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Comparative Studies on the Cysteine Proteinase Inhibitory Capacity of Mammalian Blood

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Saito, A. and Kitano, Y. *Comparative Studies on the Cysteine Proteinase Inhibitory Capacity of Mammalian Blood.* Tohoku J. Exp. Med., 2000, **190** (2), 83-92 —— The inhibition of cysteine proteinase (papain) by human, bovine, horse, rabbit, guinea pig, rat, hamster, and mouse plasma, and pig, sheep, goat, and dog sera was investigated. The rat and mouse plasma and the pig serum showed such high inhibitory capacity as 813, 380, and 508%, respectively, of the human plasma. On the other hand, the horse, guinea pig, and rabbit plasma indicated 36, 40, and 54%, respectively, of the human plasma. A cysteine proteinase inhibitor (s) was separated from α-macroglobulin by Sephacryl S-300, and the inhibitory activity of all of the mammalian plasma/sera except for the guinea pig plasma appeared at around the 50-150-kDa region. The guinea pig plasma exhibited well-resolved two peaks of 100 and 200 kDa. ———— cysteine proteinase inhibitor; mammalian plasma/serum; papain inhibitory capacity

Mammalian blood is capable of interfering with cysteine proteinases as well as serine proteinases (Järvinen 1976; Sasaki et al. 1977; Travis and Salvesen 1983). Cysteine proteinases, represented by papain and ficin, are inhibited by the two groups of plasma proteins: Cystatin and α-macroglobulin families (Barrett et al. 1982; Salvesen et al. 1986; Müller-Esterl 1987). The cystatin family dose not inhibit proteinases other than cysteine proteinases, but α-macroglobulin interacts with not only cysteine proteinases but also serine, aspartic and metallo proteinases (Barrett and Starkey 1973). The mechanism by which the proteinase is inhibited is different between the two inhibitor groups, and the structure of cystatin is distinct from that of α-macroglobulin (Barrett and Starkey 1973; Barrett et al. 1986; Müller-Esterl et al. 1986; Barrett 1987; Turk and Bode 1991). In contrast to plasma serine proteinase inhibitors (serpin), most of which have their target proteinases and inactivate them effectively, the in vivo roles of plasma cysteine

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proteinase inhibitors (CPIs) have not been fully understood, since cysteine proteinases or their zymogens have not been found in the blood. Cysteine proteinases such as cathepsin B, H, and L are localized in the lysozyme and would not be released unless the cells are impaired. CPI might work, therefore, at the site of tissue injury or against exogenous cysteine proteinases secreted by bacteria or parasites (Travis and Salvesen 1983). Participation of endogenous CPIs as well as cathepsins B, H, and L in the prognosis on cancer has been recently reviewed (Kos and Lah 1998).

Plasma CPI, now designated as cystatin type 3 (Barrett et al. 1986), was found to be identical to kininogen, a precursor of a vasoactive peptide such as bradykinin, based on the nucleotide and the amino acid sequence analyses (Ohkubo et al. 1984). The three homologous domains were confirmed in the heavy chain of low molecular weight kininogen (LK), although two of them were responsible for the inhibition of papain and cathepsin L (Salvesen et al. 1986; Müller-Esterl 1987). Interestingly, the highest inhibitory activity was exhibited by the isolated heavy chain rather than the intact forms of LK and high molecular weight kininogen (HK) (Higashiyama et al. 1986). Not only human but also bovine and rat kininogen have been investigated in great detail at the molecular level (Sueyoshi et al. 1987; Enjoji et al. 1988). In particular, rats have the characteristic kininogen, an acute phase protein termed T-kininogen (Okamoto and Greenbaum 1983), in addition to HK and LK, indicating that the physiological mechanism of CPI might be somewhat different between the human being and rat.

Takahara et al. (1983) investigated the trypsin-inhibiting capacity of 12 mammalian sera and found several-fold difference in their activities. They also demonstrated gel filtration of the rodent sera to distinguish between α-1-antitrypsin and α-macroglobulin activities. We have extended the lines of the research to CPI, since detailed studies on this inhibitor have been limited to human, bovine, and rat blood, in spite of the use of various mammals as model animals for diverse physiological and pathological conditions associated with human diseases. In this paper we compared the papain-inhibiting capacity of 12 mammalian plasma/sera and analyzed the molecules responsible for the inhibitory activity by gel chromatography. We observed marked species difference in the inhibitory capacity as well as elution profile of CPI from a column of Sephacryl S-300.

