

Chemerin158K Protein Is the Dominant Chemerin Isoform in Synovial and Cerebrospinal Fluids but Not in Plasma^{*[5]}

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Background: Chemerin is a chemokine/adipokine whose activity depends on proteolytic processing.

Results: Specific ELISAs demonstrate that in plasma the precursor is dominant, whereas in synovial fluid from arthritis patients and CSF from glioblastoma patients, chem158K dominates. Low levels of active chem157S were found.

Conclusion: Chemerin proteolysis occurs during inflammation.

Significance: This is the first report about levels of different chemerin isoforms in biological samples.

Chemerin is a chemoattractant involved in immunity that may also function as an adipokine. Chemerin circulates as an inactive precursor (chem163S), and its activation requires proteolytic cleavages at its C terminus, involving proteases involved in coagulation, fibrinolysis, and inflammation. However, the key proteolytic steps in prochemerin activation *in vivo* remain to be established. Previously, we have shown that C-terminal cleavage of chem163S by plasmin to chem158K, followed by a carboxypeptidase cleavage, leads to the most active isoform, chem157S. To identify and quantify the *in vivo* chemerin isoforms in biological specimens, we developed specific ELISAs for chem163S, chem158K, and chem157S, using antibodies raised against peptides from the C terminus of the different chemerin isoforms. We found that the mean plasma concentrations of chem163S, chem158K, and chem157S were 40 ± 7.9 , 8.1 ± 2.9 , and 0.7 ± 0.8 ng/ml, respectively. The total level of cleaved and non-cleaved chemerins in cerebrospinal fluids was $\sim 10\%$ of plasma levels whereas it was elevated ~ 2 -fold in synovial fluids from patients with arthritis. On the other hand, the fraction of cleaved chemerins was much higher in synovial fluid and cerebrospinal fluid samples than in plasma ($\sim 75\%$, 50% , and 18% respectively). Chem158K was the dominant chemerin isoform, and it was not generated by *ex vivo* processing, indicating that cleavage of prochemerin at position Lys-158, whether by plasmin or another serine protease, represents a major step in prochemerin activation *in vivo*. Our study provides the first direct evidence that chemerin undergoes extensive proteolytic processing *in vivo*, underlining the importance of measuring individual isoforms.

The chemerin gene was originally identified as a novel retinoid-responsive gene in psoriatic skin lesions (1). Chemerin is a secreted ligand of the orphan G protein-coupled receptor chemokine-like receptor 1, chemokine (C-C motif) receptor-like 2, and G protein-coupled receptor 1 (2). Various cell types involved in innate and adaptive immunity, including immature plasmacytoid dendritic cells, myeloid dendritic cells, macrophages, and natural killer cells, express chemokine-like receptor 1, and chemerin is a chemoattractant that promotes the recruitment of these cells to lymphoid organs and sites of tissue injury (3–5). Chemerin is also an adipokine that regulates adipocyte development and metabolic function and may play a role in glucose tolerance, obesity, and hyperlipidemia (6). Elevated levels of chemerin have been found in patients with diabetes (7–10) and fatty liver disease (11, 12), consistent with its role as an adipokine whereas its levels are also raised in patients undergoing chronic hemodialysis (13, 14) as well as those with Crohn disease (15) and chronic hepatitis C (16), in line with its suggested function in inflammation. However, these studies have all been carried out using ELISAs that detect total chemerin levels.

Chemerin is translated as a 163-amino acid pre-proprotein subsequently secreted as a 143-amino acid precursor (prochemerin, chem163S) following removal of the N-terminal signal peptide (2, 17). Prochemerin chem163S has low biological activity and requires further extracellular C-terminal proteolytic processing to achieve its full biological activity. These cleavages have been studied *in vitro* where a variety of enzymes involved in coagulation, fibrinolytic and inflammation such as FXIIa, plasmin, carboxypeptidase B2 (also known as thrombin-activatable fibrinolysis inhibitor), or elastase can proteolyze chemerin, giving rise to either active or inactive chemerins (2, 4, 17–19). Plasmin and elastase cleavage gives rise to chemerin21-158 (chem158K) and chemerin21-157 (chem157S), respectively, whereas proteinase 3 and mast cell chymase give rise to inactive chemerin21-155 (chem155A) and chemerin21-154 (chem154F), respectively (3, 20, 21). However, the catalytic efficiencies of most of these proteolytic cleavages of prochemerin

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have not been characterized, and whether they represent physiological activation or inactivation steps *in vivo* remains unclear.

An alternative approach has been to purify chemerin from biological fluids. Different C termini were found in the purified chemerin dependent on the source from which it was isolated as well as the methods used to isolate it, *e.g.* chemerin isoforms terminating at Ser-157, Ala-155, and Phe-154 were found in ascitic fluids, serum, and hemofiltrate, respectively (15). This approach allows identification of the chemerin isoforms but requires a large amount of starting material and is not amenable for characterization of the chemerin isoforms present in routine clinical samples.

In the accompanying paper (26) we show that the most active form of chemerin is chem157S in both chemotaxis and Ca^{2+} mobilization assays, whereas chem158K and chem163S have substantially lower activity (26). To identify and quantify the *in vivo* chemerin isoforms, we have developed specific ELISAs for each of these three chemerin isoforms. Here, we communicate the first data on the presence of the different chemerin isoforms in plasma, synovial fluid, and cerebrospinal fluid (CSF)³ samples and demonstrate that in synovial fluid and CSF samples, extensive proteolytic processing of prochemerin occurs, with chem158K representing the dominant isoform, indicating that this is a key step in the activation of prochemerin *in vivo*.

