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ANTI-PLATELET EFFECTS OF ENZYMATIC HYDROLYSATES OF COLLAGEN AND COLLAGEN-RELATED PEPTIDES

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Backgrounds and Objectives:

Laudano and Doolittle[1] reported that such synthetic peptides as Gly-Pro-Arg corresponding to the N-terminal tripeptide of fibrin α chain[2], Gly-Pro-Arg-Pro and Gly-Pro-Arg-Sar (Sar stands for sarcosine) inhibited fibrin polymerization. Plow and Marguerie[3] and Plow *et al.*[4] reported that Gly-Pro-Arg-Pro inhibited the interaction of fibrinogen with its platelet receptor, resulting in inhibition of platelet aggregation. We noticed that the sequence of Gly-Pro-Arg exists in interstitial collagen; eight units of the sequence Gly-Pro-Arg in each α 1 chain of rat and calf skin collagens[5]. The present paper describes (1) inhibition of fibrinogen/thrombin clotting by enzymatic hydrolysate of collagen and peptides containing Gly-Pro-Arg, (2) inhibition of *in vitro* platelet aggregation by enzymatic hydrolysate of collagen and the peptides containing Gly-Pro-Arg and (3) effects of Gly-Pro-Arg and collagen hydrolysate on endotoxin-induced DIC in rats.

Materials and Methods:

Materials: Thrombin (from bovine plasma), fibrinogen (from bovine plasma), collagen (from bovine achilles tendon), collagenase (from *Clostridium histolyticum*), thermolysin, Gly-Pro-Arg-Pro, Gly-Pro-Hyp were purchased from Sigma. Pepsin and trypsin were purchased from Boehringer-Mannhein Biochemica.

Syntheses of peptides: The peptides such as Gly-Pro-Arg, Gly-Pro-Arg-Gly, Gly-Pro-Arg-Gly-Pro, Gly-Pro-Arg-Pro-Pro, Gly-Pro-Arg-Pro- Pro-Pro, Sar-Pro-Arg, Gly-Ala-Arg, Gly-Pro-Lys and Ala-Gly-Pro-Arg were synthesized with a solid-phase peptide synthesizer (Applied Biosystems).

Hydrolysate of porcine skin collagen by proteinases: Collagen was hydrolyzed by collagenase, thermolysin, trypsin and pepsin. Each hydrolysate was boiled, ultrafiltered and desalted as previously described[6].

Fibrinogen/thrombin clotting assay: The clotting time was determined at 37℃ after addition of bovine thrombin to bovine fibrinogen solutions containing the synthetic peptides or collagen hydrolysates.

***Platelet aggregation* in vitro:** Rat and human PRPs were obtained and adjusted to about 300,000 platelets/$\mu\ell$ with PPP with an automatic particle counter (Erma, Co. Ltd.). Platelet aggregation was measured by the turbidimetric method[7] with an automatic aggregometer (Mebanics, Inc.).

***Platelet adhesion* in vitro:** Rat blood was passed through a collagen-coated bead column (ISK, Co., Ltd.) with or without Gly-Pro-Arg. Residual platelets in the blood passed through the column were counted with the automatic particle counter.

Anti-platelet effect of intravenously administered Gly-Pro-Arg on DIC model: Experimental DIC (disseminated intravascular coagulation) in rat was induced by repetitive intravenous administrations of endotoxin by the method described by Yoshikawa *et al.*[8] with a minor modification and was assessed by the platelet counts.

Anti-platelet effect of orally administered Gly-Pro-Arg and collagen hydrolysates on DIC model: DIC was moderately induced by intravenous administration of endotoxin by the method described by Teng *et al.*[9] with a minor modification and was assessed by the platelet counts. A commercial EPA remedy, Epadel™ (Mochida Pharmaceutical Co.), was used as a control.

