Proteolysis of AA Amyloid Fibril Proteins by Matrix Metalloproteinases-1, -2, and -3

Barbara Stix,* Thilo Kähne,[†] Knut Sletten,[‡]
John Raynes,[§] Albert Roessner,* and
Christoph Röcken*

From the Institute of Pathology* and Institute of Experimental Internal Medicine,[†] Otto-von-Guericke-University of Magdeburg, Magdeburg, Germany; the Biotechnology Center of Oslo,[‡] University of Oslo, Oslo, Norway; and the Department of Infectious and Tropical Diseases,[§] Immunology Unit, London School of Hygiene and Tropical Medicine, London, England

We recently demonstrated the presence of matrix metalloproteinases (MMPs)-1, -2, and -3 in AA amyloid deposits, which lead us to speculate that MMPs may participate in amyloidogenesis by either processing the precursor protein, or by degrading the amyloid deposits. Here we investigated this theory by determining the ability of MMP-1, -2, and -3 to degrade human acute-phase serum amyloid A (SAA) and human AA amyloid fibril proteins (AFPs). The following *in vitro* degradation experiments were performed: using either recombinant MMP-1, -2, or -3 and SAA as a substrate; using either recombinant MMP-1, -2, or -3 and AFP as a substrate; and using THP-1 cells as the protease source and AFP as the substrate. All three MMPs were able to cleave SAA and AFP within the region spanning residues 51 to 57. The following cleavage sites were identified: at 57 to 58 for MMP-1; at 7 to 8 and 51 to 52 for MMP-2; at 7 to 8, 16 to 17, 23 to 24, 51 to 52, 55 to 56, 56 to 57, and 57 to 58 for MMP-3. Cell culture experiments showed that THP-1 cells were able to degrade AFPs. Degradation was significantly delayed after addition of a general metalloproteinase inhibitor (*o*-phenanthroline) to dextran sulfate-stimulated cells. This is the first study to show that human SAAs and AFPs are susceptible to proteolytic cleavage by MMPs. Immunocytochemistry and electron microscopy showed that degradation takes place in the pericellular or extracellular compartment. (*Am J Pathol* 2001, 159:561–570)

Physiological processes that involve remodeling of the extracellular matrix, such as wound healing, embryogenesis, angiogenesis, and the female reproductive cycle, require the activity of matrix metalloproteinases (MMPs). This group of proteases degrades basement membranes and connective tissue and plays an essential role in the

homeostasis of the extracellular matrix. An imbalance in the expression and/or activity of MMPs can have important consequences in the development of diseases such as cancer and Alzheimer's disease, a cerebral amyloid disease.^{1–4} Amyloidoses are characterized by local, organ-limited, or generalized proteinaceous deposits of autologous origin showing fibrillar protein deposits with specific tinctorial and structural properties.^{5–7} Approximately 45% of all generalized amyloidoses are secondary or AA amyloidosis,^{8–11} and the acute-phase protein serum amyloid A (SAA) is the precursor of the AA fibril protein deposited in this disease. In the West, AA amyloidosis is most commonly related to rheumatoid arthritis.^{8–11} In a recent immunohistochemical investigation we showed that MMP-1, -2, and -3 were present in AA amyloid (AA) deposits and that all three were localized within the deposits independent of the organs involved (liver, kidney, or spleen).¹² These findings suggest that MMPs may participate in amyloidogenesis by either processing the precursor protein SAA, by degrading the amyloid deposits, or by remodeling the interstitial matrix after amyloid deposition.¹ We did not find any MMPs within immunoglobulin-associated AL amyloid deposits, therefore it seems unlikely that the presence of MMPs within the deposits is an essential prerequisite for tissue remodeling during amyloid deposition. Alternatively, we hypothesize that MMPs may be involved in the processing of the precursor (SAAs) or fibril proteins (AAs) in AA amyloidosis. Therefore we investigated the ability of MMP-1, -2, and -3 to degrade SAA and AA amyloid fibril proteins (AFPs) *in vitro*, which may indicate a possible involvement in the pathology of AA amyloidosis.

Materials and Methods

Materials

Recombinant human MMP-1, -2, and -3, and fluorogenic MMP substrate (DNP-Pro-Leu-Gly-Trp-Ala-D-Arg-NH₂) were purchased from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). Human THP-1 cells were pur-

Supported by grants from the Deutsche Forschungsgemeinschaft (grant no. RO 1173/3-1 and SFB 387), Bonn Bad-Godesberg, Germany; and the Wilhelm Vaillant-Stiftung, München, Germany.

Accepted for publication April 19, 2001.

Address reprint requests to PD Dr. med. Christoph Röcken, Institute of Pathology, Otto-von-Guericke-University, Leipziger Str. 44, D-39120 Magdeburg, Germany. E-mail: christoph.roecken@medizin.uni-magdeburg.de.

chased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), and the RPMI cell culture medium was obtained from PAA (Martinsried, Germany). Fetal calf serum, dextran sulfate (DS), lipopolysaccharide (LPS), *o*-phenanthroline, the protease inhibitor set, *p*-aminophenylmercuric acetate, and NaN_3 were all from Sigma (Deisenhofen, Germany). The cathepsin/subtilisin inhibitor and pepstatin A were purchased from Calbiochem-Novabiochem, and E64 was from Bachem (Heidelberg, Germany). Monoclonal antibodies against MMP-1 (clone 41-1E5, corresponding to residues 332 to 350 of human MMP-1), MMP-2 (clone 42-5D11, corresponding to residues 524 to 539 of human MMP-2), MMP-3 (clone 55-2A4 or clone B) were purchased from Oncogene (Cambridge, England) and amyloid A (clone mc1) was obtained from DAKO (Hamburg, Germany). The secondary biotinylated rabbit anti-mouse antibody, streptavidin conjugated with alkaline phosphatase, and the chromogen 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium were purchased from BioTrend Chemikalien GmbH (Cologne, Germany).

