Published in final edited form as:

Arch Biochem Biophys. 2013 July 15; 535(2): 111–114. doi:10.1016/j.abb.2013.03.008.

Collagen degradation by tumor-associated trypsins

Lynn S. Mirigian^a, Elena Makareeva^a, Hannu Koistinen^b, Outi Itkonen^b, Timo Sorsa^c, Ulf-Håkan Stenman^b, Tuula Salo^d, and Sergey Leikin^{a,*}

^aNational Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, United States ^bDepartment of Clinical Chemistry, University of Helsinki, Helsinki, Finland and Helsinki University Central Hospital, Helsinki, Finland ^cDepartment of Oral and Maxillofacial Diseases, Institute of Dentistry, Helsinki University Central Hospital (HUCH), University of Helsinki, Helsinki, Finland and Institute of Dentistry, Helsinki University, Helsinki, Finland ^dDepartment of Diagnostics and Oral Medicine, Institute of Dentistry, and the Oulu Center for Cell-Matrix-Research, University of Oulu, Oulu, Finland; Oulu University Hospital, Oulu, Finland; Institute of Dentistry, University of Helsinki, Helsinki, Finland

Abstract

In normal soft tissues, collagen is degraded primarily by collagenases from the matrix metalloproteinase family. Yet, collagenase-like activity of tumor-associated isoforms of other enzymes might be involved in cancer invasion as well. In the present study, we systematically examined collagen degradation by non-sulfated isoforms of trypsins, which were proposed to possess such an activity. We found that non-sulfated trypsin-1, -2, and -3 were able to cleave non-helical and unfolded regions of collagen chains but not the intact triple helix, similar to sulfated trypsins produced by the pancreas. Trypsin-2 sulfation did not affect the cleavage rate either. An apparent triple helix cleavage by tumor-associated trypsin-2 reported earlier likely occurred after triple helix unfolding during sample denaturation for gel electrophoresis. Nevertheless, tumor-associated trypsins might be important for releasing collagen from fibers through telopeptide cleavage as well as for degrading unfolded collagen chains, e.g. after initial cleavage and destabilization of triple helices by collagenases.

Keywords

Collagen degradation; collagenolysis; trypsin; tumor-associated trypsin; matrix metalloproteinases

Introduction

Enzymatic degradation of fibrillar collagens is crucial for normal remodeling of connective tissues as well as a variety of pathological processes, e.g. tissue repair after injury, tumorigenesis, and fibrosis [1]. The triple helical structure of collagen makes it highly resistant to degradation by common proteases. Mammalian enzymes designed to cleave the triple helix are collagenases MMP1, MMP8, and MMP13, transmembrane collagenase MMP14, and gelatinase MMP2, all of which belong to the matrix metalloproteinase

*Corresponding author: National Institute of Child Health and Human Development, National Institutes of Health, Bldg. 9, Rm. 1N-111, Bethesda, MD 20892. leikins@mail.nih.gov. Phone: 301-594-8314. FAX: 301-402-0292.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(MMP)¹ family [2]. Collagen triple helix is also cleaved by cathepsin K [3] and neutrophil elastase [4, 5]. However, the role of these enzymes in collagen turnover is more limited, e.g. cathepsin K degrades collagen primarily in acidic environments within lysosomes or bone resorption pits created by osteoclasts [1, 6].

Collagen triple helix cleavage by collagenolytic MMPs occurs at a specific site [7]. The cleavage may be prevented or significantly slowed down by alterations of this site [8, 9]. Surprisingly, these alterations do not cause major disruptions in collagen turnover in mice [8, 9]. The latter observation may be explained by other mechanisms of collagen turnover. In particular, collagen triple helices may be released from fibers upon telopeptide cleavage by MMPs, cathepsins or other enzymes, followed by resorption into cells and degradation within lysosomes (see, e.g., [1, 10–12] and references therein). Alternatively, triple helices released from fibers are expected to denature within several hours due to their low stability at body temperature, after which they can be degraded by nonspecific proteinases [13].

It is unclear whether other mammalian enzymes have physiological collagenase activity, particularly the ability to cleave collagen triple helices at sites other than those cleaved by MMPs, like bacterial collagenases do. Such enzymes may be especially important in cancer invasion, which requires degradation of collagen fibers in tumor and host stroma [14, 15].

