Azurocidin induces elastinolytic activity of medullasin

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Abstract: Addition of azurocidin, a protein in granulocytes similar to serine proteases but has no protease activity because of replacement of the active serine residue by glycine, to the incubation mixture containing medullasin induced elastinolytic activity of medullasin. Both medullasin and human leukocyte elastase were already shown to have negligible elastinolytic activity (Aoki, Y. et al. J. Biochem. 114, 122, 1993). Elastinolytic activity of medullasin was induced dose-dependently by the addition of azurocidin. Medullasin activity determined by using apo-ornithine transaminase or casein as substrates or that by N-methoxy-succinyl-(Ala)₂-Pro-Val-p-nitroanilide as substrate remained unchanged when azurocidin was added to the tube containing medullasin. Therefore, azurocidin is considered to cause an appearance of elastinolytic activity of medullasin without affecting the protease activity of it.

Key words: Medullasin; azurocidin; elastinolytic activity; orcein-elastin.

Introduction. Neutral serine proteases in granulocytes such as leukocyte elastase and proteinase-3(AGP7) were reported to degrade elastin fiber and play a significant role in causing emphysema in vivo.1–4 Also cathepsin G was reported to cause potentiation of elastinolytic activity of human leukocyte elastase.5 To the contrary, medullasin, a serine protease in granulocytes which is similar to leukocyte elastase in that its amino acid sequence from N-terminal to Glu²¹⁸ is identical to elastase has negligible elastinolytic activity.6–9 Because the sonicated mixture of human granulocytes showed elastinolytic activity when orcein-elastin was employed as substrate, the origin of elastinolytic activity of granulocytes was investigated by purifying proteases and proteins from granulocytes.

Materials and methods. Materials. Bovine neck ligament was purchased from Tokyo Shibaura Meat Examination Center. Papain (2x crystallized) was purchased from MP Biochemicals, Inc. Collagenase from Clostridium histolyticum (type II), α-chymotrypsin from bovine pancreas (type II), insoluble collagen from bovine Achilles tendon, pancreas elastase (type IV) and orcein (synthetic, certified by the Biological Stain Commission) were obtained from Sigma. Sephadex G-75 and Resource ETH column were purchased from GE Healthcare Bio-Science Corp, NJ, USA. Biorex 70 resin was obtained from Bio-Rad Lab., California, USA.

Methods. Medullasin was purified and further crystallized from human bone marrow cells by the method described.6 Cathepsin G was purified as described earlier.10 Purification of azurocidin. Azurocidin was purified from human bone marrow cells. Bone marrow cells were obtained from ribs resected during operation of patients with lung disease after obtaining informed consent and stored frozen. They were sonicated, centrifuged and extracted with 1 M potassium phosphate buffer (pH 7.0) containing both 1 mM EDTA and 1 mM p-chloromercuribenzoic acid at 37 °C for 30 min. From the precipitate azurocidin was extracted with 5 M sodium acetate buffer (pH 4.0) containing 0.05 M sucrose and 1 mg/ml sodium deoxycholate for 20 min on ice with vigorous agitation and centrifuged. The extract was applied on Sephadex G-75 column equilibrated with 0.5 M sodium acetate buffer (pH 5.0) and 0.1 M NaCl. Fractions containing cathepsin G activity were con-
centrated by ultrafiltration, and dialyzed against 0.05 M potassium phosphate buffer (pH 7.0). They were applied on Biorex 70 column equilibrated with 0.05 M potassium phosphate buffer (pH 7.0) and eluted with linear gradient from 0.05 M potassium phosphate buffer (pH 7.0) to 0.2 M potassium phosphate buffer (pH 7.0) containing 2 M NaCl. Fractions containing cathepsin G activity were collected and concentrated by ultrafiltration. They were applied to Resource ETH column equilibrated with 0.1 M sodium acetate buffer (pH 5.0) containing 2.4 M ammonium sulfate. Azurocidin was eluted by linear gradient from 0.1 M sodium acetate buffer (pH 5.0) containing 2.4 M ammonium sulfate to 0.05 M sodium acetate buffer (pH 5.0). The fractions containing azurocidin were further applied on the same column and eluted by the same method described above to obtain pure azurocidin.