Methods

Preparation of Serum Amyloid A

Plasma was obtained by plasmapheresis and measured for SAA by enzyme-linked immunosorbent assay (ELISA).¹³ Plasma with a minimum SAA concentration of 100 $\mu\text{g}/\text{ml}$ was used for SAA purification using hydrophobic interaction chromatography, gel filtration, and ion-exchange chromatography as described,¹³ with the modification that 10 mmol/L ethylenediaminetetraacetic acid (EDTA) was added to plasma before it was run through the hydrophobic interaction column. SAA purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4% stacking gel and 16% (acrylamide:bisacrylamide 19:1 v/v; Severn Biotech, Ltd., Worcestershire, Great Britain) resolving gel.

Preparation of Amyloid Fibril Proteins

Amyloid fibril proteins were prepared as described by Skinner and colleagues.¹⁴ Briefly, this was as follows: ~6 g of amyloidotic tissue from a spleen (~10% of the splenic tissue was replaced by amyloid), which had been stored at -80°C , was homogenized in 60 ml of aqueous 0.9% NaCl solution for 30 seconds using an Ultra-Turrax T8 (IKA-Labortechnik, Staufen, Germany). The homogenate was centrifuged at 10,000 rpm for 30 minutes at 4°C . The supernatant was discarded and the pellet was homogenized in 60 ml of 0.9% NaCl for 30 seconds, centrifuged, resuspended for 15 seconds in 60 ml sodium citrate buffer (0.05 mol/L sodium citrate, 0.01 mol/L Tris, pH 8.0), and centrifuged again. Subsequently the supernatant was discarded and the pellet was resuspended in 60 ml of 0.9% NaCl solution for 15 seconds and centrifuged. The last washing step was repeated until the optical density (OD 280) of the supernatant was below 0.1. Thereafter the pellet was resuspended in 60 ml of

deionized water and centrifuged for 200 minutes at 10,000 rpm (4°C); this step was repeated three times. The final pellet had a whitish top layer and contained AA fibril proteins (AFPs) as demonstrated by SDS-PAGE, Western blotting, amino acid sequencing, and mass spectrometry. This was stored at -20°C until further use.

Degradation Experiments with Recombinant MMP-1, -2, and -3

Before the degradation experiment, MMP-1 was activated with 1 mmol/L *p*-aminophenylmercuric acetate for 60 minutes at 37°C . MMP-2 and MMP-3 did not necessitate activation before the degradation experiments. Degradation experiments with recombinant MMPs were performed as follows: samples from the pellet containing AFP were dissolved in water by heating for 30 minutes at 100°C before the supernatant was sterile filtrated (Millex HV, 0.45 μm ; Millipore, Eschborn, Germany) and concentrated to 6 $\mu\text{g}/\mu\text{l}$ (whole protein). Ten μl of the concentrated sample were mixed with 5 μl of protease and 20 μl of reaction buffer (containing 20 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L CaCl_2 , and 1 mmol/L ZnCl_2 , pH 7.6), and then made up to a volume of 40 μl with deionized water. The MMPs were used in a concentration of 0.15 $\mu\text{mol}/\text{L}$ or 0.30 $\mu\text{mol}/\text{L}$ and the degradation reaction was performed at 37°C for 18 hours before being stopped by the addition of EDTA (2.86 mmol/L). Incubation in the absence of MMPs or in the presence of 2.86 mmol/L EDTA served as controls. For the degradation experiments with SAA, SAA was dissolved in 4 mol/L of urea and subsequently dialyzed against deionized water. SAA was diluted with reaction buffer to a final concentration of 20 $\mu\text{mol}/\text{L}$. For mass spectrometry, aliquots were removed after 0 minutes, 90 minutes, and 18 hours. All degradation experiments were performed in triplicate. The activity of MMPs (30 $\mu\text{mol}/\text{L}/200 \mu\text{l}$ of either MMP-1, MMP-2, or MMP-3) was tested by fluorospectroscopy (excitation 280 nm, emission 360 nm; Spectramax Gemini dual-scanning microplate spectrofluorometer; Molecular Devices Corp., Sunnyvale, CA) using a 80 $\mu\text{mol}/\text{L}$ of DNP-PLGWAR (200 μl) fluorogenic substrate. All MMPs were active and degraded the substrate.

Degradation Experiments with THP-1 Cells

Four days before the degradation experiments, a THP-1 cell suspension was seeded in a 5-ml culture chamber (Falcon, Becton-Dickinson, Heidelberg, Germany) with an average cell density of 80,000 cells/ml of RPMI medium containing 10% fetal calf serum. Three days later, the cells were transferred into serum-free medium and stimulated with 10 $\mu\text{mol}/\text{L}$ DS or 5 $\mu\text{g}/\text{ml}$ LPS for 24 hours. On the day of the degradation experiments, the cell medium was changed and AFP (final concentration, 150 $\mu\text{g}/\text{ml}$) was added. After 6, 24, and 48 hours, 500- μl samples were removed from the THP-1 cell suspension and homogenized by sonication in the presence of 2 mg/ml of a protease inhibitor set, before centrifugation at 14,000 rpm for 10 minutes at room temperature. The

resulting supernatant was stored at -20°C until further use (the pellet did not contain any AA immunoreactive material as tested by Western blotting and ELISA). Incubations without prestimulation and in the presence of *o*-phenanthroline (10 mmol/L), pepstatin A, E64, or cathepsin/subtilisin inhibitor served as controls. Incubation without AFP served as further negative control. All experiments were performed in triplicate.