Materials and Methods

Materials

Bovine (*Bos taurus*) and horse (*Equus caballus*) plasma and sheep (*Ovis aries*), goat (*Capra hircus*), pig (*Sus scrofa*), and dog (*Canis familiaris*) sera were products of Irvine Scientific (Santa Ana, CA, USA). Human (*Homo sapiens*) plasma was obtained from a healthy adult. Rat (*Rattus rattus*, SD), mouse (*Mus*

musculus, ICR), hamster (*Mesacricetus auratus*), guinea pig (*Cavia porcellus*), and rabbit (*Oryctolagus cuniculus*) blood were withdrawn from an artery or the heart of healthy individual animals. EDTA was used as anticoagulant to 1 mM at final concentration. Papain, porcine trypsin, and soybean trypsin inhibitor were products of Sigma Chemical Co. (St. Louis, MO, USA), and Remazol Brilliant Blue hide powder was from Nacalai Tesque (Kyoto). Sephacryl S-300 and marker proteins for gel chromatography calibration were obtained from Amersham Pharmacia Biotech (Tokyo). The active site of papain was titrated with E-64 (Barrett et al. 1982).

Inhibition of papain

Various amounts of mammalian plasma/sera (0.2–20 μl) were preincubated with 0.2 ml of 50 mM Tris-HCl, pH 8.0, containing 250 mM methylamine for 30 minutes at 56°C (Minakata et al. 1982). Papain (0.195 μg) and 50 μl of the activating reagent consisting of 50 mM cysteine and 20 mM of EDTA were added to the above solution and the mixture was kept at 56°C for 15 minutes. Hide powder suspension (0.25 ml) was added and agitated for 10 minutes at room temperature (Barrett 1981). The reaction was stopped with 0.25 ml of 30% acetic acid, and the mixture was centrifuged; an absorbance at 595 nm of the supernatant was then measured. A linear portion in the plot of the residual activity versus the amount of the plasma/sera was extrapolated to zero activity, and the amount of papain inhibited by a given amount of the plasma/sera was calculated. Protein concentration of the plasma/sera was measured according to the Lowry-Folin method (Lowry et al. 1951) with bovine serum albumin as standard.

Gel chromatography on Sephacryl S-300

The sample (0.5 ml of plasma/sera) was subjected to gel filtration on Sephacryl S-300 (1.5×90 cm) preequilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. The column was run with the same buffer at a flow rate of 23 ml/hour and fractionated by 1 ml. Absorbance at 280 nm of the fractions was measured, and the papain inhibitory activity of an aliquot of the fraction was assayed as previously described. Trypsin-protein amidase (TPA) activity, characteristic of α-macroglobulin as well as murinoglobulin, was measured as previously described (Saito and Sinohara 1985a, b) based on the method of Ganrot (1966).

Results

CPI activity of the mammalian blood

Mammalian plasma contains a significant amount of α-macroglobulin, which interferes with the measurement of the papain inhibitory activity of CPI. To minimize the effect of α-macroglobulin, the plasma/serum was preincubated with 250 mM methylamine (Minakata et al. 1982). α-Macroglobulin of most of the

TABLE 1. *Papain inhibitory capacity of mammalian plasma and sera*

	Papain inhibitory capacity[a]		Protein concentration (mg/ml)
	(μg/ml)	(%)	
Human plasma	32	100	70.5
Bovine plasma	58	180	59.5
Horse plasma	12	36	72.4
Rabbit plasma	17	54	63.3
Guinea pig plasma	15	46	62.9
Hamster plasma	63	198	95.2
Rat plasma	268	837	84.8
Mouse plasma	122	380	62.9
Pig plasma	163	508	111.9
Sheep serum	312	98	80.0
Goat serum	34	105	96.2
Dog serum	24	74	80.0

[a]The inhibitory activity is shown by the amount of active papain inhibited by 1 ml of the plasma or serum. The relative inhibitory capacity to the human plasma is also indicated.

species is irreversibly inactivated with a nucleophile such as methylamine (Swenson and Howard 1979). Table 1 shows the papain inhibitory capacity of 12 mammals in the presence of methylamine. However, α-macroglobulin which was resistant to methylamine treatment (Motoshima et al. 1988; Tamamizu et al. 1989) may participate in the papain inhibitory activity and increase the apparent activity of CPI, since papain entrapped in α-macroglobulin could not react with a high molecular weight chromogenic substrate, hide powder (Barrett 1981). Therefore, the CPI level as summarized in Table 1 might be somewhat higher than that for CPI alone in some of the species. The papain inhibitory capacity of the mammals showed significant species difference, from 35% of the human plasma for the horse to 837% for the rat. The protein concentration of the plasma/serum was in the range of 60–96 mg/ml except for the pig serum (112 mg/ml), and the papain inhibitory capacity was not necessarily proportional to the protein concentration. For example, the protein concentration of the mouse plasma was lower than that of the human, but the inhibitory capacity of the mouse was 3.8 times that of human plasma.