EXPERIMENTAL PROCEDURES

Antibody Preparation—Rabbit polyclonal antibodies were raised against peptides derived from the C termini of the human chemerin sequence, ¹⁵¹CGQFAFSKALPRS¹⁶³ (anti-chem163S), ¹⁵¹CGQFAFSK¹⁵⁸ (anti-chem158K), and ¹⁴⁹KCGQFAFS¹⁵⁷ (anti-chem157S) that were conjugated to keyhole limpet hemocyanin according to a standard protocol (Covance, Denver, PA). To isolate specific antibodies from the antisera, positive selection affinity chromatography using the cognate peptide conjugated to Sepharose was performed. Typically, rabbit antiserum (~200 ml) was diluted 5-fold with PBS, filtered through a 0.22- μm filter, and applied to a 2.5-ml column with the cognate peptide coupled to amino-link agarose (AminoLink kit; Thermo Scientific, Rockford, IL) equilibrated with PBS. After extensively washing with PBS, bound antibody was eluted with 0.1 M glycine and 250 mM NaCl, pH 3.0, and 1-ml fractions were collected in tubes containing 50 μl of 1 M Tris-HCl, pH 8.5, to neutralize the pH. Fractions containing the antibody were identified by their reactivity toward the cognate peptide in a direct ELISA. The fractions with the highest reactivity were pooled and subjected to negative selection affinity chromatography by adsorption on amino-link columns coupled with the noncognate chemerin peptides to remove cross-reactive antibodies.

Direct Peptide ELISA to Determine Antibody Reactivity—Peptide (1 $\mu\text{g}/\text{ml}$) corresponding to a chemerin isoform in 0.1 M NaHCO_3 , pH 8.6, was coated directly onto a 96-well ELISA plate at room temperature for 2 h. Nonspecific binding sites were blocked

by incubation with 1% BSA in PBS for 1 h. Antibody fractions from the cognate peptide affinity chromatography were diluted with 1% BSA in PBS and incubated for 1 h followed by washing and incubation with peroxidase-conjugated goat anti-rabbit IgG (100 ng/ml) in PBS with 1% BSA (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. After washing, tetramethylbenzidine substrate (Alpha Diagnostic International, San Antonio, TX) was incubated for 20 min followed by the addition of Stop solution (Alpha Diagnostic International) and measurement of absorbance at 450 nm.

Preparation of Glutathione S-Transferase (GST)-tagged Human Chemerins—cDNA (Open Biosystems, Huntsville, AL) encoding human chem163S, chem158K, and chem157S lacking the 20-amino acid signal peptide were cloned into pGEX 6P-3 vector (GE Healthcare) and transformed into BL21 Star (DE3) competent cells (Invitrogen). The resultant expression plasmids were verified by sequencing. The proteins were produced in 2 \times YT medium at 37 °C for 4 h. Cells were pelleted before suspension in STE buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5) containing 1 mM DTT, 5 units/ml rLysozyme (Novagen, Madison, WI), benzonase nuclease, and Complete protease inhibitor (Roche Applied Science). The insoluble proteins were solubilized using a rapid GST inclusion body solubilization and renaturation kit (Cell Biolabs, San Diego, CA). The cleared supernatants were loaded onto a glutathione-Sepharose 4B column equilibrated with STE buffer. After exhaustive washing with STE buffer, GST-fused chemerins were eluted with 10 mM reduced glutathione. The recovered proteins were >90% pure as determined by silver staining following separation on SDS-PAGE.

Western Blot Analysis of Anti-chemerin IgGs—Purified recombinant GST-chem163S, GST-chem158K, and GST-chem157S (~280 ng each) were separated by SDS-PAGE under reducing conditions followed by Western blotting with each of the purified anti-chem163S, anti-chem158K, or anti-chem157S IgG (500 ng/ml). The blot was developed with peroxidase-conjugated goat anti-rabbit antibody (100 ng/ml) and detected using ECL (GE Healthcare) detection.

Specific ELISAs for Human Chem163S, Chem158K, and Chem157S—A commercially available mouse monoclonal anti-human chemerin antibody (R&D Systems, Minneapolis, MN) was used as the capturing antibody. The antibody (4 $\mu\text{g}/\text{ml}$) was coated in PBS buffer onto ELISA plates, and nonspecific binding sites were blocked with BSA as described above for the direct peptide ELISA. Purified recombinant chem163S, chem158K, and chem157S, prepared as described in the accompanying paper (26), were used as standards to construct the calibration curves. Samples and standards were diluted with 1% BSA in PBS and incubated in the wells for 2 h. After washing with 0.05% Tween 20 in PBS, the samples were incubated with the specific cognate antibodies (500 ng/ml). In the case of anti-chem163S, 10 $\mu\text{g}/\text{ml}$ chem158K peptide was added to remove the residual cross-reactivity observed with chem158K, and in the case of anti-chem157S, 10 $\mu\text{g}/\text{ml}$ chem163S peptide was added to remove the residual cross-reactivity with chem163S. In the peptide competition ELISA, 10 $\mu\text{g}/\text{ml}$ cognate or noncognate peptides (chem163S, CGQFAFSKALPRS; chem158K, CGQFAFSK; chem157S, KKGQFAFS; chem156F, PHSFYF-

³ The abbreviations used are: CSF, cerebrospinal fluid; GBM, glioblastoma; OA, osteoarthritis; OPN, osteopontin; PPACK, H-D-Phe-Pro-Arg-chloromethyl ketone; PsA, psoriatic arthritis; RA, rheumatoid arthritis.