SDS-PAGE and Western Blotting

Proteins were resolved in 16.5% polyacrylamide gels according to Schagger and Jagow¹⁵ and visualized by staining with Coomassie blue. For Western blotting, proteins on unstained polyacrylamide gels were transferred onto a polyvinylidene difluoride membrane [Immobilon-P^{SQ} (polyvinylidene difluoride), pore size 0.1 μm ; Millipore, Bedford, MA] using the tank-blotting system from Bio-Rad Laboratories (München, Germany) according to the manufacturer's instructions. Transferred proteins were visualized by Coomassie blue staining. Immunostaining of the proteins was performed using anti-amyloid A antibodies (dilution 1:200). Free binding sites of the membrane were blocked overnight at room temperature with phosphate-buffered saline (PBS) containing 3% bovine serum albumin and 0.05% Tween-20. Incubation with the primary antibody was performed at 37°C for 90 minutes. After extensive washing in Tris-buffered saline supplemented with 0.05% Tween-20, the secondary biotinylated rabbit anti-mouse antibody was administered for 30 minutes at 37°C (dilution, 1:3000). Immunostaining was visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

Solid Phase Binding Assay

An ELISA was used to determine the concentration of AFP during degradation experiments with THP-1 cells. The assay protocol followed that used for antibody capture assays.¹⁶ The wells of polystyrene microtiter plates (96 well; Costar, Cambridge, MA) were each coated with 50 μl of the supernatant from the homogenized cell suspensions. After 2 hours of incubation at room temperature, the plates were washed three times with PBS (10 minutes each time), then incubated with 3% bovine serum albumin in PBS containing 0.1% Tween-20 and 0.2% NaN_3 for 2 hours (room temperature) to block residual hydrophobic surfaces. Incubation with anti-amyloid A antibody (dilution 1:400) was performed overnight at 4°C . Plates were washed thoroughly with PBS, incubated with alkaline phosphatase-conjugated secondary antibody (dilution 1:5000, 2 hours at room temperature, rabbit anti-mouse immunoglobulin G; Bio-Rad), and then washed again in the same way. Bound immunoglobulin G was detected by the EIA Kit (Bio-Rad) according to the manufacturer's instructions. Absorbance was measured at 405 nm using an ELISA plate reader and the amount of bound ligand was determined by subtracting the optical density of the blank wells, in which the ligand was omitted. The data were analyzed using the graphic program

Sigma Plot (Jandel Scientific, Chicago, IL). For direct quantitation, AFP standards (1 to 8 $\mu\text{g}/\text{ml}$, whole protein content), which had been isolated from human spleen, were precoated onto wells of the same plates as the test solutions to generate a standard curve. Duplicate assays were performed throughout.

Amino Acid Sequencing

For amino acid sequencing the polyvinylidene difluoride-electroblotted bands were cut out and placed into a 477A protein sequencer connected to a 120A PTH amino acid analyzer (Applied Biosystems, Foster City, CA). The samples were analyzed for 7 to 11 cycles.

Mass Spectrometry

Cleavage sites were determined using a matrix-assisted laser-desorption/ionization time-of-flight mass spectrometer (Reflex III; Bruker Daltonics, Germany). Human SAA or AFP were incubated with either MMP-1, MMP-2, or MMP-3 as described above and 0.5- μl aliquots were removed at the time points indicated. The samples were subsequently co-crystallized with 0.5 μl of 3,5-dimethoxy-4-hydroxycinnamic acid (20 mg/ml) in 70% acetonitrile on a SCOUT 384 matrix-assisted laser-desorption/ionization target. The mass spectrometry was performed on an matrix-assisted laser-desorption/ionization time-of-flight mass spectrometer in linear mode with internal calibration. The BioTools 1.0 software (Bruker Daltonics) was used for the annotation of the SAA fragments; the accepted mass tolerance was 100 ppm.

Immunocytochemistry

Immunocytochemistry was performed using cell pellets obtained by cytocentrifugation onto precoated glass slides (5 minutes, 1500 rpm) (Cytospin 3; Shandon GmbH, Frankfurt/Main, Germany). The morphology and structural integrity of the cells was investigated using May-Grünwald-Giemsa stain. MMPs were detected with monoclonal antibodies directed against MMP-1 (dilution 1:100), MMP-2 (1:20), and MMP-3 (1:20). AFPs were localized with anti-amyloid A antibody (1:600). THP-1 cells were identified as macrophages using an antibody directed against CD68 (monoclonal, dilution 1:40; DAKO, Glostrup, Denmark). Before immunostaining the slides were pretreated with 10 mmol/L EDTA (twice for 10 minutes in a 450 W microwave oven). Immunoreactions were visualized with the avidin-biotin-peroxidase complex method applying a Vectastain avidin-biotin complex-alkaline phosphatase kit (distributed by Camon, Wiesbaden, Germany). Neufuchsin or 3,3'-diaminobenzidine tetrahydrochloride served as chromogens. The cells were counterstained with hematoxylin.