Recent studies reported efficient cleavage of type I collagen triple helices by trypsin-2 from human adenocarcinoma cells [16] and type II collagen triple helices by trypsin-2 from urine of a pancreatitis patient [17]. However, bovine pancreatic trypsin did not cleave these triple helices under the same conditions, consistent with previous reports of collagen triple helix resistance to trypsin [18]. The collagenase-like activity could therefore be a specific feature of human trypsin-2 [16, 17]. Furthermore, trypsin-1, -2 and -3, i.e., cationic-, anionic- and mesotrypsin, have somewhat different activities, which might be further modified by sulfation of pancreatic, but not tumor-associated isoforms [19–21].

Here, we examined type I collagen susceptibility to human trypsin-isoenzymes more systematically. Our experiments revealed no triple helix cleavage by non-sulfated trypsin-1, -2, and -3 and suggested that the cleavage observed in [16] occurred after triple helix unfolding during sample denaturation for electrophoresis (before inactivation of more stable trypsin). We found that sulfated and non-sulfated trypsin-2 had similar activity; both cleaved only non-helical, unfolded regions of collagen chains.

Materials and methods

Human collagen, consisting of ~90% type I and 10% type III collagens, was purified by ammonium sulfate precipitation, pepsin treatment, and salt fractionation from cell culture media of human foreskin fibroblasts (CRL 2127, ATCC) as previously described [22]. Bovine serum albumin (BioPharm Laboratories) and collagen were fluorescently labeled with Alexa Fluor 546 (Invitrogen) and Cy5 (GE Healthcare), correspondingly [22]. Recombinant human trypsinogens were prepared and activated with enterokinase as described in [23]. Activity of the resulting trypsin was evaluated with a chromogenic peptide substrate (S-2222, Chromogenix). Sulfated trypsinogen-2 was purified from 10 mL of pancreatic juice by affinity chromatography using monoclonal trypsinogen-2 antibody coupled to cyanogen bromide-activated Sepharose [19]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) on precast 4–12% Bis-Tris mini gels

¹Abbreviations used: MMP, matrix metalloproteinase; BSA, bovine serum albumin; AF, Alexa Fluor dyes; SDS, sodium dodecyl sulfate; LDS, lithium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HAc, acetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

(Invitrogen) before and after enterokinase processing revealed complete activation and only minimal degradation of the enzymes. Collagen cleavage was performed in 50 mM Tris, 150 mM NaCl, 5 mM CaCl₂, 0.05% Brij 35, pH 7.5 and analyzed by SDS/PAGE on precast 3–8% Tris-acetate or 4–12% Bis-Tris mini gels (Invitrogen) after denaturation of sample aliquots in lithium dodecyl sulfate (LDS) gel loading buffer (Invitrogen) supplemented with acetic acid (HAc), EDTA, or HAc and EDTA. Fluorescently labeled collagen and BSA were visualized by scanning the gels in a FUJI FLA5000 fluorescence scanner (FUJI Medical Imaging). Intensities of gel bands were measured with the Science Lab software supplied with the scanner.

Results

To characterize collagenase activity of trypsins, we prepared mixtures of Cy5-labeled collagen (~0.1 mg/ml) with AF-546-labeled bovine serum albumin (BSA, ~0.01 mg/ml) and incubated them with 10, 100 or 1000 nM of activated trypsins at 4 or 20 °C. After the incubation, sample aliquots were mixed with a gel loading buffer containing acetic acid (HAc) or EDTA, denatured and analyzed by SDS/PAGE. We observed no significant cleavage of type I collagen (even after 24 h incubation at 20 °C), provided that trypsin was inhibited by 0.5 M HAc prior to sample denaturation (Figure 1). We also observed no cleavage of homotrimeric type I collagen (data not shown) or type III collagen ($\alpha 1(\text{III})_3$ band in Fig. 1). The observed cleavage of BSA in each of the samples confirmed that the trypsins were active during the incubation. The results were similar for non-sulfated trypsin-1, -2 (Try-2), and -3, as well as sulfated trypsin-2 (Try-2S).