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PGQFAF; chem154F, PHSFYFPGQF; chem152G, EDPHSFY-FPG) were incubated with specific anti-chemerin antibodies. The plates were then processed as described for the direct peptide ELISA. The concentrations of chemerin isoforms were calculated from the calibration curves of the purified chemerin standards.

Detection of Chemerin Isoforms in Human Samples—Human samples were obtained under protocols approved by Stanford University Medical Center or Partners Healthcare Institutional Review Boards. Informed consent was obtained from donors of plasma CSF samples while synovial fluid samples were obtained under a protocol for discarded specimens. Blood was drawn from volunteers into Na-Citrate tubes (BD Biosciences), and plasma was prepared. In some experiments H-D-Phe-Pro-Arg-chloromethyl ketone (PPACK; Calbiochem) was included in the Na-Citrate collection tubes. Plasma (2 ml) was mixed with 100 μ l of heparin-agarose (Sigma) and complete protease inhibitor (Roche Applied Science). After incubation at 4 °C for 2 h, the heparin-agarose beads were pelleted and washed extensively with PBS, and chemerin was eluted with 0.8 M NaCl in PBS, all in the presence of Complete protease inhibitor. The eluates were diluted with 1% BSA in PBS for assay in the specific ELISAs described above.

CSF and synovial joint samples were stored frozen at -80 °C until the time of analysis. After thawing, 400 μ l of a CSF sample, or 30 μ l of a synovial fluid sample diluted to 400 μ l with PBS, was mixed with 50 μ l of heparin-agarose and Complete protease inhibitor before heparin-agarose adsorption was performed as described above and the eluates assayed by the specific ELISAs.

Statistics—Comparison of two samples was by Student's *t* test; multigroup comparisons were by Kruskal-Wallis analysis. The analysis was carried out using Prism v5 (GraphPad, La Jolla, CA). Values of $p < 0.05$ were considered significant.

RESULTS

Generation and Characterization of Specific Antibodies for Chem163S, Chem158K, and Chem157S—Because the activity of the different chemerin isoforms varies significantly, measurements of total levels of chemerin in biological fluids do not give a complete description of the status of the chemerin system. We therefore developed a panel of ELISAs capable of specifically detecting individual chemerin isoforms in human samples using the methodology we had employed previously to develop specific ELISAs for osteopontin (OPN) isoforms (22). Peptide antigens were used to immunize rabbits to raise specific antibodies directed against the different C-terminal sequences of chem163S, chem158K, and chem157S. Specific rabbit anti-chem163S, anti-chem158K, and anti-chem157S IgGs were purified by positive selection by binding to the cognate peptide-conjugated Sepharose followed by negative selection to remove any cross-reacting antibodies using the noncognate peptide-conjugated Sepharose. The specificity of these anti-chemerin isoform IgGs was demonstrated by Western blotting in which each antibody only recognized its cognate protein and not the other two chemerin isoforms (Fig. 1A).

Development of ELISAs Specific for Human Chem163S, Chem158K, and Chem157S—The specific antibodies, anti-chem163S, anti-chem158K, and anti-chem157S were used to develop sandwich ELISAs specific for human chem163S, chem158K, and chem157S, respectively. A commercially available monoclonal antibody that recognizes all three human chemerin isoforms was used as the capture antibody. Recombinant chemerin proteins chem163S, chem158K, chem157S, and chem155A produced as described in the accompanying paper (26) were used as standards. Recombinant chem163S, but not recombinant chem158K, chem157S, or chem155A, was detected in a dose-dependent manner by anti-chem163S specifically, with a lower limit of detection of 0.2 ng/ml (Fig. 1B, left). Likewise, anti-chem158K specifically recognized recombinant chem158K and not chem163S, chem157S, or chem155A (Fig. 1B, center) whereas anti-chem157S specifically recognized recombinant chem157S and not chem163S, chem158K, or chem155A. Both of these ELISAs also had similar sensitivity to the chem163S ELISA with a lower limit of detection of 0.2 ng/ml (Fig. 1B, right).

To confirm the specificity of the ELISAs, competitive inhibition with the cognate peptide was performed. The C-terminal peptide from human chem163S specifically inhibited the signal from recombinant chem163S protein in the ELISA for chem163S, whereas the C-terminal peptides corresponding to chem158S and chem157S did not (Fig. 1C, left). In a similar fashion, the C-terminal peptides of human chem158K and chem157S specifically inhibited the signals from chem158K and chem157S proteins in the ELISAs for chem158K and chem157S, respectively (Fig. 1C, center and right).

Other chemerin isoforms have been reported such as chem156F, chem154F, and chem152G (20, 21). We tested whether those isoforms would be detected by the specific ELISAs by including the noncognate C-terminal peptides corresponding to chem152G, chem154F, and chem156F to the specific ELISAs. They did not inhibit the signal from recombinant chem163S protein in the ELISA for chem163S, nor did they inhibit chem158K or chem157S protein signals in the ELISAs for chem158K or chem157S (supplemental Fig. 1). These data show that the ELISAs for the different chemerin isoforms have good sensitivity and specificity.

