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COMPARISON OF SNAKE VENOM REPROLYSIN AND MATRIX METALLOPROTEINASES AS MODELS OF TNF-α CONVERTING ENZYME

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Abstract. The reprolysin ht-d was compared to several human MMPs for the ability to cleave a peptide substrate representing the processing site of human pro-TNF. The rank order of inhibitor potency for a series of hydroxamic acids was also compared among these enzymes and for inhibition of TNF release from human white blood cells. The results suggest that ht-d is a better model TNF convertase than are the human MMPs.

Tumor necrosis factor alpha (TNF) is a potent proinflammatory cytokine that stimulates a variety of signals in cells replete with either of two cell surface TNF-receptors.[1-3] TNF is expressed as a 26 kDa precursor, which is released from the cell surface as a soluble, 17 kDa mature cytokine.[3] Processing of the 26 kDa precursor to the 17 kDa mature form is facilitated by specific proteolytic cleavage between Ala76 and Val77 of TNF.[4] The specific proteinase that catalyzes the conversion of 26 kDa TNF to the 17 kDa form in vivo has yet to be identified, but some properties of this TNF-α converting enzyme (TACE) have been revealed through biochemical and pharmacological studies.[5-7] Recent studies from several groups have demonstrated that the activity of the enzyme can be inhibited by hydroxamate-containing compounds, designed as inhibitors of the matrix metalloproteinases (MMPs), in vivo, in cellular assays, and in cell-free assays with partially purified enzyme.[5-7] Recently, Moss et al. reported that human TACE displays amino acid sequence homology to a family of metalloproteinase, known as the reprolysins, that are distinct from the MMPs.[8] The hemorrhagic toxin from western diamondback rattlesnake (*Crotalus atrox*) venom, ht-d, is a prototypical member of the reprolysin family that has been studied in detail.[9-10] Studies of the substrate specificity of ht-d reveal a strong preference for alanine at the P1 site and an aliphatic amino acid at the P1' site; an Ala-Leu bond was the most rapidly cleaved substrate within synthetic peptides and within the insulin B chain.[11] This substrate preference suggests that the Ala-Val bond of 26 kDa TNF might likewise be a preferred cleavage site for ht-d. Based on these observations we have investigated whether the snake venom reprolysin ht-d might serve as a useful model of human TACE, and report the results of these studies here.

Material and Methods

Substrates. The MCA and DNP labeled peptides (>95% purity) were purchased from Bachem and QCB, respectively. ^{3}H-Leu labeled 26 kDa TNF was prepared by in vitro transcription/translation of the cDNA for human TNF using a rabbit reticulocyte lysate kit (Promega), according to the manufacturers instructions. All of the substrates were stored as concentrated stock solutions at -20 °C until use.

Enzymes. Recombinant human MMP1, MMP3, MMP8, and MMP9 were expressed and purified at DuPont Merck Research Laboratories. Details of the expression and purification of each of these were similar to those reported for MMP9.[12] Human MMP2 was purified from HT-1080 fibrosarcoma cells, as previously described.[13] All of the MMPs were activated with APMA (4-aminophenylmercuric acetate), as previously described.[12] Ht-d was purified as previously described.[14]

Enzyme Assays. Fluorogenic assays using the MCA-labeled peptides were performed as previously described[15,16] at a final substrate concentration of 10 μM. A reversed-phase HPLC assay of TNF converting activity was performed with the peptide DNP-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-NH_2 as substrate. Enzyme (320 nM) and substrate (150 μM) were mixed together in enzyme buffer with a cocktail of protease inhibitors (50 μg/mL N-methoxysuccinyl-A-A-P-V-chloromethyl ketone; 100 μg/mL α-1 antitrypsin; 10 μg/mL leupeptin; 5 μg/mL aprotinin; 2 μM bestatin; and 1 mM PMSF) and incubated at 37 °C for varying lengths of time. After a specific incubation time, an aliquot of the reaction mixture was removed and mixed with 6 N HCl to quench the reaction. This mixture was then injected onto a Zorbax Rx-C18 column (4.6 x 250 mm), and the components were eluted with a linear gradient from 0 to 70% acetonitrile in aqueous 0.1% TFA (trifluoroacetic acid). The initial velocity of the enzymatic reaction was measured as the area under the product peak per unit time. The product peak was identified by its coincident retention time with that of a genuine sample of the synthesized product peptide DNP-Pro-Leu-Ala-Gln-Ala-COOH (QCB). Confirmation that enzymatic cleavage of the substrate peptide resulted in lysis of the correct scissile bond was obtained by collecting the fraction containing the product peak and subjecting the sample to mass spectral analysis.

