequence we tried to demonstrate the formation of CML-vimentin aggresomes in human facial skin biopsies in vivo. We investigated skin sections obtained from 3 human donors hoping to detect aggresomes as an indicator of an accumulating life-long damage. Vimentin was found in the dermal part of the human skin representing a marker for dermal fibroblasts (46, 47) (Fig. 8). Strikingly we were able to detect aggregated vimentin (Fig. 8). This clearly points out the in vivo relevance of the aggresome formation as a consequence of life-long protein glycation in terms of CML-vimentin.

**DISCUSSION**

Our experiments clearly show that CML-vimentin is formed in human skin fibroblasts in vivo. Contradictory to the current view (8) it was shown for the first time that the structure of vimentin plays a dominant role in the susceptibility of this protein to non-enzymatic glycation, especially when compared with other proteins with longer half-lives and similar expression levels. Until now the longevity of proteins was discussed to be among the most important factors in the glycation reaction (8) because of the nonspecific character of the Maillard reaction. Vimentin plays a dominant...
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FIGURE 8. Vimentin is forming aggresomes in vivo. Human facial skin biopsies were analyzed. One section of this biopsy is shown here. The biopsies were embedded in paraffin. Sections of 5 μm were prepared and stained for vimentin (green) to examine whether aggregated vimentin can be detected in vivo. Cell nuclei are stained with DAPI (blue). The identified aggresomes are indicated by arrows. The upper right panel shows the negative control stained only with secondary antibody.

role in CML formation being the major target for this type of glycation in primary human fibroblasts. However, the high degree of CML modification of vimentin is not due to its slow turnover. Although degradation of actin is slower than vimentin degradation, actin, being expressed in a similar rate (35), does not show any detectable level of CML (Fig. 1b). The modification sites of vimentin are predominantly found in its linker regions (37), reflecting the exposure of these segments to the surrounding environment. This exposure makes the affected amino acids susceptible to glycation finally leading to the structural breakdown of the vimentin filament. In this context Thornalley et al. (36) reported that glycation of albumin by methylglyoxal is enhanced at amino acid residues with high surface exposure caused by their accessibility.

Intermediate filaments in general are characterized by a strong structural similarity. This similarity is based on the presence of a centrally located rod domain consisting of the coiled-coil domains 1A, 1B, 2A, and 2B (Fig. 3b). These domains are separated by the linkers L1, L12, and L2 (38). The linker L1 acts as a flexible hinge to separate the domains 1A and 1B (39). This flexibility is required to enable proper dimerization of intermediate filaments needed for spreading of the filament system. The glycation of the linker region by CML might therefore result in the loss of this flexibility. Herrmann et al. (48) demonstrated that the exchange of a single amino acid in the linker regions L1 or L12 is able to block the spreading of various filament systems. Smith et al. (38) put this fact down to the loss of flexibility in the linker regions. The glycation of an amino acid residue in the linker region is additionally addicted to the composition of the nearby amino acid residues due to the fact that the reactivity of the residues depends on the availability of free electron pairs of the nitrogen of the amino groups and secondary factors such as steric constraints (36, 49).

The structural similarity of the family of intermediate filaments suggests that other filament systems like desmin in muscle cells, keratins in epithelial cells or neurofilament triplet proteins in neurons (22) could be affected by a high degree of glycation as well. The high expression level and wide distribution of intermediate filaments in many vertebrate and invertebrate cells (22) makes them an interesting target for further studies linking the mechanism of glycation to several diseases. Strikingly, aggregates of neurofilament proteins, type IV intermediate filaments, were found in Parkinson disease (4, 50). Furthermore aggregated type III intermediate filament protein GFAP was reported to aggregate in Alexander disease (51). The fact that no CML glycation of keratins was detected in primary human keratinocytes (Fig. 1a) may be caused by the fast turnover of keratinocytes resulting in a shorter life time of the cells. Additionally, other AGE modifications may be present instead of CML while a strong colocalization of CML with other AGEs was reported (52). We were able to prove this colocalization in fibroblasts while we detected not only CML at the protein vimentin, but also CEL, pentosidine, and pyrraline.

CML modification in general contributes to the enhanced stiffness found in vivo for skin and other fibroblast containing tissues in aging (9). In the study presented here we were able to demonstrate that fibroblasts lose their contractile capacity due to glycation of vimentin (Fig. 5). A loss of contractile capacity results in the diminished ability of dermal fibroblasts to reorganize collagen fibers, but the reorganization of collagen is essential for tissue development and tissue repair to rearrange the extracellular matrix (53). The aggregates of vimentin we detected in old human skin give strong evidence that vimentin aggregation occurs in vivo with all consequences described before. Interestingly recent literature reports that vimentin-deficient cells exhibit a different mechanical stiffness compared with vimentin-containing cells (54, 55). Inhibition of glycation of vimentin might, therefore, provide a new target to retard the intrinsic aging of various tissues. Notably vimentin plays a role in wound healing (23, 24) while fibroblasts deficient in vimentin exhibit a reduced ability to reorganize collagen fibers after injury. The impaired wound healing in diabetes may, therefore, partly be based on the high glycation of vimentin according to a increased glycation rate in diabetes in general (56) because of the higher glucose level. Interestingly CML levels in collagen, which is an extracellular AGE target, were described to be accelerated in diabetes and aging, leading to further tissue stiff-
ering (12). With regard to wound healing recent findings demonstrated that the vimentin intermediate filament is significantly involved in the process of lymphocyte adhesion to endothelial cells during diapedesis (57).

The identification of vimentin as a major damaged protein in aggresomes supports the theory that protein aggregates directly enhance aging of tissues (45, 58, 59), linking aging to glycation. At the same time the role of vimentin as a cage (42) that surrounds other damaged proteins in aggresomes needs to be reconsidered, because we were able to show that vimentin itself is the damaged protein inside the aggresome. The aggregation of CML-modified proteins inside the aggresome and thus the separation of highly glycated proteins from the remaining protein pool may represent a cellular attempt to minimize the damage for nonglycated, functional active proteins. This proposal is supported by the fact that other AGEs than CML, like pentosidine or crosslines, play an important role in the formation of AGEs in vivo (7, 8).

Interestingly an extraordinary production of vimentin was reported previously for senescent fibroblasts (60). This may be a cellular response to compensate the damaged CML-vimentin resulting in the de novo production of vimentin to maintain function. We were able to show that exemplarily in vivo, when vimentin aggresomes were found along with distributed vimentin throughout the cytosol. This indicates a chronic process of vimentin modification during the lifetime of the donor, and in parallel the adaptation of the fibroblast vimentin expression to this condition.

Altogether our study is the first report that identifies vimentin as the major target of CML glycation in human fibroblasts and demonstrates that features in protein structure are most important for the formation of AGEs in vivo. The modified vimentin is non-functional, insufficiently degraded and accumulates in aggresomes. We give strong evidence that this process occurs also in vivo.

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