Detection of Chem163S, Chem158K, and Chem157S Levels in Human Plasma—Having established the specificity of the ELISAs, we used them to determine the levels of chem163S, chem158K, and chem157S in human plasma from normal volunteers. In the initial experiments to determine levels of chemerin isoforms in plasma, very high values for chem163S were detected (supplemental Fig. 2). Analysis of plasma by Western blotting using the anti-163S antibody showed the presence of a high molecular mass cross-reactive substance (supplemental Fig. 3A, lane 3). To circumvent this problem, a heparin adsorption step was introduced before the ELISA that separates the interfering substance while allowing quantitative recovery of chemerin isoforms upon elution from the heparin-agarose beads (supplemental Fig. 3B). The chemerin detection antibody from R&D did not recognize this high molecular mass band (supplemental Fig. 3C, lane 2), but it recognized recombinant chem163S, chem158K, chem157S, and chem155A pre-

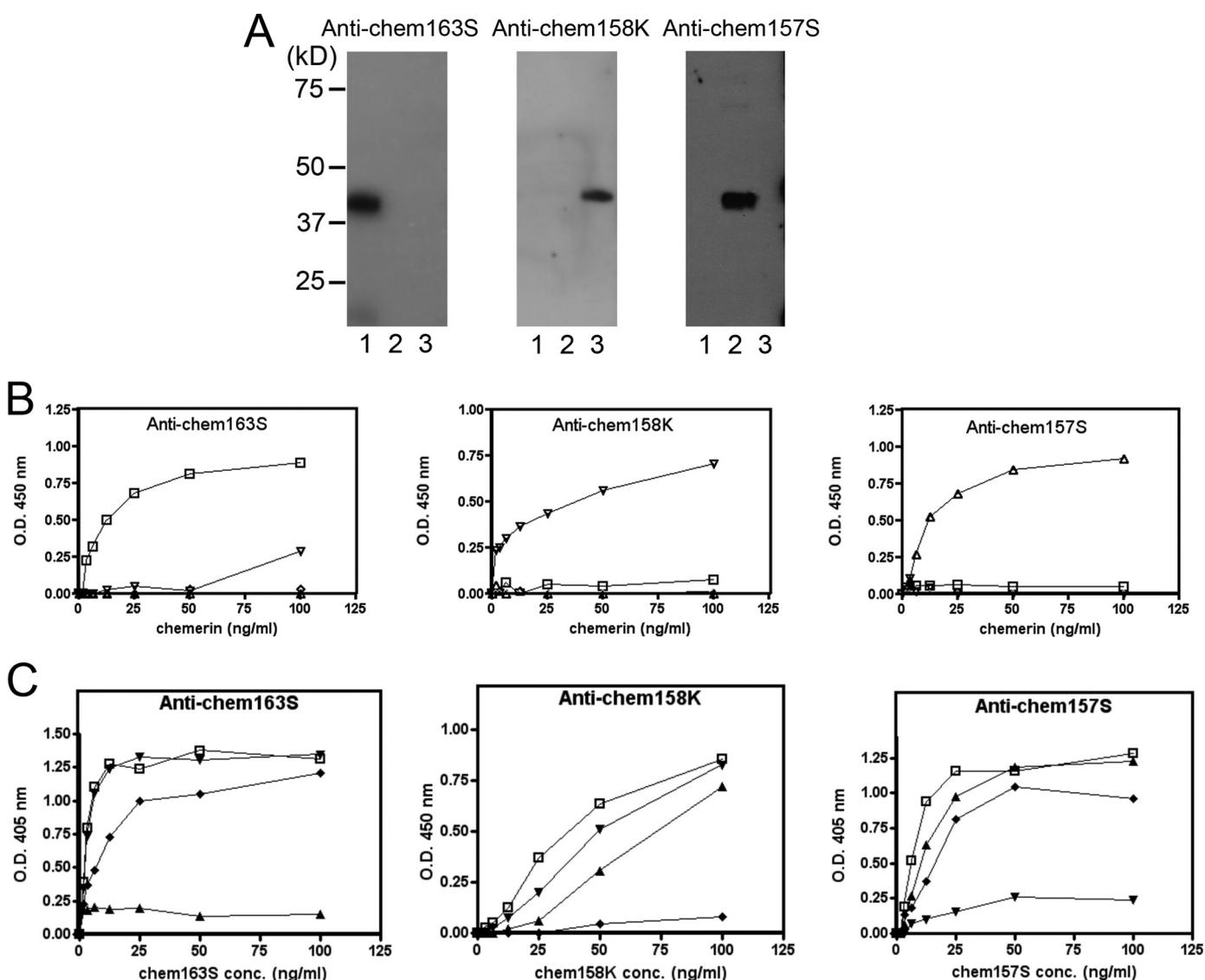


FIGURE 1. **Characterization of specific antibodies against recombinant chemerin isoforms.** A, purified antibodies specific for different chemerin isoforms were characterized by Western blot analysis of recombinant GST-chem163S (left), GST-chem157S (right), and GST-chem158K (center) with anti-chem163S, anti-chem157S, and anti-chem158K, as described under "Experimental Procedures." Molecular mass markers are shown on the left of the panels. B, recombinant chem163S (□), chem158K (▽), and chem157S (△), and chem155A (◆) by anti-chem163S, anti-chem158K, and anti-chem157S were detected using specific ELISAs as described under "Experimental Procedures." None of the three antibodies detects chem155A (◆). C, cognate peptide competes in the ELISA, but the noncognate peptides do not. C-terminal chem163S peptide (▲) specifically inhibited the signal from recombinant chem163S by itself (□) in the chem163S ELISA, but the C-terminal chem158K peptide (◆), chem157S peptide (▼) did not compete (left). C-terminal chem158K peptide (◆) and chem157S peptide (▼) specifically inhibited the signals from chem158K by itself (□) and chem157S by itself (□) proteins in the ELISAs for recombinant chem158K and chem157S, respectively (center and right), but the noncognate peptides did not compete. Values are the mean of duplicates.

pared as described in the accompanying paper (supplemental Fig. 3D), showing that the high molecular mass band was not related to chemerin.