**Determination of protease activity.** Medullasin activity was determined by measuring the degradation of apo-ornithine transaminase as described. The activity was also measured by employing N-methoxysuccinyl-(Ala)$_2$-Pro-Val-p-nitroanilide as substrate. Cathepsin G activity was determined by using N-succinyl-(Ala)$_2$-Pro-Phe-p-nitroanilide as substrate.

Caseinolytic activity of proteases was determined by incubating 5 mg heat-denatured casein in 0.5 ml of 0.1 M Tris-HCl (pH 8.0) in the presence of proteases at 37°C. The reaction was terminated by adding 0.2 ml of 20% TCA and the absorbance at 280 nm in the supernatant was measured to determine caseinolytic activity of proteases.

**Determination of elastinolytic activity of proteases.** Elastin fibers were prepared from bovine neck ligament by the method of Partridge et al. They were stained with orcein by the method of Sachar et al. Elastinolytic activity of proteases were determined by a slightly modified method of Sachar et al. The incubation mixture contained 6 mg orcein-elastin and various amounts of proteases in 0.5 ml of 0.05 M Tris-HCl (pH 8.0). Tubes were incubated at 25°C for 24–48 hours. After centrifugation at 10,000 g for 5 min, the absorbance at 590 nm of the supernatant was measured to determine elastinolytic activity of proteases. When the activity of proteases degrading unstained elastin fibers as substrate was determined, the activity was measured by determining the absorbance at 280 nm in the supernatant.

**Determination of amino acid sequence of proteases and azurocidin.** Proteases such as medullasin and cathepsin G were identified by determining amino acid sequence around N-terminal and measuring the activity against synthetic substrates specific to proteases. Azurocidin was identified by determining amino acid sequence around N-terminal and by applying on polyacrylamide disc gel electrophoresis. Amino acid sequence of proteases and azurocidin was determined by using Shimadzu protein sequencer (PPSQ-21).

**Polyacrylamide disc gel electrophoresis.** Polyacrylamide disc gel electrophoresis was carried out according to the method of Reisfeld et al. Samples dissolved in 40 µl of 10% sucrose solution were layered on the top of the gel. Electrophoresis was performed for 90 min at a constant current of 3 mA per tube. Proteins were detected by staining with amido black and destaining in 7% acetic acid.

**Results. Purity of granulocyte proteases and azurocidin.** Purified medullasin, cathepsin G and azurocidin were applied on polyacrylamide disc gel electrophoresis at pH 4.3. As shown in Fig. 1 both medullasin and azurocidin gave a single band on the gel. Electrophoresis of cathepsin G showed three isozyme bands as described earlier.

**Amino acid sequences around N-terminals.** Results of determination of amino acid sequence around N-terminals of proteases and azurocidin by using Shimadzu protein sequencer (PPSQ-21) are
shown in Table I. Amino acid sequence around N-terminals of medullasin and cathepsin G were same as those reported earlier.\textsuperscript{14, 15} Also that of azurocidin was the same as described previously.\textsuperscript{16}

**Elastinolytic activity of proteases in granulocytes.** Elastinolytic activities of proteases in granulocytes and azurocidin determined by using orcein-elastin as substrate are summarized in Table II. As shown in this table both medullasin and cathepsin G revealed negligible elastinolytic activity. Addition of cathepsin G to the incubation mixture containing medullasin failed to induce elastinolytic activity of it. Also azurocidin which is not a protease showed negligible elastinolytic activity. Elastinolytic activity of these proteases was almost same or below that of papain, chymotrypsin and collagenase which are considered to have no elastinolytic activity.

**Proteolytic activity of proteases in granulocytes measured using unstained elastin fibers as substrate.** When unstained elastin fibers were employed as substrate and proteolytic activity of proteases in granulocytes was determined by measuring the increase in absorbance at 280 nm in the supernatant, an apparent proteolytic activity was observed as shown in Table III.

**Synergistic effect of proteases and azurocidin in granulocytes on the digestion of orcein-elastin as substrate.** Table IV shows the effect of the mixture of proteases and azurocidin on the elastinolytic activity of proteases measured with orcein-elastin as substrate. Addition of azurocidin to the incubation mixture containing cathepsin G induced negligible elastinolytic activity. To the contrary, addition of azurocidin to the incubation mixture containing medullasin induced a significant elastinolytic activity of medullasin. The effect of azurocidin was dose-dependent as shown in this table.