Electron Microscopy

For electron microscopy cells were fixed in a mixture of 2% formalin/2.5% glutaraldehyde (pH 7.2, overnight at

4°C) and then in 3.125% glutaraldehyde (7 hours at 4°C). After standard procedures of tissue processing for electron microscopy, the cells were finally embedded in Lowicryl using the K4M kit (Plano, Wetzlar, Germany). Samples for postembedding immunoelectron microscopy were not postfixed with OsO₄. Polymerization took place throughout 24 hours at -30°C and was initiated by UV light. Semithin sections (1 µm) were stained with toluidine blue. Ultrathin sections (80 to 120 nm) were mounted on copper grids and counterstained with 3% aqueous uranyl acetate (30 minutes at room temperature) and contrasted with 1% aqueous lead citrate (15 minutes at room temperature).

For postembedding immunoelectron microscopy, ultrathin sections (120 nm) were mounted on formvar-coated nickel grids (200 mesh; Plano). Immunoelectron microscopy was performed in triplicate as described previously.¹⁷ The specificity of immunostaining was controlled by omitting the primary antibody. The sections were air-dried and examined using a Zeiss EM900 electron microscope.

Results

Three sets of experiments were performed to test the putative role of MMP-1, -2, and -3 in the pathology of AA amyloidosis.

Degradation of SAA by MMP-1, -2, and -3

The first set of experiments was performed to determine whether the precursor protein of AA amyloidosis is susceptible to proteolytic cleavage by MMPs. Purified, delipidated human acute-phase SAA (20 µmol/L) was incubated with 0.30 µmol/L of either recombinant MMP-1, -2, or -3 at 37°C overnight. SDS-PAGE, Western blotting, and mass spectrometry were applied to identify proteolytic fragments and cleavage sites. After SDS-PAGE bands of ~4 to 5 kd were detected (Figure 1A). Fragments and cleavage sites were identified using mass spectrometry. Three fragments were annotated after degradation with MMP-1: fragment 1 to 57, 58 to 104, and a fragment of 2726.5 Da corresponding to either 7 to 29, 8 to 30, or 9 to 31 (Figure 2). The latter fragment could not be further specified. The fragment spanning 58 to 104 most likely correlates with the band identified by Coomassie blue staining (Figure 1A). The amount of the N-terminal fragment spanning 1 to 57 (Figure 2) was probably too low to be detected either by Coomassie blue or silver staining (Figure 1A). Three fragments were annotated after degradation with MMP-2: 1 to 51, 52 to 104, and 8 to 55. Four fragments were annotated after degradation with MMP-3: 8 to 55, 57 to 104, 58 to 104, and 74 to 104 (Figure 2). The fragments generated by MMP-2 or -3 probably are not separated by SDS-PAGE or the amount was too low to be detected either by Coomassie blue or silver staining (Figure 1A). After degradation with MMP-1, MMP-2, or MMP-3 the amount of SAA was reduced accordingly. However, SAA was still detectable after 18 hours of incubation (Figures 1 and 2).

Figure 1. *In vitro* degradation experiments revealed that recombinant MMP-1 (lane 1), -2 (lane 2), and -3 (lane 3) degrade SAA (A) and AFP (B and C). Incubation without MMPs served as a control (lanes SAA and AFP). AFP was prepared from the spleen of a patient who suffered from generalized AA amyloidosis (B and C). After SDS-PAGE and Western blotting three bands were identified (lane AFP). *In vitro* degradation experiments revealed that recombinant MMP-1 (lane 1), -2 (lane 2), and -3 (lane 3) degrade AFP generating smaller AA-immunoreactive fragments (C). *In vitro* degradation was performed at pH 7.4 for 18 hours at 37°C using 0.30 µmol/L of proteases. Incubation without MMPs served as a control (lanes SAA and AFP). SDS-PAGE and Coomassie blue staining (A and B); Western blotting with anti-amyloid A antibody (C).

Degradation of AFPs by MMP-1, -2, and -3

In almost all patients with AA amyloidosis the fibril protein is an N-terminal cleavage product of the precursor protein that is generated by proteolysis.^{5,18} Thus, the isolated fibril protein differs from the precursor protein in that it lacks between 18 and 60 amino acid residues at the C-terminus and, therefore, precursor and fibril proteins may not be accessible to the same proteases. With the second set of experiments we tested whether recombinant MMP-1, -2, and -3 are able to cleave AFP. AFP was prepared from the spleen of a patient who had suffered from generalized AA amyloidosis. After SDS-PAGE and immunoblotting, three AA-immunoreactive bands of ~6, 7, and 9 kd were detected (Figure 1, B and C). Amino acid sequencing for seven and eight cycles showed that all three bands were homologous with the N-terminus of serum amyloid A (SAA) starting with serine in position 2. Using mass spectrometry the following eight N-terminally intact fragments were annotated: 2 to 21, 2 to 64, 2 to 65, 2 to 66, 2 to 67, 2 to 68, 2 to 76, and 2 to 86 (Figure 3). The fragment spanning residues 2 to 86 corresponds to the 9-kd band (Figure 1B), 2 to 76 corresponds to the 7-kd band, and 2 to 64, 2 to 65, 2 to 66, 2 to 67, and 2 to 68 probably are not separated by SDS-PAGE and corre-

Figure 2. *In vitro* degradation experiments using SAA as a substrate were analyzed by mass spectrometry and showed that each protease has its own proteolytic profile. *In vitro* degradation was performed at pH 7.4 for 18 hours at 37°C. Incubation without MMPs served as a control (control). SAA was incubated with 0.30 μ mol/L MMP-1 (+MMP-1), -2 (+MMP-2), or -3 (+MMP-3).

spond to the 6-kd band (Figure 1B). It was interesting to note that the purified AFP contained a fragment corresponding to residues 2 to 21, which was probably too small to be retained in the gel.