We observed collagen triple helix degradation consistent with that reported in Ref. [16] only when the trypsins were not inhibited by acetic acid. Apparently, the degradation occurred during sample denaturation rather than the preceding 24 h incubation at 4 or 20 °C. It has been reported that collagen denatures at or below 40–42 °C, depending on the heating rate [13], while human trypsin is stable and remains active at least up to 60 °C [24]. Thus, denatured collagen molecules could be rapidly degraded before trypsin inactivation in the process of the sample heating for denaturation. Neither the sample loading buffer nor EDTA (up to 125 mM) prevented the degradation.

Next, we examined collagen cleavage by sulfated and non-sulfated isoforms of trypsin-2 at 37 °C (Fig. 2). In these experiments, the reaction was stopped by mixing sample aliquots with LDS gel loading buffer containing both HAc and EDTA. Treatment with 100 nM trypsin-2 and -2S caused significant degradation of type III collagen within 15 min, but almost no degradation of type I collagen even after 2 h incubation. We observed very rapid, complete cleavage of type III collagen and slow degradation of type I collagen with 1000 nM trypsin-2 and -2S. No large intermediate cleavage products of either type III or type I collagen chains, like those in samples denatured without HAc (Fig. 1), were observed.

Discussion

Our experiments demonstrate that sulfated and non-sulfated human trypsins are capable of cleaving only nonhelical or unfolded regions of type I and type III collagens. Degradation of the helical regions by human tumor-associated trypsin 2 but not bovine pancreatic trypsin reported in Ref. [16] apparently resulted from insufficient inhibition of the enzyme in the gel-loading buffer rather than from its collagenase activity. Lower stability and more significant loss of activity above the triple helix unfolding temperature in the gel-loading buffer with EDTA were likely responsible for preventing the cleavage by bovine trypsin at the same conditions.

It is important to keep in mind that collagen triple helix stability varies significantly with the amino acid sequence along the 300-nm-long molecule [25]. Weaker regions of the molecule undergo local unfolding and refolding, sometimes referred to as microunfoldings [26–29]. While unfolded, these regions become susceptible to cleavage by general proteases, including trypsins [26, 29, 30]. At or below room temperature such cleavage of type I collagen is slow and inefficient because of infrequent local unfolding and short lifetimes of unfolded conformations, although some cleavage is observed at high enzyme concentrations [31, 32].

The rate of local unfolding of collagen, the lifetimes of unfolded conformations, and the rate of cleavage at the unfolded sites rapidly increase with temperature [32]. At 37 °C, collagen triple helices not incorporated into fibers are thermodynamically unstable and denature with the characteristic time from several hours to several days, depending on the species [13]. Initial, local unfolding and cleavage of the least stable triple helix regions produces fragments that are even less thermally stable than full-length molecules [26, 29, 30]. Rapid denaturation of these fragments at 37 °C promotes further cleavage of all susceptible peptide bonds.

The slow collagen degradation by trypsin observed at 37 °C (Fig. 2) is consistent with the nonspecific cleavage at microunfoldings sites. In particular, less thermally stable type III collagen was degraded faster than more stable type I collagen. Rapid unfolding and subsequent degradation of the initial cleavage products likely resulted in final products that were too short to be visible on the gel. In contrast, fast collagen degradation during denaturation of samples, in which trypsin-2 was not inhibited by HAc, was less complete due to trypsin inactivation by heating shortly after thermal unfolding of collagens, resulting in large degradation products clearly visible in EDTA lanes in Fig. 1.

It is worthwhile recalling that local triple helix unfolding also promotes assembly of fibers, in which the molecules are stabilized and prevented from further unfolding and proteolytic cleavage [26]. However, we completely inhibited fiber formation in our experiments by utilizing pepsin-treated collagen and 50 mM Tris in the reaction buffer.

In conclusion, tumor-associated trypsins do not appear to have collagenase activity. Still, they might participate in physiological collagen degradation by releasing the triple helices from fibers through telopeptide cleavage and subsequently cleaving unfolded regions of collagen chains as well as by activating proMMPs [16, 33, 34].