**Effect of azurocidin on the protease activity of medullasin.** Medullasin activity failed to change when azurocidin was added to the incubation mixture containing medullasin. The activity determined by measuring the degradation of apo-ornithine transaminase or by caseinolytic activity was not affected by the addition of azurocidin. Also medullasin activity measured by using N-methoxysuccinyl-(Ala)\textsubscript{2}-Pro-Val-p-nitroanilide as substrate remained unchanged when azurocidin was added to the incubation mixture containing medullasin (Data not shown).

**Discussion.** Medullasin activity in granulocytes was elevated in patients with various inflammatory diseases such as multiple sclerosis\textsuperscript{17, 18} and rheumatoid arthritis.\textsuperscript{19}

The protease induced inflammation by injuring endothelial cells in vessels and causing accumulation of macrophages and granulocytes.\textsuperscript{20} Medullasin also plays important roles in the regulation of both immunity and nonspecific self defense mechanism (biophylaxis). It enhances human natural killer cell activity by causing the maturation of immature natural killer cells,\textsuperscript{21, 22} induces activated killer cells from CD16 positive cells which injure every malignant cell,\textsuperscript{23, 24} and increases DNA synthesis of human lymphocytes.\textsuperscript{25} Medullasin also potentiates cytostatic activity and superoxide production of monocytes.\textsuperscript{26} Certain bacterial toxin such as Shiga toxin 2 exerts its effect through alteration of medullasin activity in granulocytes.\textsuperscript{27}

We have reported that both medullasin and human leukocyte elastase are essentially devoid of elastinolytic activity.\textsuperscript{7} As shown in Results both medullasin and cathepsin G lacked elastinolytic activity, and addition of cathepsin G to the incubation mixture containing medullasin failed to induce elastinolytic activity of the protease. Elastinolytic activity of these proteases were same or below that of proteases such as papain, collagenase or \(\alpha\)-chymotrypsin which are said to be devoid of elastinolytic activity as shown in Results.

To the contrary, several papers reported that human leukocyte elastase showed elastinolytic activity.\textsuperscript{1–8} In these papers, however, elastinolytic activity was determined by measuring the increase in absorbance at 280 nm of the supernatant by employing unstained elastin fibers as substrate or by measuring desmosine released from elastin preparations by ELISA method.\textsuperscript{28} Amino acid compositions of elastin and collagen are quite similar in that they contain large quantities of Gly, Ala, and Pro residues and lack Trp. Therefore, contamination of collagen fibers in the preparation of elastin is not

| Table I. Analysis of amino-acid sequence around N-terminals of proteases and azurocidin |
|-----------------------------------------------|
| Protein     | Amino Acid Sequence                  |
|--------------|--------------------------------------|
| Medullasin   | Ile Val Gly Gly Arg Arg               |
| Cathepsin G  | Ile Ile Gly Gly Arg Glu               |
| Azurocidin   | Ile Val Gly Gly Arg Lys               |

\begin{align*}
\text{Terminals of medullasin and cathepsin G were same as those reported earlier.}^{14, 15} \text{ Also that of azurocidin was the same as described previously.}^{16}
\end{align*}
Table II. Elastinolytic activity of proteases

| Proteases added | Concentration (µg/ml) | Orcein-elastin digested* /24 hour | % of elastase |
|-----------------|-----------------------|----------------------------------|--------------|
| Elastase        | 5                     | 6.72±0.2 mg                      | 100          |
| Medullasin      | 50                    | 16.5±0.4 µg                      | 0.026        |
| Cathepsin G     | 50                    | 16.5±0.4 µg                      | 0.023        |
| Medullasin      | 50                    |                                  |              |
| + Cathepsin G   | 50                    | 17.0±0.4 µg                      | 0.027        |
| Azurocidin      | 50                    | 16.0±0.4 µg                      | 0.024        |
| Papain          | 50                    | 40.5±0.8 µg                      | 0.060        |
| Chymotrypsin    | 50                    | 43.0±1.0 µg                      | 0.064        |
| Collagenase     | 50                    | 40.0±1.1 µg                      | 0.060        |

*Mean±SD (N=3)