Degradation experiments revealed that recombinant human MMP-1, -2, and -3 all degrade AFPs (Figure 1, B and C). The same banding pattern was generated using either 0.15 μ mol/L or 0.30 μ mol/L samples of MMPs (data not shown). Whereas MMP-1 and MMP-3 generated fragments of approximately the same size, a slightly smaller polypeptide was found after proteolysis with MMP-2. In each case the degradation was incomplete and after overnight incubation fibril proteins were still detectable by immunoblotting (Figure 1C). However, the amount of detectable AFP was reduced accordingly. No degradation was observed in the absence of MMPs or in the presence of an inhibitor (EDTA). The fragments generated were immunoreactive for AA amyloid (Figure 1C). Amino acid sequencing for seven and eight cycles showed that all additional bands occurring after proteolysis revealed a sequence of SAA starting with serine in position 2. Cleavage sites were identified using mass spectrometry. MMP-1 generated a fragment spanning

position 2 to 57 (Figure 3). MMP-2 generated a fragment spanning 2 to 51 (Figure 3). Five fragments were found after degradation with MMP-3: 2 to 16, 2 to 23, 2 to 51, 2 to 56, and 2 to 57 (Figure 3). After degradation with all three MMPs, the amount of the seven largest fibril proteins was reduced accordingly. However, degradation was not complete and all fibril proteins were detectable after 18 hours of incubation (Figure 3). None of the MMPs tested here cleaved the peptide spanning 2 to 21 (Figure 3).

Degradation of AFPs by THP-1

In vivo an individual protease is embedded in a complex network of activating and inactivating factors, such as specific compartments, pH, salt concentrations, presence of metal ions, other proteases, and protease inhibitors. The activity of a particular protease depends on the specific constellation of these activating and inactivating factors as well as accessibility of the substrate. Therefore, a third set of experiments was performed, using cell culture to test whether metalloproteinases, both in general and in a more complex system, may participate in the

Figure 3. *In vitro* degradation experiments using AFP as a substrate were analyzed by mass spectrometry and showed that each protease has its own proteolytic profile. *In vitro* degradation was performed at pH 7.4 for 18 hours at 37°C. Incubation without MMPs served as a control (control). AFP was incubated with 0.30 μ mol/L MMP-1 (+MMP-1), -2 (+MMP-2), or -3 (+MMP-3).

degradation of AFP. A monocyte/macrophage cell line (THP-1) was chosen because macrophages are commonly found in many different amyloid diseases, including AA amyloidosis, and it has been proposed that they are involved in amyloidogenesis. They synthesize a broad range of proteases that may process the precursor protein to generate the fibril protein, and they may also be involved in the degradation of the deposits.^{19–22} Before the degradation experiments, MMPs were up-regulated by stimulating THP-1 cells with either 10 μ mol/L of DS or 5 μ g/ml of LPS. Unstimulated cells served as controls. An increased amount of MMP-1, -2, and -3 protein was detected immunocytochemically in DS-stimulated cells (Figure 4). After 24 hours of stimulation, AFP (150 μ g/ml) was added to the culture medium and its subsequent degradation was assessed by ELISA using an antibody directed against amyloid A.

Degradation experiments with THP-1 cells showed that unstimulated cells degraded AFP by ~24, 39, and 55% after 6, 24, and 48 hours, respectively. After similar time intervals, LPS-stimulated THP-1 cells degraded AFP by 36, 43, and 64%, and DS-stimulated cells degraded AFP

by 16, 50, and 74%. These differences were not statistically significant (Figure 5). However, after the addition of o-phenanthroline, a general MMP inhibitor, the degradation of AFP was significantly delayed in DS-stimulated THP-1 cells as compared to unstimulated cells ($P = 0.043$) and DS-stimulated cells without the inhibitor ($P = 0.006$) (Figure 5). All other protease inhibitors tested, ie, pepstatin A, E64, and a cathepsin/subtilisin inhibitor (alone or in combination), had no effect on the degradation (data not shown).

Immunocytochemistry (Figure 6A) and immunoelectron microscopy (Figure 6B) were applied to identify the compartment where degradation took place. Immunostaining for AA amyloid was confined to THP-1 cells at 6, 24, and 48 hours and localized at the cell surface or cell membrane (Figure 6A, arrows). In addition, patchy immunoreactive deposits were identified that were located in close proximity to or enclosed by THP-1 cells (Figure 6A, asterisk). However, immunostaining gradually diminished throughout time. No immunostaining was found after incubation in the absence of AFP (Figure 6B). Immunoelectron microscopy with gold-labeled secondary antibodies

Figure 4. The expression of MMP-1, -2, and -3 in THP-1 cells was demonstrated by immunocytochemistry. THP-1 cells were cultured for 24 hours in serum-free medium, and afterwards incubated with DS (10 μ mol/L; **middle lane**) and LPS (5 μ g/ml; **right lane**), respectively. No stimulation served as a control (**C; left lane**). Immunostaining with anti-MMP-1, anti-MMP-2, or anti-MMP-3. 3,3'-Diaminobenzidine tetrahydrochloride (brown color; MMP-1) or Neufuchsin (red color; MMP-2, MMP-3) served as chromogens. Hematoxylin counterstain. Original magnifications, $\times 400$.

helped to identify the patchy immunoreactive deposits as AA immunoreactive fibrillar material (Figure 6C). Occasionally gold particles were found at the cell surface, but no immunolabeling was found intracellularly.