Acknowledgments

The authors thank Ms. Maarit Leinimaa and Ms. Annikki Löfhjelm for excellent technical assistance. This work was funded in part by the Intramural Research Program of the National Institute of Child Health and Human Development, National Institutes of Health, USA, Helsinki University Central Hospital, the Finnish Cancer Foundation, the Academy of Finland, Sigrid Juselius Foundation and Finska Läkaresällskapet.

References

1. Song F, Wisithphrom K, Zhou J, Windsor LJ. *Front Biosci.* 2006; 11:3100–3120. [PubMed: 16720379]
2. Nagase H, Visse R, Murphy G. *Cardiovascular research.* 2006; 69:562–573. [PubMed: 16405877]
3. Garner P, Borel O, Byrjalsen I, Ferreras M, Drake FH, McQueney MS, Foged NT, Delmas PD, Delaisse JM. *The Journal of biological chemistry.* 1998; 273:32347–32352. [PubMed: 9822715]
4. Kafienah W, Buttle DJ, Burnett D, Hollander AP. *Biochem J.* 1998; 330(Pt 2):897–902. [PubMed: 9480907]

5. Mainardi CL, Hasty DL, Seyer JM, Kang AH. The Journal of biological chemistry. 1980; 255:12006–12010. [PubMed: 6254989]
6. Costa AG, Cusano NE, Silva BC, Cremers S, Bilezikian JP. Nat Rev Rheumatol. 2011; 7:447–456. [PubMed: 21670768]
7. Nagase H, Woessner JF Jr. The Journal of biological chemistry. 1999; 274:21491–21494. [PubMed: 10419448]
8. Han S, Makareeva E, Kuznetsova NV, DeRidder AM, Sutter MB, Losert W, Phillips CL, Visse R, Nagase H, Leikin S. The Journal of biological chemistry. 2010; 285:22276–22281. [PubMed: 20463013]
9. Liu X, Wu H, Byrne M, Jeffrey J, Krane S, Jaenisch R. The Journal of cell biology. 1995; 130:227–237. [PubMed: 7790374]
10. Krane SM, Byrne MH, Lemaitre V, Henriot P, Jeffrey JJ, Witter JP, Liu X, Wu H, Jaenisch R, Eeckhout Y. The Journal of biological chemistry. 1996; 271:28509–28515. [PubMed: 8910479]
11. Li Z, Yasuda Y, Li W, Bogyo M, Katz N, Gordon RE, Fields GB, Bromme D. The Journal of biological chemistry. 2004; 279:5470–5479. [PubMed: 14645229]
12. Madsen DH, Ingvarsen S, Jurgensen HJ, Melander MC, Kjoller L, Moyer A, Honore C, Madsen CA, Garred P, Burgdorf S, Bugge TH, Behrendt N, Engelholm LH. The Journal of biological chemistry. 2011; 286:26996–27010. [PubMed: 21652704]
13. Leikina E, Merts MV, Kuznetsova N, Leikin S. Proc Natl Acad Sci U S A. 2002; 99:1314–1318. [PubMed: 11805290]
14. Egeblad M, Rasch MG, Weaver VM. Curr Opin Cell Biol. 2010; 22:697–706. [PubMed: 20822891]
15. Liotta LA. Cancer Res. 1986; 46:1–7. [PubMed: 2998604]
16. Moilanen M, Sorsa T, Stenman M, Nyberg P, Lindy O, Vesterinen J, Paju A, Konttinen YT, Stenman UH, Salo T. Biochemistry-Us. 2003; 42:5414–5420.
17. Stenman M, Ainola M, Valmu L, Bjartell A, Ma GF, Stenman UH, Sorsa T, Luukkainen R, Konttinen YT. Am J Pathol. 2005; 167:1119–1124. [PubMed: 16192646]
18. Bruckner P, Prockop DJ. Anal Biochem. 1981; 110:360–368. [PubMed: 7015914]
19. Itkonen O, Helin J, Saarinen J, Kalkkinen N, Ivanov KI, Stenman UH, Valmu L. Febs J. 2008; 275:289–301. [PubMed: 18076654]
20. Koivunen E, Huhtala ML, Stenman UH. The Journal of biological chemistry. 1989; 264:14095–14099. [PubMed: 2503510]
21. Szepessy E, Sahin-Toth M. Febs J. 2006; 273:2942–2954. [PubMed: 16759229]
22. Makareeva E, Cabral WA, Marini JC, Leikin S. The Journal of biological chemistry. 2006; 281:6463–6470. [PubMed: 16407265]
23. Koistinen H, Koistinen R, Zhang WM, Valmu L, Stenman UH. Neuroscience. 2009; 160:97–102. [PubMed: 19249338]
24. Anderson RA Jr, Mack SR, Beyler SA, Zaneveld LJ. Comp Biochem Physiol B. 1984; 77:707–713. [PubMed: 6428804]
25. Makareeva E, Mertz EL, Kuznetsova NV, Sutter MB, DeRidder AM, Cabral WA, Barnes AM, McBride DJ, Marini JC, Leikin S. The Journal of biological chemistry. 2008; 283:4787–4798. [PubMed: 18073209]
26. Kadler KE, Hojima Y, Prockop DJ. The Journal of biological chemistry. 1988; 263:10517–10523. [PubMed: 3392022]
27. Privalov PL. Adv Protein Chem. 1982; 35:1–104. [PubMed: 6762066]
28. Privalov PL, Tiktopulo EI, Tischenko VM. J Mol Biol. 1979; 127:203–216. [PubMed: 430563]
29. Ryhanen L, Zaragoza EJ, Uitto J. Arch Biochem Biophys. 1983; 223:562–571. [PubMed: 6344798]
30. Westerhausen A, Kishi J, Prockop DJ. The Journal of biological chemistry. 1990; 265:13995–14000. [PubMed: 2116413]
31. Kuznetsova N, Leikin S. The Journal of biological chemistry. 1999; 274:36083–36088. [PubMed: 10593890]