Separation of CPI and α-macroglobulin activities by gel filtration

Because the participation of α-macroglobulin in the papain inhibitory capacity as listed in Table 1 was not clear in some of the species, the separation of α-macroglobulin from CPI was carried out by Sephacryl S-300. Fig. 1 shows the chromatograms of the 9 mammals whose papain inhibitory activities were in the range similar to the human range, and Fig. 2 shows the elution pattern of the rat,

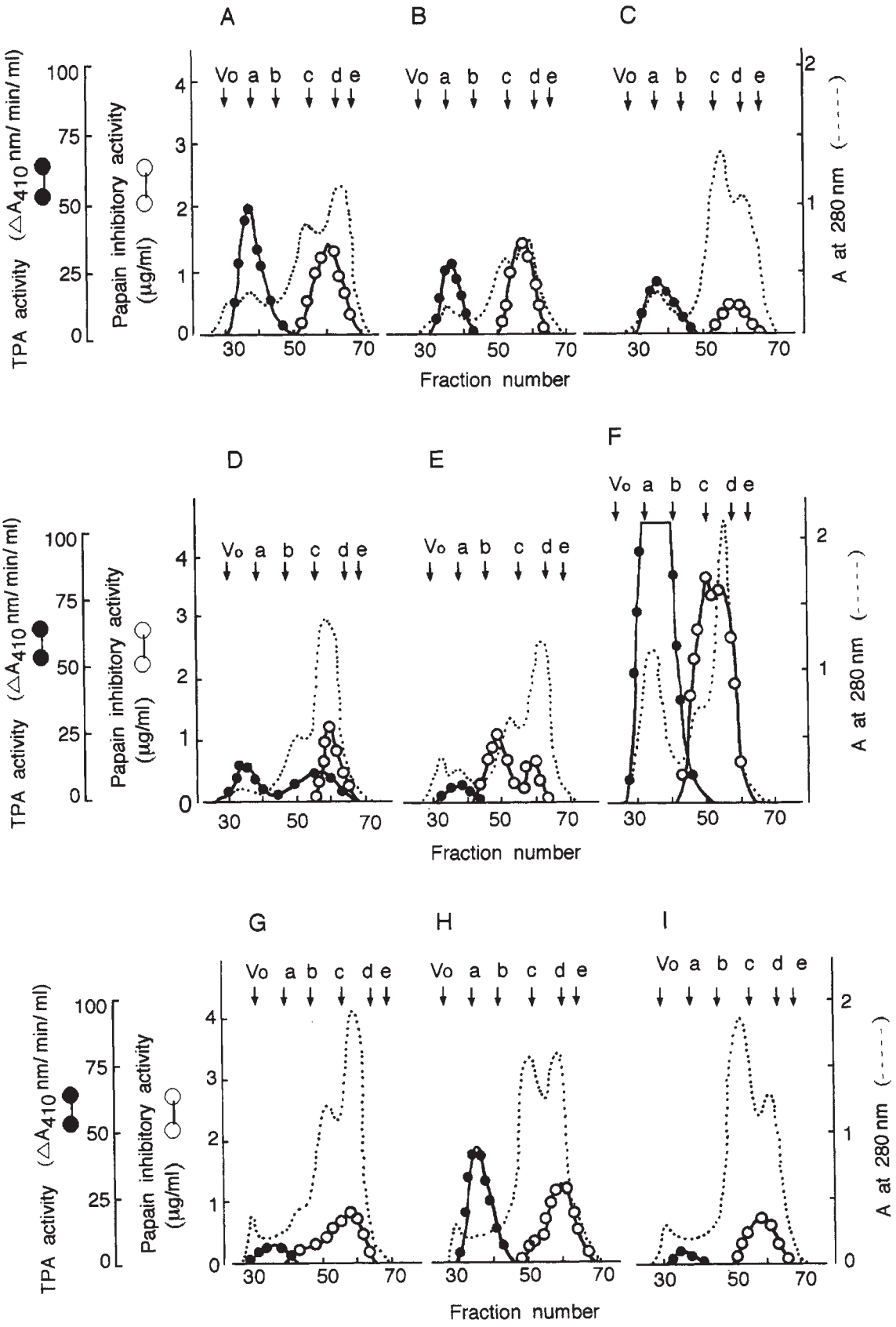
mouse, and pig, whose inhibitory activities were several-fold higher than that for human beings. α-Macroglobulin activity, which appeared at the void fraction, was clearly separated from the papain inhibitory activity, and the degree of papain inhibition was almost parallel to the values in Table 1. Human plasma contains two kinds of CPI: HK and LK, whose molecular weights are 108 kDa and 78 kDa, respectively (Kato et al. 1981). However, the human plasma (Fig. 1A) showed a broad peak of papain inhibition ranging from 50 kDa to 150 kDa. Cystatin C, another type of CPI with 13 kDa, occurs in many biological fluids such as seminal plasma, cerebrospinal fluid and plasma but the concentration in normal plasma is less than 1% of kininogen (Barrett et al. 1986). Sasaki et al. (1977) also found a single peak of CPI in human plasma by gel filtration on Sephadex G-200. Bovine and rat blood contain plural forms of kininogen having different molecular weight (Kato et al. 1981; Enjoji et al. 1988; Motoshima et al. 1988), but they were indistinguishable under these experimental conditions (Figs. 1B and 2B). It is apparent that guinea pig plasma showed two distinct peaks responsible for papain inhibition, one being 200 kDa and the other 100 kDa (Fig. 1E). Shoulders were observed in the papain inhibitory activity of the sheep and goat sera (Figs. 1G and H), but not of other mammals.