Discussion

Patients suffering from rheumatoid arthritis exhibit significantly increased serum levels of MMP-1, -2, -3, and -9 compared to healthy controls, and the serum level of MMP-3 correlates with the C-reactive protein level and the erythrocyte sedimentation rate.^{23–25} MMP-3 is not an independent marker of joint disease in rheumatoid arthritis, but is correlated with systemic inflammation.²⁶ Inflammatory cytokines (such as interleukin-1, interleukin-6, tumor necrosis factor- α) are potent inducers of some MMPs including MMP-1, -3, and -9, and they may increase transcription by as much as 100-fold.^{27–29} The production and secretion of MMP-2 and -3 can be stimulated by the acute-phase protein SAA.²⁷ Thus, AA amyloidosis occurs under conditions that are known to generate significantly increased serum and tissue levels of MMPs, as

compared with healthy control individuals, and it is tempting to speculate that these MMPs may influence the pathogenesis of AA amyloidosis.

Previously we have shown that MMP-1, -2, and -3 are present within AA deposits,¹² and we hypothesize that MMPs may be involved in the processing of the precursor (SAA) or fibril proteins (AFP). In the present study we believe that we are the first to show that human SAA and AFP are indeed susceptible to proteolytic cleavage by MMPs. Both, SAA and AFPs were degraded by MMP-1, -2, and -3 generating fragments of different sizes. Proteolysis was neither uniform nor complete, and although each protease had its own pattern of cleavage sites some were generated by more than one of the MMPs (Figures 2 and 3). For instance redundancy was observed for MMP-1 and MMP-3 both cleaving between position 57 and 58, and for MMP-2 and MMP-3 both cleaving between residues 7 and 8, 51 and 52 as well as between 55 and 56. The cleavage sites for SAA and AFP also showed similarities: between residue 57 and 58 for MMP-1, between 51 and 52 for MMP-2, and between 56 and 57, and 57 and 58 for MMP-3. All MMPs share the propensity to

Figure 5. Degradation of AFP by THP-1 cells. Cells were stimulated with DS (A) or with LPS (B). Incubation in the absence of THP-1 cells served as a control. Degradation was inhibited by the addition of *o*-phenanthroline (oP). Degradation was assessed by ELISA as described in Material and Methods. **Filled circle**, control (RPMI + DS or LPS + AFP); **filled inverted triangle**, THP-1 + AFP; **filled square**, THP-1 + DS or LPS + AFP; **filled diamond**, THP-1 + DS or LPS + AFP + oP. Each point represents the mean value $\pm$ SEM of at least three independent experiments. *, $P < 0.05$.

cleave SAA and AFP within the region spanning residues 51 to 57. Similar results have been reported by Mitchell and colleagues³⁰ who proposed that MMP-1 and -3 are able to degrade rabbit acute-phase SAA3 within the region spanning residues 50 to 57.

However, despite similarities degradation of SAA and AFP also showed differences. MMP-2 and MMP-3 generated fragments from SAA, which we were unable to find after degradation of AFP, whereas MMP-3 generated fragments from AFP, which we could not detect after degradation of SAA. These differences may be related to differences in the secondary or tertiary structure. AFP lacked the Arg1 and the cleavage within the N-terminal half of the protein such as between residues 7 and 8, 15 and 16, and 23 and 24 may require an intact N-terminus. However, future investigations will have to clarify this issue.

The evaluation of the degradation results by SDS-PAGE, immunoblotting, and mass spectrometry was not strictly comparable, because the size of the peaks obtained by mass spectrometry do not necessarily correlate

directly with the amount of protein or peptide present on the target (see Figures 1, 2, and 3).

The results of our study show that 1) SAA and AFP seem to have overlapping but probably not completely identical substrate characteristics; 2) whereas all three MMPs have partly similar but not completely identical proteolytic profiles; and 3) MMP-1, -2, and -3 do not lead to complete degradation of SAA or AFP. The latter finding is supported by our third set of experiments; cell culture experiments revealed that degradation of AFP by DS-stimulated THP-1 cells was delayed in the presence of a general metalloproteinase inhibitor. Degradation was not entirely blocked by *o*-phenanthroline, which indicated that other proteases, besides metalloproteinases, are involved in the degradation process. However, as yet, we are unable to identify any additional specific protease group by the use of protease inhibitors such as pepstatin A (aspartate proteases such as cathepsin D), E64 (cysteine proteases such as cathepsin B and K), and cathepsin/subtilisin inhibitor (cysteine and serine proteases). Based on this observation one may speculate that different proteases are able to degrade AFP sufficiently. We have already identified four proteases [ie, MMP-1, -2, -3 (present investigation) and cathepsin K³¹] that are able to degrade AFP. However, if redundancy is present for the degradation of SAA and AFP, then why does AA occur or persist? It is possible that the degrading system becomes imbalanced during the course of AA deposition or that the presence of protease inhibitors such as α 2-macroglobulin, which have been detected in AA deposits,¹² may prevent sufficient degradation. To the best of our knowledge, AFP ending at position 51, 56, 57, or 58 (as generated here by degradation with MMPs) have not been described, which would have indicated *in vivo* activity of MMPs. This may be interpreted in three ways: 1) MMP-1, -2, and -3 are inactive *in vivo*; 2) MMP-1, -2, and -3 cleave at different sites *in vivo* to those *in vitro*; 3) or a fibril protein, that has been cleaved by MMPs is rapidly degraded by other proteases and escapes detection. Further investigations are needed to clarify this issue.