Using the revised method, the levels of the different chemerin isoforms were measured in plasma from normal volunteers (Fig. 2). The plasma level for chem163S was 40 ± 7.9 ng/ml (mean \pm S.D., $n = 9$), chem158K level was 8.1 ± 2.9 ng/ml, whereas chem157S was barely detectable at 0.7 ± 0.8 ng/ml. To exclude the possibility that the chem158K resulted from plasmin cleavage of chem163S occurring during or after the blood draw, blood was drawn into tubes with and without 0.1 mM PPACK, an inhibitor of plasmin, thrombin, and other serine proteases. The levels of chem163S, chem158K, and chem157S were not altered by the presence of PPACK. Because

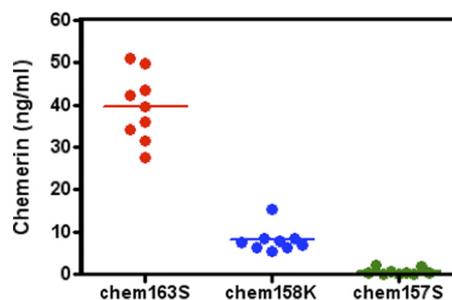


FIGURE 2. **Levels of chemerin isoforms in human plasma.** Chem163S, chem158K, and chem157S levels in normal human plasma ($n = 9$) were determined using specific ELISAs as described under "Experimental Procedures." Horizontal lines show the mean.

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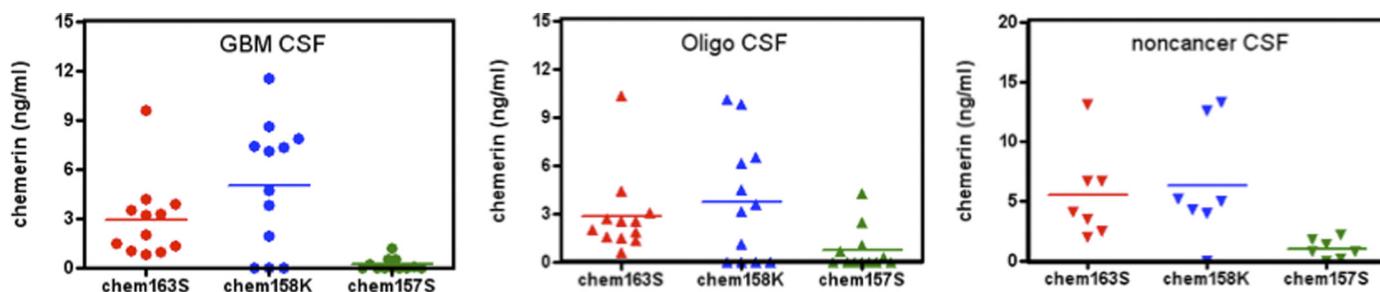


FIGURE 3. Levels of chemerin isoforms in CSF of glioblastoma, oligodendrocytoma, and miscellaneous CNS disease. Chem163S, chem158K, and chem157S levels in CSF from GBM ($n = 12$), oligodendrocytoma (*Oligo*) ($n = 12$), and miscellaneous (*noncancer*) CNS disease patients ($n = 7$) were determined using specific ELISAs as described under "Experimental Procedures." Horizontal lines show the mean.

plasmin is an enzyme that has been suggested to be responsible for chem163S cleavage to chem158K, we measured plasmin-anti-plasmin levels in these samples by ELISA. The levels of plasmin-anti-plasmin in all samples were within the normal range for human plasma and no different if PPACK was included during the blood draw (data not shown). Taken together, these data suggest that the detectable level of chem158K in plasma did not result from *ex vivo* activation of plasminogen and processing of chem163S by plasmin or other serine proteases.

Detection of Chemerin Isoforms in CSF Samples from Patients with CNS Disease—Next, we measured the levels of chemerin isoforms in CSF samples of patients with malignant glioblastoma (GBM), oligodendrocytoma, and miscellaneous CNS diseases that included one sample each from a patient with migraine, pseudotumor cerebri, meningioma (negative cytology), cylindroma necrosis postradiation therapy (negative cytology), malignant hypertension, ascending sensory loss, and bilateral Vth nerve palsies (negative cytology) (Fig. 3). In GBM CSF, the levels for chem163S, chem158K, and chem157S were 3 ± 2.4 , 5.1 ± 3.9 , and 0.2 ± 0.3 ng/ml, respectively (mean \pm S.D., $n = 12$). In oligodendrocytoma CSF samples ($n = 12$), the corresponding levels were 2.9 ± 2.5 , 3.8 ± 3.8 , and 0.7 ± 1.3 ng/ml, whereas in the miscellaneous CNS disease (noncancer CNS disease) CSF samples ($n = 7$), the levels were 5.5 ± 3.8 , 6.3 ± 4.8 , and 1.0 ± 0.8 ng/ml, respectively. There were no significant differences among the three disease groups. In all of these CSF samples, the level of chem163S was much lower ($\sim 10\%$) than that in normal plasma. On the other hand, the fraction of chem163S that had been processed, represented as the ratio of chem158K to chem163S, was significantly higher. The possibility of *ex vivo* plasmin processing of chem163S was tested by inclusion of PPACK when a CSF sample was freshly collected, but again there was no difference noted in the levels of the chemerin isoforms (data not shown), similar to the situation in plasma, indicating that chem158K did not arise from *ex vivo* proteolytic processing.