α-Macroglobulin activity of rat plasma showed two peaks (Fig. 2B), which are due to α-macroglobulin and murinoglobulin (α-1-inhibitor-3), respectively (Saito and Sinohara 1985b). Other rodents such as mouse (Saito and Sinohara 1985a), guinea pig (Suzuki and Sinohara 1986), and hamster (Miyake et al. 1993) also contain respective murinoglobulins, but their amounts were not so copious as rat murinoglobulin. Mammals other than rodents exhibited a single peak of TPA activity, indicating that high concentration of murinoglobulin is unique to rat plasma. Rabbit plasma showed TPA activity in a small molecular region in addition to α-macroglobulin (Fig. 1D). We have found the activity due to the S-1 isoform of α-1-antitrypsin, whose molecular weight was around 48 kDa (Saito and Sinohara 1988).

Discussion

Takahara et al. (1983) investigated the trypsin inhibitory capacity of the 12 mammalian sera and found the relative capacity of mouse, rat, and guinea pig sera were 442, 278, and 301%, respectively, of human serum. The serum trypsin inhibitory capacity of the other mammals was over the range (84–152%) similar to that of the human serum. Therefore, the mouse and rat are the mammals whose plasma exhibited the highest inhibitory capacity against the cysteine proteinase (papain) as well as the serine proteinase (trypsin). The horse and guinea pig showed the smallest inhibitory capacity against papain, but they inhibited trypsin more effectively than the human sample.

The exact correlation between plasma CPI and kininogen in various mammals has not been clarified, but Karlsrud et al. (1996) demonstrated that the


A
B
C
D
E
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G
H
I
Vo a b c d e
TPA activity ($\triangle A_{410}$ nm/min/ml)
Papain inhibitory activity (μg/ml)
A at 280 nm (- - - -)
Fraction number
100
75
50
25
0
4
3
2
1
0
2
1
30
50
70

Fig. 1.