The cell culture experiments presented here indicate that degradation of AFP may take place initially in the extracellular or at least pericellular compartment. AA immunoreactive material was not found within THP-1 cells, and immunostaining was confined to the cell surface or to large extracellular patches of fibrillar material (Figure 6). The main site of activity of the MMPs is the peri- or extracellular compartment.^{28,29} In addition, it was recently shown that murine acute phase SAA and murine AA fibril proteins bind to a cell surface receptor of advanced glycation end products (RAGE), which is a signal transduction receptor and does not lead to the internalization of SAA/AA fibril proteins.³² However, after binding to RAGE, signal transduction is mediated by p21^{ras}, mitogen-activated kinases, ERK1 and ERK2^{32,33} that are known to be involved in the regulation of MMP genes.³⁴ Blocking the binding of acute phase SAA or AA fibril protein to RAGE delays the onset of AA amyloidosis.³² Whether this effect is mediated through regulation of MMP genes and hence protein expression and activity needs to be determined.

Figure 6. Immunocytochemistry (**A** and **B**) and immunoelectron microscopy (**C**) were applied to identify a putative compartment of degradation. Immunostaining was confined to the cell surface or cell membrane (**A**, arrows) and to patchy extracellular deposits (**A**, asterisk). No immunostaining was found after incubation in the absence of AFP (**B**). Immunoelectron microscopy with gold-labeled secondary antibodies showed AA-immunoreactive fibrillar material (**C**). Immunostaining with anti-amyloid A antibody. Original magnifications: $\times 400$ (**A** and **B**), $\times 30,000$ (**C**).

In summary, the putative interaction between SAA/AFP and MMPs seems to be complex. SAA can induce synthesis and secretion of MMPs²⁷ at concentrations that are equivalent to those observed in serum and synovial fluid samples of patients with inflammatory arthritis.^{27,35} MMPs, in turn, are able to cleave SAA and AFP. Therefore, MMPs may be involved in the pathology of AA amyloidosis by partially degrading SAA or AA deposits. MMPs also degrade basement membranes and connective tissue and play an essential role in the homeostasis of the extracellular matrix. The deposition of amyloid disrupts the integrity of the vascular and interstitial matrix, which again, may modify the activity of MMPs. Thus, investigation of the putative role of MMPs in AA amyloidosis merits further attention.

Acknowledgments

We thank Mrs. Mansfeld, Mrs. Müller, and Mrs. Schmitz for their excellent and skillful assistance.

References

1. Forget MA, Desrosiers RR, Beliveau R: Physiological roles of matrix metalloproteinases: implications for tumor growth and metastasis. *Can J Physiol Pharmacol* 1999, 77:465–480
2. Curran S, Murray GI: Matrix metalloproteinases in tumour invasion and metastasis. *J Pathol* 1999, 189:300–308
3. Nagase H, Woessner Jr JF: Matrix metalloproteinases. *J Biol Chem* 1999, 274:21491–21494
4. Yoshiyama Y, Asahina M, Hattori T: Selective distribution of matrix metalloproteinase-3 (MMP-3) in Alzheimer's disease brain. *Acta Neuropathol* 2000, 99:91–95
5. Husby G, Marhaug G, Dowton B, Sletten K, Sipe JD: Serum amyloid A (SAA): biochemistry, genetics and the pathogenesis of AA amyloidosis. *Amyloid* 1994, 1:119–137
6. Glenner GG: Amyloid deposits and amyloidosis (part 2 of 2). The β -fibrilloses. *N Engl J Med* 1980, 302:1333–1343
7. Glenner GG: Amyloid deposits and amyloidosis (part 1 of 2). The β -fibrilloses. *N Engl J Med* 1980, 302:1283–1292
8. Linke RP, Nathrath WBJ, Eulitz M: Classification of amyloid syndromes from tissue sections using antibodies against various amyloid fibril proteins: report of 142 cases. *Amyloidosis*. Edited by GG Glenner, EF Osserman, EP Benditt, E Calkins, AS Cohen, D Zucker-Franklin. New York, London, Plenum Publishing Corporation, 1986, pp 599–605
9. Hoshii Y, Takahashi M, Ishihara T, Uchino F: Immunohistochemical classification of 140 autopsy cases with systemic amyloidosis. *Pathol Int* 1994, 44:352–358
10. Röcken C, Schwotzer E, Linke RP, Saeger W: The classification of amyloid deposits in clinicopathological practice. *Histopathology* 1996, 29:325–335
11. Stregle RJ, Saeger W, Linke RP: Diagnosis and immunohistochemical classification of systemic amyloidosis. Report of 43 cases in an unselected autopsy series. *Virchows Arch* 1998, 433:19–27
12. Müller D, Roessner A, Röcken C: Distribution pattern of matrix metalloproteinases (MMP-1, -2, -3, and -9), tissue inhibitors of matrix metalloproteinases (TIMP-1 and -2), and α 2-macroglobulin in cases of generalized AA and AL amyloidosis. *Virchows Arch* 2000, 437: 521–527
13. Raynes JG, McAdam KP: Purification of serum amyloid A and other high density apolipoproteins by hydrophobic interaction chromatography. *Anal Biochem* 1988, 173:116–124
14. Skinner M, Shirahama TS, Cohen AS, Deal CL: The association of amyloid p-component (AP) with amyloid fibril: an updated method for amyloid fibril protein isolation. *Prep Biochem* 1983, 12:461–476
15. Schägger H, Jagow G: Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 1987, 166:368–379
16. Harlow E, Lane D: Antibodies. A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1988
17. Röcken C, Roessner A: An evaluation of antigen retrieval procedures for immunoelectron microscopical classification of amyloid deposits. *J Histochem Cytochem* 1999, 47:1385–1394