Detection of Chemerin Isoforms in the Synovial Fluid of Arthritis Patients—Based on our previous observation that proteolytic cleavage of OPN in synovial fluids was much higher in rheumatoid arthritis (RA) than in either osteoarthritis (OA) or psoriatic arthritis (PsA) (22), we hypothesized that the inflammatory environment in RA would also lead to higher levels of processing of chemerin. Therefore, we investigated the chemerin isoform levels in synovial fluid samples of patients

with RA, OA, and PsA (Fig. 4). In RA synovial fluid samples, the levels of chem163S, chem158K, and chem157S were 12 ± 22.3 , 56 ± 42.9 , and 9.3 ± 10.5 ng/ml (mean \pm S.D., $n = 23$), respectively. In OA synovial fluid samples ($n = 23$), the corresponding levels were 9.0 ± 7.2 , 74.1 ± 74.4 , and 6.1 ± 9.9 ng/ml; and in PsA samples ($n = 13$), they were 1.1 ± 2.3 , 58.9 ± 36.6 , and 3.2 ± 6.2 ng/ml, respectively. It is striking that the total chemerin levels (chem163S plus chem158K plus chem157S) found in the synovial fluid samples of all three types of arthritis patients were much higher than in CSF samples from CNS disease and were approximately 2-fold higher than that of plasma samples of normal people. The chem158K form was the dominant isoform of chemerin in all disease groups, and there was no significant difference among RA, OA, and PsA. We tested whether the chem158K had been generated *ex vivo* in the synovial fluid samples by inclusion of PPACK in the collecting tubes, and we found that the presence of PPACK did not alter the level of chem158K, suggesting that it had not been generated *ex vivo*.

Comparison of Cleaved Chemerins in Human Plasma, Inflammatory CSF, and Synovial Fluid Sample—We compared the fraction of the total chemerin that had been cleaved in the different types of human fluid samples. Using chem163S, chem158K, and chem157S levels determined by the specific ELISAs, the fraction of cleaved chemerins (chem158K plus chem157S) was calculated for each sample. Because the levels of chemerin isoforms were not different among the CSF or synovial fluid samples from different disease states, the CSF and synovial fluid samples were analyzed together as two separate groups and compared with plasma (Fig. 5). The fraction of cleaved chemerin was 0.18 in plasma whereas it was ~ 0.5 in inflammatory CSF samples, and it approached 0.75 in the synovial fluid samples with the means varying significantly when analyzed by the Kruskal-Wallis test ($p < 0.0001$), thus indicating that significant cleavage and processing of chemerin occur in these extravascular compartments in inflammatory diseases.

DISCUSSION

Prochemerin (chem163S) can be cleaved in its C-terminal domain by different serine and cysteine proteases *in vitro*, generating a series of chemerin isoforms with different levels of activity (Fig. 6) (23). To investigate which are the relevant forms of chemerin *in vivo*, we have developed ELISAs to measure the different chemerin isoforms in biological samples. The ELISAs are based on polyclonal antibodies raised against peptides that represent the C termini of the different isoforms and possess

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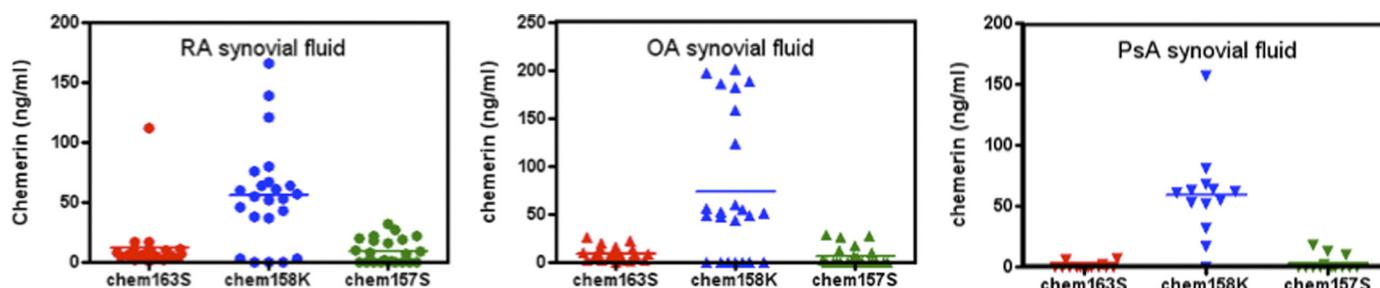


FIGURE 4. Levels of chemerin isoforms in synovial fluid of patients with RA, OA, and PsA. Chem163S, chem158K, and chem157S levels in synovial fluid of patients with RA ($n = 23$), OA ($n = 23$), and PsA ($n = 13$) were determined using specific ELISAs as described under "Experimental Procedures." Horizontal lines show the mean.