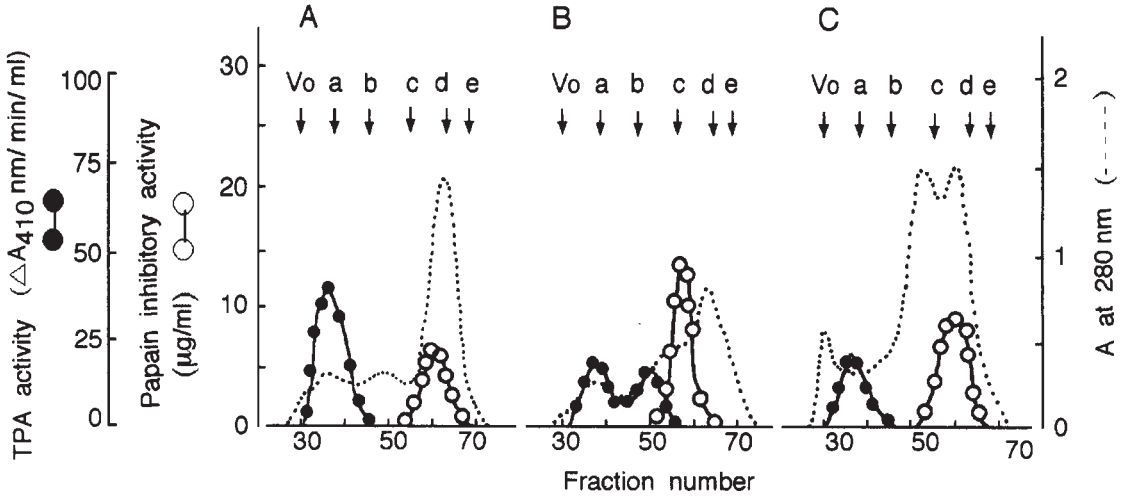

Fig. 2. Elution profile of mouse plasma (A), rat plasma (B), and pig serum (C) from a column of Sephacryl S-300. The illustrations of the chromatograms are the same as those in the legend to Fig. 1 except for the scale of papain inhibitory activity.

amount of human kininogen could be estimated by measuring the papain inhibitory activity of acid-treated plasma in the presence of methylamine. By assuming a 1 : 1 stoichiometry between papain and kininogen, they evaluated the CPI level of normal plasma to be 3.2 μM.

Yamamoto (1987) reported that the molecular weights of guinea pig HK and LK were 100 kDa and 60 kDa, respectively. Yoshida et al. (1989) isolated HK and LK from guinea pig plasma with Sephadex G-150. The LK fraction was further separated into two forms, LK1 and LK2, by an Affi-Gel Blue® column (Nippon Bio-Red Laboratories, Tokyo). The molecular weights of purified HK, LK1, and LK2 were 80 kDa, 65 kDa, and 60 kDa, respectively, although the difference in the estimated molecular weight of HK between the two groups was not clear. Guinea pig plasma CPI that appeared at 200 kDa by gel filtration (Fig. 1E) was not in accord with the HK previously obtained and might be due to the unique structure of the molecule or the aggregation during chromatography. Tani et al. (1987) isolated HK from pig plasma by successive chromatographies on DEAE-Sephadex A-50 and Zn-chelate Sepharose 4B. The yield of HK was 31 mg from 1 liter of plasma with a recovery of 75%. The molecular weight of pig HK was 99 kDa under reducing conditions in SDS-PAGE, but the same sample showed

Fig. 1. Elution profiles of mammalian plasmas and sera from a column of Sephacryl S-300. A: human plasma B: bovine plasma C: horse plasma D: rabbit plasma E: guinea pig plasma F: hamster plasma G: sheep serum H: goat serum I: dog serum. The chromatographies were carried out as described in the text using 0.5 ml of the sample. Papain inhibitory activity (○) and TPA activity (●) were measured as in the text. Broken lines denote UV absorbance at 280 nm. The standard proteins for the calibration of the column are indicated at the top of each figure; V_0, 2000 kDa (blue dextran); *a*, 669 kDa (thyroglobulin); *b*, 440 kDa (ferritin); *c*, 158 kDa (aldolase); *d*, 67 kDa (albumin); *e*, 43 kDa (ovalbumin).

200 kDa under non-reducing conditions. Sugo et al. (1981) purified 150 mg of horse HK from 8.5 liter of the plasma and found that horse HK was similar to bovine HK in molecular weight (78 kDa) and amino acid composition, but the characterization of HK as CPI has not been investigated. Recently, Mashiko and Takahashi (1997) noted the two types of kininogen from dog plasma, one being plasma kallikrein susceptible and the other not, and purified HK using Zinc-chelating Sepharose 6B and ion-exchange chromatographies. The molecular weight of dog HK was 125 kDa and inhibited papain and ficin but did not inhibit bromelain.

The results described here show that the papain inhibitory capacity of mammalian plasma/serum is considerably different from species to species with respect to not only the degree of the inhibition but also the kind of molecules responsible for the inhibition. These differences reflect the presence of a variety of kininogen molecules and their physiological functions in the mammalian blood. Therefore, one should be careful in applying data obtained using the blood of the experimental animals to human subjects (Takahara et al. 1983).

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