32. Kuznetsova NV, McBride DJ, Leikin S. J Mol Biol. 2003; 331:191–200. [PubMed: 12875845]
33. Sorsa T, Salo T, Koivunen E, Tyynela J, Kontinen YT, Bergmann U, Tuuttila A, Niemi E, Teronen O, Heikkila P, Tschesche H, Leinonen J, Osman S, Stenman UH. The Journal of biological chemistry. 1997; 272:21067–21074. [PubMed: 9261109]
34. Vilen ST, Suojanen J, Salas F, Risteli J, Ylipalosaari M, Itkonen O, Koistinen H, Baumann M, Stenman UH, Sorsa T, Salo T, Nyberg P. Cancer Invest. 2012; 30:583–592. [PubMed: 22909050]

Highlights

We examined collagen degradation by non-sulfated trypsins commonly found in tumors.

Non-sulfated trypsins -1,-2, and -3 cleave only unfolded regions of collagen.

Trypsin-2 sulfation does not affect its collagenolytic activity.

Collagen cleavage by trypsin-2 reported earlier involved prior helix unfolding.

Figure 1.

Collagen cleavage by Try-2 and Try-2S at 20 °C. **A.** Gel electrophoresis of collagen-BSA mixtures incubated with different concentrations of Try-2 for 24 h at 20 °C; 4–12% Bis-Tris gradient mini gel. **B.** Gel electrophoresis of collagen-BSA mixtures incubated with 100 nM of Try-2 or Try-2S; 3–8% Tris-acetate gradient mini gel. Cy5-labeled collagen chains and their degradation products appear in red. AF-546-labeled BSA and its degradation products appear in green. SDS/PAGE sample buffer contained either EDTA or HAc.

Figure 2.

Collagen cleavage by Try-2 and Try-2S at 37 °C. **A** and **B**. Gel electrophoresis of collagen incubated with 100 and 1000 nM Try-2S for 0 to 120 min at 37 °C; 3–8% Tris-Acetate gradient mini gels; sample buffer contained both EDTA and HAc. Bands corresponding to $\alpha 1(\text{III})_3$ trimers of type III collagen chains and $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$ monomers of type I collagen chains were visualized at the same scanner settings on multiple gels and stitched together without any image processing. No collagen degradation products were visible in any of the gels. **C** and **D**. Fractions of uncleaved type I and type III collagens calculated from intensities of the corresponding bands on the gels shown in panels B and A as well as similar Try-2 gels. Try-2 cleavage is shown with circles (○) and Try-2S with squares (□). All experiments were performed in triplicate. The error bars show the corresponding standard deviations.