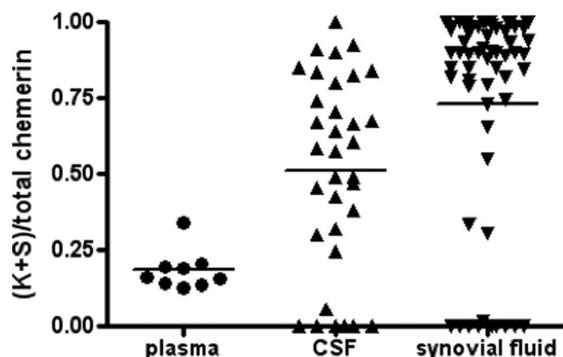


FIGURE 5. Fraction of cleaved chemerin in human plasma, CSF of CNS disease, and synovial fluid of arthritis. Ratio of cleaved chemerin levels (sum of chem158K plus chem157S) to total chemerin (sum of chem163S, chem158K, and chem157S) in plasma (●), CSF (▲), and synovial fluid (▼) is shown. All samples from Figs. 3, 4, and 5 have been included. Horizontal lines show the mean.

specificity for a single chemerin isoform as demonstrated by Western blot analysis and specific competitive inhibition by the cognate C-terminal peptide but not the noncognate peptides in the ELISAs (Fig. 1). The lack of competition by the noncognate peptides suggests that the key determinants recognized by the antibodies are the C-terminal amino acids, as we have found previously in epitope mapping of the specific antibodies for the different proteolytically cleaved OPN isoforms (18).

The ELISAs proved to have excellent specificity and sensitivity for the different chemerin isoforms allowing for the first time determination of the levels of three key chemerin isoforms, the precursor, chem163S, and two cleavage products, chem158K and chem157S. Importantly, the sensitivity of each of the ELISAs is ~ 0.2 ng/ml (~ 0.012 nM) whereas 50% of the effective concentration (EC_{50}) for the most active isoform, chem157S, is 1.17 ± 0.74 nM in a calcium mobilization assay. Thus, the range of the ELISAs covers the concentrations at which we have shown chemerin to be active (26).

Use of these ELISAs in our studies showed that in normal human plasma samples, chem163S, the prochemerin form, dominated, whereas chem158K represented only a small percentage of the total chemerin, and chem157S was barely detectable. Thus, under normal conditions, most of the chemerin present in plasma was uncleaved and therefore inactive. The low but reproducible level of chem158K suggests that there is a constitutive level of processing of the inactive chem163S in plasma at base line. The protease(s) responsible for this cleavage remains to be established.

On the other hand, CSF samples from patients with a variety of CNS diseases and synovial fluids from arthritis patients showed a completely different picture. The total level of cleaved and noncleaved chemerins was significantly lower ($\sim 15\%$) in the CSF than plasma whereas it was ~ 2 -fold higher than the plasma level in synovial fluid. Even more striking was the fraction of cleaved chemerins present in these samples from patients with inflammatory diseases. In normal plasma, only about 18% of chemerin is cleaved, whereas the fraction was $\sim 50\%$ in CSF samples and approached $\sim 75\%$ in the arthritis synovial fluid samples. Thus, considerable proteolysis of the intact chemerin is occurring in these two extravascular compartments. The possibility that chemerin is synthesized and processed locally in these compartments is supported by the significant differences between the total amount of chemerin as well as the extent of its cleavage that was observed in the CSF and synovial fluid samples.

It is notable that in both the synovial and CSF compartments, chem158K was the dominant form. We have investigated the possibility of *ex vivo* plasmin processing by comparison of samples collected with and without PPACK and found that no detectable chemerin processing occurred. It is therefore likely that a high fraction of chemerin is cleaved in these compartments, as a result of the inflammatory milieu in arthritis for the synovial samples and glioma and other inflammatory CNS diseases for the CSF samples. *In vitro* studies have shown that either plasmin or tryptase can cleave the five C-terminal amino acids from chem163S resulting in chem158K (23). Although the activity of chem158K is low (26), the levels present in the synovial fluid samples, but not the CSF samples, are high enough to suggest that they may be sufficient to contribute a significant amount of chemerin activity without further processing. Although chem158K can be efficiently generated by plasmin cleavage *in vitro* (19), the protease(s) involved *in vivo* remains to be established. Because prochemerin can be cleaved by a large variety of proteases, the high levels of chem158K present in CSF and synovial fluids provide *in vivo* validation of the importance of cleavage at lysine 158, by plasmin or other serine proteases, at these extravascular sites, and it is probable that active chemerin, chem157S, is generated via this intermediate (Fig. 6).

Other chemokines such as CCL15 and CCL23 also require proteolytic processing to generate the isoforms that possess maximum activity (24), although these cleavages are at the N terminus rather than the C terminus. Analysis of synovial fluids, some from patients with RA, for cleavage of CCL15 and CCL23