Table III. Digestion of unstained elastic fibers by proteases determined by the increase in the absorbance at 280 nm of the supernatant

| Proteases                  | Elastin fibers digested* /24 hours (mg) | % of elastase |
|----------------------------|----------------------------------------|--------------|
| Elastase (2.5 µg/ml)       | 2.24 ± 0.07                            | 100          |
| Medullasin (50 µg/ml)      | 0.97 ± 0.03                            | 2.17         |
| Cathepsin G (50 µg/ml)     | 0.34 ± 0.02                            | 0.77         |

*Mean±SD (n=3)

Table IV. Induction of elastinolytic activity of medullasin by azurocidin

| Proteases or azurocidin added | Orcein elastin digested* /24 hours | % of elastase |
|-------------------------------|-----------------------------------|--------------|
| Elastase (5 µg/ml)            | 6.75 ± 0.2 mg                      | 100          |
| Medullasin (50 µg/ml)         | 16.0 ± 0.4 µg                      | 0.024        |
| Cathepsin G (50 µg/ml)        | 17.5 ± 0.4 µg                      | 0.026        |
| + Azurocidin (50 µg/ml)       | 16.5 ± 0.4 µg                      | 0.025        |
| Azurocidin (50 µg/ml)         | 49.5 ± 0.7 µg                      | 0.147        |
| Medullasin (50 µg/ml)         | 175 ± 20 µg                        | 0.259        |
| + Azurocidin (25 µg/ml)       | 380 ± 30 µg                        | 0.563        |

*Mean±SD (n=3)

easily excluded by determining amino acid composition. Collagen fibers are easily digested by various proteases including medullasin as shown previously. Therefore, it is extremely difficult to conclude that certain proteases digested elastin fibers by determining the increase of absorbance at 280 nm in the supernatant. Also the ELISA method employed in these papers determining the release of desmosine from elastin preparations does not accurately shows elastinolysis, since the ELISA method
employed determining desmosine cross-reacts with pyridinoline released from collagen fibers. Elastin fibers stained with orcein are considered to be the appropriate substrate for the determination of elastinolytic activity of proteases, because the dye, orcein, specifically binds to elastin.\textsuperscript{28, 29} Also in certain papers pure proteases were not employed in the experiments.\textsuperscript{1–3} The report that elastinolytic activity of human leukocyte elastase was potentiated significantly by cathepsin G\textsuperscript{5} and that showing only a small increase\textsuperscript{39} might reflect incomplete purity of proteases, because the presence of azurocidin caused the appearance of elastinolytic activity of medullasin as shown in the present report. Also complete elimination of azurocidin from protease preparations was not so easy in the present study, because it behaves similarly to medullasin and cathepsin G in Sephadex G-75 column, Biorex 70 column and Resource ETH column. Although three to four isozymes of azurocidin were reported previously,\textsuperscript{16, 31} only one band of azurocidin was observed on polyacrylamide disc gel electrophoresis in the present study as shown in Results. Therefore, protein bands apparently considered as isozymes might not be isozymes, but contaminated proteins.

Azurocidin in granulocytes which has similar active center to serine proteases but lacks protease activity is considered to play antimicrobial activity in vivo.\textsuperscript{15, 31} Results described in the present report suggest that azurocidin plays an important role in the digestion of elastin fibers besides its antimicrobial activity. As shown in Results protease activity of medullasin measured by employing apo-ornithine transaminase or casein as substrate and that using N-methoxysuccinyl-(Ala)\textsubscript{2}-Pro-Val-p-nitroanilide as substrate failed to change when azurocidin was added to the incubation mixture. Therefore, the effect of azurocidin is speculated that elastin fibers attached by azurocidin or those attached by medullasin-digested azurocidin may cause alteration of the sensitivity to medullasin, the mechanism of which will be further investigated in the future.

Medullasin plays various roles in biophylaxis as described above. However, the protease itself is devoid of elastinolytic activity. The present results showed that medullasin plays elastinolytic activity in cooperation with azurocidin. Granulocytes contain a large amount of medullasin and azurocidin. Therefore, it is considered that both medullasin and azurocidin released simultaneously from granulocytes play important roles in the development of lung emphysema, vascular injury or inflammation through digestion of elastin.

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