To determine whether ht-d would recognize full length 26 kDa TNF as a substrate, and process it to the 17 kDa mature form of the cytokine, the following assay was performed. HT-d (320 nM) and ^{3}H-Leu TNF were mixed together in enzyme buffer supplemented with 0.25% Tween-20, 15% (v:v) glycerol, and the protease cocktail described above, and incubated at 37 °C for 90 min. After incubation the reaction was quenched by adding an equal volume of 4X reducing SDS sample buffer (Novex). Samples were then electrophoreses on 14% acrylamide gels and analyzed by autoradiography as previously described.[5]

Assay of TNF Release from Whole Human Blood. Whole blood assays were performed using a modification of the procedure reported by Desch et al.[17] TNF was quantified by ELISA, using a monoclonal antibody to human TNF.

Inhibitor Synthesis. All of the hydroxamic acids tested here were synthesized by the Medicinal Chemistry Department of DuPont Merck Research Laboratories. Details of the synthesis of individual compounds will be described in a separate communication.

Results and Discussion

Two peptide substrates were used to characterize the MMPs and ht-d. One of these peptides is a permissive substrate that is efficiently cleaved by all of the human MMPs that we have tested. Its sequence has been previously reported by Knight et al.[15]: MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2, where MCA is the highly fluorescent 7-methoxycoumarine-4-yl acetyl group, and DPA is a fluorescence quenching N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl group. We refer to this peptide as the "Gly-Leu peptide" from here on. The second peptide was designed to bracket the scissile bond of TNF and utilize the same fluorogenic properties of the Gly-Leu peptide. Its sequence is as follows: MCA-Pro-Leu-Ala-Gln-Ala-Val-DPA-Arg-Ser-Ser-Ser-Arg-NH_2. This peptide is referred to here as the "Ala-Val peptide". The ability of each of these peptides to serve as substrates for various human MMPs and ht-d was tested as described under Material and Methods, and the results are summarized in Figure 1, where the relative efficiencies of proteolysis are plotted as the natural logarithim of the initial velocity ratio for cleavage of the Ala-Val and Gly-Leu peptide. As seen in Figure 1, enzymes that prefer the Ala-Val peptide as substrate display a positive value for the natural logarithm of this ratio, while enzymes preferring the Gly-Leu peptide display a negative value. All of the human MMPs display between a 2- and 15-fold preference for the Gly-Leu peptide. In stark contrast to this behavior, ht-d displays about a 7-fold preference for the Ala-Val peptide. To verify that ht-d cleaves this peptide at the Ala-Val bond we have synthesized the same peptide with a dinitrophenyl chromophore replacing the MCA group, and without the DPA quencher, and used this to determine the cleavage products after proteolysis by reversed-phase HPLC and mass spectrometry. For both ht-d and MMP3, the cleavage products result from proteolysis of the Ala-Val bond, as expected (data not shown).

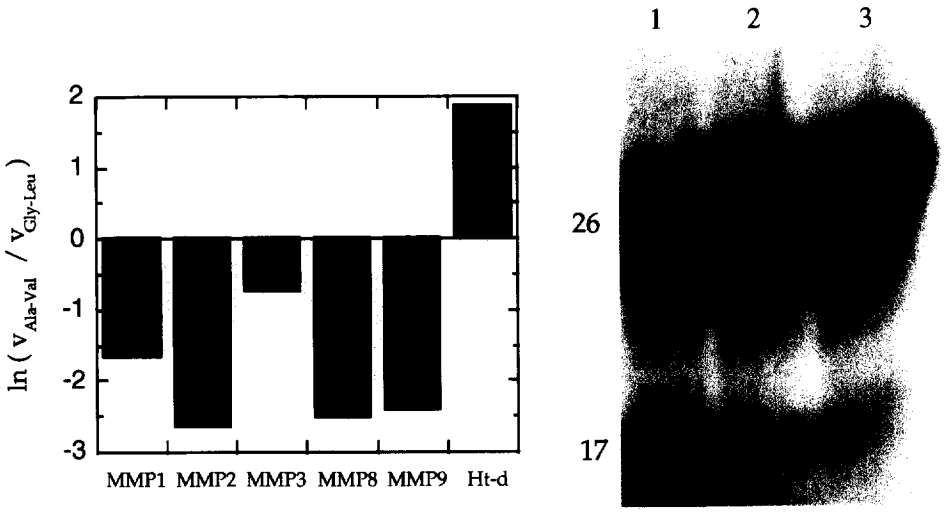

Figure 1 (left). Histogram of the relative effectiveness of MMPs and ht-d for cleavage of the Ala-Val and Gly-Leu peptides. See text for details. Figure 2 (right) Autoradiograph of TNF processing by ht-d. Lane 1, TNF after incubation with ht-d. Lane 2, as in lane 1 but in the presence of 1 μM compound 1. Lane 3, TNF substrate alone.