Results and Discussions:

The peptides containing Gly-Pro-Arg and hydrolysates of collagen by collagenase, thermolysin and trypsin inhibit fibrinogen/thrombin clotting (data not shown). Gly-Pro-Arg, Gly-Pro-Arg-containing peptides and the collagen hydrolysate inhibited aggregation of the rat and human platelets (Figs. 1 and 2). Other Gly-Pro-Arg analogues in which a single amino acid is replaced from Gly-Pro-Arg, such as Sar-Pro-Arg, Gly-Pro-Lys and Gly-Ala-Arg or Ala-Gly-Pro-Arg had no inhibitory activity on human platelet aggregation (data not shown). These results suggest that the sequence Gly-Pro-Arg is prerequisite for inhibition of human platelet aggregation and that extension of the peptide chain toward its N terminus deprives Gly-Pro-Arg of its inhibitory activity. In the DIC-induced rats, the platelet markedly decreased, whereas i.v. administration of Gly-Pro-Arg inhibited the decrease in platelet counts (Fig. 3). Similarly, oral administration of the collagen hydrolysates by collagenase or thermolysin inhibited the decrease in the platelet

counts in DIC-induced rats. Suppression of DIC by the collagen hydrolysates by collagenase was almost equal to that by EPA (Fig. 4). Passing through the collagen-bead column, it was shown that Gly-Pro-Arg prevented adhesion of platelet from 58.4±5.9 % (n=9) to 42.1±9.8 % (n=6) ($p<0.01$). Since inhibition of blood hemostasis in DIC syndrome is caused by various mechanisms such as inhibition of fibrinogen/thrombin clotting, platelet aggregation and platelet adhesion, Gly-Pro-Arg and the collagen hydrolysates may effectively exert the anti-platelet and anti-thrombosis actions *in vivo*.

Conclusions:

We found the following; (1) Gly-Pro-Arg-containing peptides and hydrolysates of collagen by collagenase, thermolysin and trypsin inhibited fibrinogen/thrombin clotting; (2) Gly-Pro-Arg-containing peptides and hydrolysates of collagen by thermolysin inhibited aggregation of rat and human platelet and; (3) intravenous and oral administration of Gly-Pro-Arg and enzymatic hydrolysate of collagen suppressed decreases in platelet counts of endotoxin-induced-DIC rats. Collagen has generally been regarded as a potent inducer of platelet aggregation. However, these findings suggest that hydrolysates of collagen and collagen-related peptides prevent platelet aggregation.

Pertinent literature:

1. Laudano, A. P., and Doolittle, R. F., *Biochemistry* **19**, 1013-1019, 1980.
2. Cottrell, B. A., and Dolittle, R. F., *Biochem. Biophys. Acta* **453**, 426-432, 1976.
3. Plow, E. F., and Marguerie, G., *Proc. Natl. Acad. Sci.* (USA) **79**, 3711-3715, 1982.
4. Plow, E. F., and Marguerie, G., and Ginsberg, M., *Biochem. Pharmacol.* **36**, 4035-4040, 1987.
5. Hulmes, D. J. S., Miller, A., Parry, D. A., Piez, K. A., and Wood-head-Galloway, J., *J. Mol. Biol.* **79**, 137-148 , 1973.
6. Maruyama, S., Nonaka, I., and Tanaka, H., *Biochim. Biophys. Acta* **1164**, 215-218, 1993.
7. Born, G. V., *Nature (London)* **194**, 927-929, 1962.
8. Yoshikawa, T., Furukawa, Y., Murakami, M., Takemura, S., and Kondo, M., *Res. Exp. Med. (Berlin)* **179**, 223-228, 1981.
9. Teng, C.M., Ko, F. N., Wang, J. P., Lin, C. N., Wu, T. S., and Huang, T. F., *J. Pharm. Pharmacol.* **43**, 667-669, 1991.