Chemerin158K Is the Dominant Form in Synovial Fluids and CSF

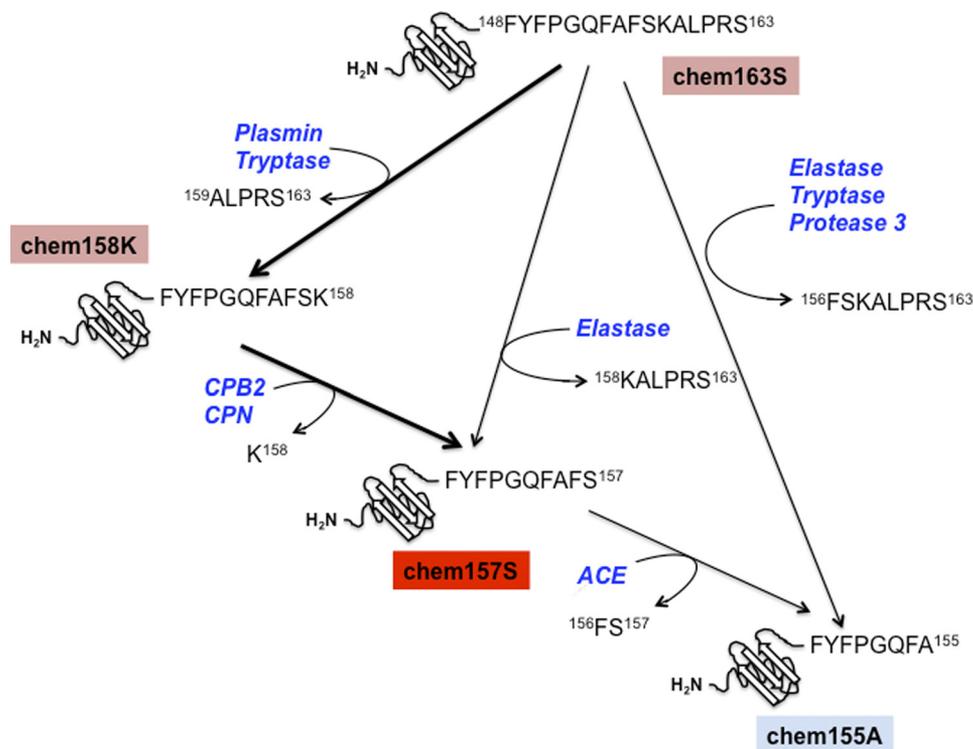


FIGURE 6. **Schematic of chemerin cleavages.** Chem163S (pink box) can be cleaved either by elastase, generating a mixture of chem157S (red box) and chem155A (blue box), by proteinase 3 and trypsin generating chem155A or by plasmin generating chem158K (pink box). Chem158K can then be cleaved by carboxypeptidases, carboxypeptidase B2 (CPB2) or carboxypeptidase N (CPN), to form the active chem157S, which is subsequently inactivated by angiotensin-converting enzyme (ACE) forming inactive chem155A. The heavier arrows represent the cleavage pathway for chem163S described in this paper. The icon for the N-terminal region of chemerin is based on the model in Zabel *et al.* (25).

demonstrated the presence of cleaved active isoforms of CCL15 and CCL23. Proteolytic processing of these G protein-coupled receptor ligands such as chemerin, CCL15, or CCL23 may represent a general mechanism to localize their effects to the site of inflammation.

When the cleavage of OPN in synovial fluids from different types of arthritis was compared, we have found previously that there was far more cleavage of OPN in RA than in OA or PsA to the thrombin-cleaved form (OPN-R) as well as the thrombin/carboxypeptidase B2 double-cleaved form (OPN-L). However, there was no difference in cleavage of chemerins when samples from the different types of arthritis were evaluated, suggesting that the regulation of proteolytic processing of OPN and prochemerin is completely different. Similarly, there was no difference in chemerin levels or in the fraction of cleaved chemerin when GBM samples were compared with oligodendrocytoma or noncancer CSF samples. The noncancer samples were from patients some of whom had inflammatory diseases such as cylindroma necrosis and postradiation therapy that may also affect levels and cleavage of chemerin. In the accompanying paper (26), analysis of gene expression shows that chemerin mRNA is increased in grade IV GBM compared with lower grade gliomas that include oligodendrocytoma or epilepsy samples. The increase in mRNA was not reflected in the levels of chemerin present in the CSF, but that may be because the GBM itself is only a minor contributor to the overall protein content of the CSF.

Surprisingly, our study found very low levels of chem157S in any of the samples. We have shown that both plasma carboxy-

peptidase N and carboxypeptidase B2 can cleave chem158K to chem157S, the fully activated isoform of chemerin (23). There were elevated levels of OPN-L in synovial fluid samples (18), as well as in GBM CSF samples (data not shown), indicating the presence of an active carboxypeptidase that should be capable of removing the C-terminal lysine from chem158K to generate chem157S. In addition, we have shown the presence of carboxypeptidase B2 protein in the synovium (18). Therefore, we would have expected to detect chem157S in these samples as both the substrate, chem158K, and the enzyme have been shown to be present. One possible explanation for this is that chem157S was rapidly cleaved further, possibly by the angiotensin-converting enzyme (6), to the inactive chem155A isoform *in vivo* (26). This may represent a physiological step to regulate the activity of this potent chemoattractant. Alternatively, chem157S may bind to cells and therefore cannot be detected in the fluid phase. We are currently developing a specific ELISA for chem155A that will help to resolve this issue.

Our study provides the first insights into the *in vivo* levels of the different chemerin isoforms and suggests that global determinations of chemerin levels may be misleading in terms of the biological potency of chemerin present. In particular, the different levels of chem158K found in different compartments emphasize that the generation of fully active chem157S is probably under tight local regulation. The determination of the different chemerin isoforms in various disease states should advance our understanding of this novel chemoattractant that acts in the interface between thrombosis, inflammation, and immunity.

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Chemerin158K Protein Is the Dominant Chemerin Isoform in Synovial and Cerebrospinal Fluids but Not in Plasma

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