18. Solomon A, Weiss DT: Protein and host factors implicated in the pathogenesis of light chain amyloidosis (AL amyloidosis). *Amyloid* 1995, 2:269–279
19. Shirahama TS, Cohen AS: Intralysosomal formation of amyloid fibrils. *Am J Pathol* 1975, 81:101–116
20. Takahashi M, Yokota T, Kawano H, Gondo T, Ishihara T, Uchino F: Ultrastructural evidence for intracellular formation of amyloid fibrils in macrophages. *Virchows Arch [A]* 1989, 415:411–419
21. Miura K, Ju ST, Cohen AS, Shirahama TS: Generation and use of site-specific antibodies to serum amyloid A for probing amyloid A development. *J Immunol* 1990, 144:610–613
22. Röcken C, Kisilevsky R: Binding and endocytosis of murine high density lipoprotein from healthy (HDL) and inflamed donors (HDL_{SAA}) by murine macrophages in vitro. A light- and electronmicroscopic investigation. *Amyloid* 1997, 4:259–273
23. Ishiguro N, Ito T, Obata KI, Fujimoto N, Iwata H: Determination of stromelysin-1, 72 and 92 kDa type IV collagenase, tissue inhibitor of metalloproteinase-1 (TIMP-1), and TIMP-2 in synovial fluid and serum from patients with rheumatoid arthritis. *J Rheumatol* 1996, 23:1599–1604
24. Keyszer G, Lambiri I, Keysser M, Keysser C, Nagel R, Burmester GR, Jung K: Matrix metalloproteinases, but not cathepsins B, H, and L or their inhibitors in peripheral blood of patients with rheumatoid arthritis are potentially useful markers of disease activity. *Ztschr Rheumatol* 1998, 57:392–398
25. Keyszer G, Lambiri I, Nagel R, Keysser C, Keysser M, Gromnica-Ihle E, Franz J, Burmester GR, Jung K: Circulating levels of matrix metalloproteinases MMP-3 and MMP-1, tissue inhibitor of metalloproteinase 1 (TIMP-1), and MMP-1/TIMP-1 complex in rheumatic disease. Correlation with clinical activity of rheumatoid arthritis versus other surrogate markers. *J Rheumatol* 1999, 26:251–258
26. So A, Chamot AM, Péclat V, Gerster J-C: Serum MMP-3 in rheumatoid arthritis: correlation with systemic inflammation but not erosive status. *Rheumatology* 1999, 38:407–410
27. Migita K, Kawabe Y, Tominaga M, Origuchi T, Aoyagi T, Eguchi K: Serum amyloid A protein induces production of matrix metalloproteinases by human synovial fibroblasts. *Lab Invest* 1998, 78:535–539
28. Yu AE, Murphy AN, Stetler-Stevenson WG: 72-kDa gelatinase (gelatinase A): structure, activation, regulation, and substrate specificity. *Matrix Metalloproteinases*. Edited by WC Parks, RP Mecham. San Diego, London, Boston, New York, Academic Press, 1998, pp 85–113
29. Nagase H: Stromelysins 1 and 2. *Matrix Metalloproteinases*. Edited by WC Parks, RP Mecham. San Diego, London, Boston, New York, Academic Press, 1998, pp 43–84
30. Mitchell TI, Jeffrey JJ, Palmiter RD, Brinckerhoff CE: The acute phase reactant serum amyloid A (SAA3) is a novel substrate for degradation by the metalloproteinases collagenase and stromelysin. *Biochim Biophys Acta* 1993, 1156:245–254
31. Röcken C, Stix B, Brömme D, Ansorge S, Roessner A, Bühling F: A putative role for cathepsin K in degradation of AA and AL amyloidosis. *Am J Pathol* 2001, 158:1029–1038
32. Yan SD, Zhu HJ, Zhu AP, Golabek A, Du H, Roher A, Yu J, Soto C, Schmidt AM, Stern D, Kindy M: Receptor-dependent cell stress and amyloid accumulation in systemic amyloidosis. *Nat Med* 2000, 6:643–651
33. Thornalley PJ: Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs. *Cell Mol Biol* 1998, 44:1013–1023
34. Westermarck J, Kahari VM: Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J* 1999, 13:781–792
35. Sukenik S, Henkin J, Zimlichman S, Skibin A, Neuman L, Pras M, Horwitz J, Shainkin-Kestenbaum R: Serum and synovial fluid levels of serum amyloid A protein and C-reactive protein in inflammatory and non-inflammatory arthritis. *J Rheumatol* 1998, 15:942–945