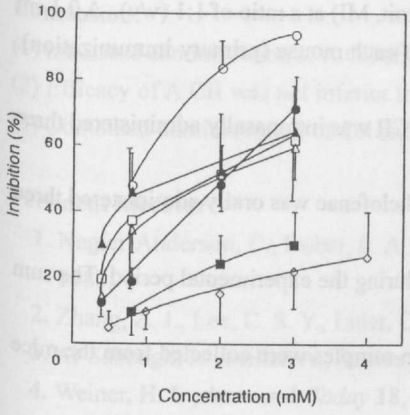

Fig. 1 Inhibition of rat platelet aggregation by synthetic peptides and a collagen hydrolysate
□,Gly-Pro-Arg ○,Gly-Pro-Arg-Gly ▲,Gly-Pro-Arg-Gly-Pro ●,Gly-Pro-Arg-Pro ■,Gly-Pro-Arg-Pro-Pro △,Gly-Pro-Arg-Pro-Pro-Pro ◇,Collagen hydrolysate by thermolysin. The concentration of the hydrolysate is represented by the concentration of glycine in a 6M HCl hydrolysate of the sample. The final concentration of ADP was 100 μM.

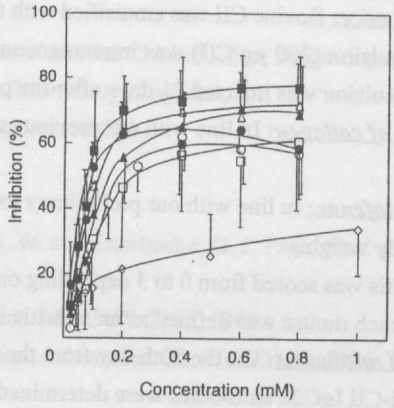

Fig. 2 Inhibition of human platelet aggregation by synthetic peptides and by a collagen hydrolysate
See the footnote of Fig. 1. The final concentration of ADP was 10 μM.

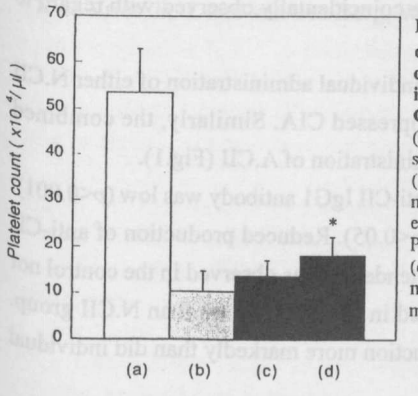

Fig. 3 Inhibition of the decrease in platelet count in the endotoxin-induced-DIC rat by intravenously administered Gly-Pro-Arg
(a), injection of physiological saline every 30 min for 4 h (n=9); (b), injection of endotoxin at 100 mg/kg (n=6); (c), injection of endotoxin 100 mg/kg, and Gly-Pro-Arg at 12.5 mg/kg (n=10); (d), injection of endotoxin at 100 mg/kg, and Gly-Pro-Arg at 25.0 mg/kg (n=6). *$p<0.01$

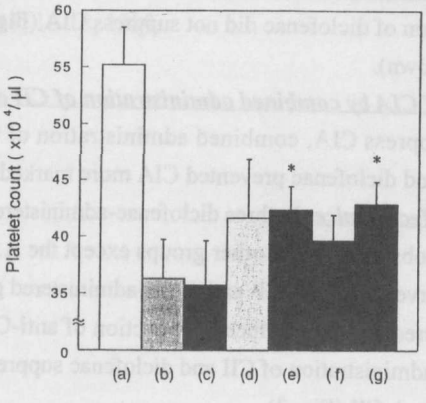

Fig. 4 Inhibition of the decrease in platelet count in the endotoxin-induced-DIC rat by orally administered Gly-Pro-Arg and collagen hydrolysates
(a), injection of physiological saline (n=9); (b), injection of endotoxin at 25.0 mg/kg (n=10); (c), Injection of endotoxin at 25.0 mg/kg and Gly-Pro-Arg at 250 mg/kg (n=8); (d), Injection of endotoxin at 25.0 mg/kg and Gly-Pro-Arg at 500 mg/kg (n=4); (e), injection of endotoxin at 25.0 mg/kg and the collagen hydrolysate by collagenase at 44.7 mg/kg (n=8); (f), injection of endotoxin at 25.0 mg/kg and the collagen hydrolysate by thermolysin at 13.3 mg/kg (n=8); (g), injection of endotoxin at 25.0 mg/kg and a commercial EPA remedy Epadel at 110 mg/kg (n=8). *$p<0.05$