The data presented in Figure 1 suggest that ht-d is a better TNF-convertase than are the human MMPs. If this is true, one would expect ht-d to also be capable of cleaving the 26 kDa TNF protein to the correct 17 kDa product. To verify this we have prepared ^{3}H-Leu labeled 26 kDa TNF and used this as a substrate for the enzyme. Figure 2 illustrates a typical autoradiograph for the conversion of 26 kDa TNF to 17 kDa TNF by ht-d. This figure further illustrates that the TNF converting activity of ht-d can be inhibited by specific hydroxamic acids. Compound **1** is a hydroxamic acid that potently inhibits MMPs and TNF release from human white blood cells. As seen in Figure 2 it also inhibits the TNF converting activity of ht-d. At a concentration of 1 μM, compound **1** reduced the production of 17 kDa TNF from the 26 kDa precursor to essentially background levels. We note that all of the MMPs studied here are also capable of cleaving 26 kDa TNF to its 17 kDa product, albeit at a significantly reduced rate relative to ht-d. We have not, however, been able to quantify accurately the relative efficiencies of cleavage of the 26 kDa TNF by the different enzymes using this radiographic assay. Therefore, the results presented in figure 2 merely confirm that ht-d is capable of processing 26 kDa TNF, but cannot be used to quantitatively distinguish this activity from that of the MMPs.

Table I. Inhibition profiles of MMPs, ht-d, and TNF release from LPS stimulated human white blood cells for a series of hydroxamic acids.

Compound	TNF release	Enzymes					
		ht-d	MMP1	MMP2	MMP3	MMP8	MMP9
	(IC_{50}, μM)	(IC_{50}, nM) [a]	(K_i, nM)	(K_i, nM)	(K_i, nM)	(K_i, nM)	(K_i, nM)
1	0.12	5	5	<1	<1	<1	2
2	0.30	3	6	2	<1	1	2
3	IA[b]	>500	30	2	63	<1	7
4	IA	>500	>500	32	21	10	14

[a]The inhibitor potency for ht-d is reported as IC_{50} values rather than K_i values because the solubility limits of the substrate precluded accurate determination of a K_m. However, the assays were performed at a substrate concentration well below the substrate K_m. Thus, the IC_{50} values reported here are most likely good approximations of the apparent K_i of the inhibitors. IC_{50} and K_i values represent the mean of 2-3 determinations each. The standard errors associated with these measurements were typically ± 10%. [b]IA = inactive in this assay at concentrations as high as 50 μM.

To further compare the activities of ht-d and the MMPs with respect to TNF conversion we have studied the effects of a series of hydroxamic acids as inhibitors of these various enzymes. Table I summarizes the results of these studies for a representative set of hydroxamic acids as inhibitors of ht-d and the MMPs, and compares them with the inhibition pattern seen for blocking release of TNF from human white blood cells after stimulation with bacterial lipopolysaccaride (LPS). As can be seen from this table, all of the inhibitors used here are potent inhibitors of the various MMPs. However, compounds **3** and **4** are inactive as inhibitors of TNF release from

white blood cells. This result indicates that the structure-activity relationships for MMP and TACE inhibition are divergent and portends the ability to design compounds that can selectively inhibit one or the other class of metalloproteinases. Interestingly, the inhibitor profile for ht-d is best correlated with that for TNF release from white blood cells, and is clearly distinct from that for the MMPs. Blockage of TNF release from cells could result from mechanisms other than inhibition of TACE. For instance, inhibition of TNF mRNA production could result in a similar reduction in TNF release from stimulated cells. The compounds described here, however are all hydroxamic acids and most likely derive their cellular effects through inhibition of the metalloproteinase, TACE. Again, these data indicate that the reprolysin ht-d is a better model of human TACE than are the human MMPs studied here.

Note

While this paper was under review, the complete amino acid sequence of human TACE was reported.[18, 19] The sequence reveals significant structural homology between the catalytic domain of TACE and ht-d, consistent with the biochemical data reported here.

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