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DIFFERENT NH_2-TERMINAL FORM WITH 12 ADDITIONAL RESIDUES OF α_2-PLASMIN INHIBITOR FROM HUMAN PLASMA AND CULTURE MEDIA OF HEP G2 CELLS

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SUMMARY: α_2-Plasmin inhibitor (α_2PI) was purified from plasma or from the culture media of Hep G2 cells by one-step immunoaffinity chromatography procedure. Majority of α_2PI purified from plasma was the previously recognized plasma α_2PI with NH_2-terminal Asn (Asn-α_2PI), whereas majority of α_2PI purified from the culture media was retaining the "pro" peptide of 12 amino acids with NH_2-terminal Met (Met-α_2PI). When Hep G2 cells were cultured in serum-free media, the α_2PI secreted to the media was totally in a form of Met-α_2PI. Incubation of Met-α_2PI with human plasma induced the complete conversion of Met-α_2PI to Asn-α_2PI. The results indicate that α_2PI is synthesized and secreted from liver cells as Met-α_2PI and Met-α_2PI is converted to Asn-α_2PI by proteolytic cleavage in plasma during the circulation.

We have expressed α_2-plasmin inhibitor (α_2PI) in baby hamster kidney (BHK) cells and found that α_2PI expressed retained a part (12 amino acids) of the leader sequence at the NH_2-terminal end (1). We then suggested that the leader sequence was composed of pre-(signal) peptide of 27 amino acids and propeptide of 12 amino acids (1), and BHK cells may have failed to recognize the Pro-Asn peptide bond between the propeptide and mature α_2PI although the leader sequence was cleaved by signal peptidase. Subsequently, Bangert et al. (2) and Enghild et al. (3) found that the "pro"-type of α_2PI ("pro"-α_2PI) was present in the purified α_2PI prepared from human blood plasma in an amount of 30-50% of the total α_2PI. Then, the questions arisen were whether the processing from "pro"-α_2PI to mature α_2PI took place in the α_2PI-producing liver cells or in the circulation or even *in vitro* by degradation of

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the protein during the purification procedures. To answer the questions, we have purified α_2PI from plasma or culture media of α_2PI-producing human liver cell line Hep G2 (4), and analyzed the NH_2-terminal sequences of these α_2PIs. For the purification, we used one-step immunoaffinity chromatography procedure (5, 6) instead of multi-step conventional purification procedures (6) to avoid possible *in vitro* degradation of the protein.

MATERIALS AND METHODS

Cell culture. Hep G2 cells were maintained in Dulbecco's modified Eagle's minimal essential medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (FCS) (General Scientific Laboratories, CA), 2mM glutamine (GIBCO), 100U/ml penicillin and 100μg/ml streptomycin (GIBCO). Hep G2 cells were also maintained in serum-free medium, ITES-eRDF (insulin 9μg/ml, transferrin 10μg/ml, ethanolamine 10μM, sodium selenite 20nM) (7).

Immunoaffinity purification of human α_2PI. Venous blood was drawn from a normal volunteer into plastic tubes containing 10% volume of 3.8% sodium citrate and centrifuged to separate plasma. α_2PI in plasma or in the culture media of Hep G2 cells, was purified by a one-step immunoaffinity chromatography procedure as already described (5). Before applied onto the column, aprotinin powder (final concentration 20U/ml) (Boeringer Mannheim, Mannheim, Germany) was added to plasma or culture media. Monoclonal antibody JTPI-1 (8), used in the immunoaffinity column procedure did not bind to either α_2PI antigen in FCS or plasmin-α_2PI complex which may form in the presence of FCS. The α_2PI concentration was immunologically measured by the enzyme-linked immunosorbent assay as previously described (8) using an α_2PI assay kit (α_2PI Teijin, EIA-B, Teijin, Tokyo).

Incubation of α_2PI purified from the serum-free culture media of Hep G2 cells with α_2PI-deficient human plasma. α_2PI-deficient human plasma was obtained by running plasma through a column for immunoaffinity chromatography of α_2PI. α_2PI purified from the serum-free culture media of Hep G2 cells was incubated at 60μg/ml with α_2PI-deficient human plasma or 0.01M phosphate buffer, pH 7.4, containing 0.15M NaCl (PBS) plus 10% volume of 3.8% sodium citrate.

SDS-PAGE analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a slab gel was carried out using a gradient separating gel of acrylamide (10% to 20%), 0.1% SDS and a stacking gel of 4% acrylamide containing 0.1% SDS according to the method of Laemmli (9). 2μg of each preparation of α_2PI was applied on the well and the proteins were stained with Coomassie blue.

NH_2-terminal-sequence analysis. α_2PIs immunopurified from three independent batches of plasma or culture media were separately combined, and 14μg of protein of each preparation were analyzed by SDS-PAGE followed by electroblotting onto polyvinylidene difluoride membrane (Immobilon) (Millipore Corp, MA) in 3-[cyclohexylamino]-1- propanesulfonic acid buffer as described previously (10). Blotted proteins were detected by staining with Coomassie Blue, and the bands were cutted and analyzed directly for the NH_2-terminal amino acid sequence by using an automatic protein sequencer (477A, Applied Biosystems, Foster City, CA).

Reagents. Unless otherwise indicated, reagents were purchased from Wako Pure Chemical Industries, Osaka, Japan.

RESULTS AND DISCUSSION

Each preparation of immunopurified α_2PI was analyzed by SDS-PAGE with reduction. While plasma α_2PI showed a major band of 67kDa and a minor band of 65kDa

(non-plasminogen-binding form) as reported (5), Hep G2 α_2PI showed a broad band of 67-70kDa (Fig.1), suggesting that Hep G2 α_2PI was larger in size than plasma α_2PI.

NH_2-terminal-sequence analysis. Analysis of the NH_2-terminal amino acid sequence of each immunopurified protein revealed two parallel sequences (Table 1). One (Sequence A) was identical to the reported sequence for plasma α_2PI starting with Asn at position 40 downstream from the NH_2-terminus of pre-α_2PI (11, 12, 13). By subtraction, a second sequence (sequence B) was identified and shown to start with the Met at position 28 downstream from the NH_2-terminus of pre-α_2PI. The results indicate the presence of two forms of α_2PI; one is the previously reported form of the inhibitor with NH_2-terminal Asn (sequence A) and the other is the one with 12 more NH_2-terminal residues (sequence B) which we have previously called "pro"-peptide (1). The total amounts of both forms were calculated as the means of the yields of the five first amino-acid residues of each sequence. The relative amounts of different forms of the inhibitor in plasma (sequence A: 66%, sequence B: 34%, Table 1 (a)) were well compatible with the values reported by Bangert et al. (62% vs. 38%) (2) or Enghild et al. (50-67% vs. 33-50%) (3). In contrast, in the conditioned media of Hep G2 cells containing FCS, α_2PI with the "pro"-peptide (sequence B) was a major form of the inhibitor (sequence A: 29%, sequence B: 71%, Table 1 (b)). When Hep G2 cells were cultured in serum-free media, the "pro"-peptide of 12 amino acids was completely retained at the NH_2-terminus of the secreted α_2PI (sequence B: 100%, Table 1 (c)). These results indicate that serum (FCS) present in the conditioned media was responsible for the cleavage of the "pro"-peptide. Furthermore, incubation of this "pro"-peptide retaining form of

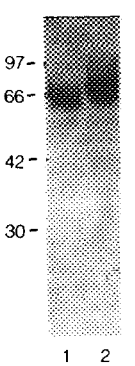

Fig. 1. Analysis of α_2PI purified from plasma or the serum-free culture media of Hep G2 cells by SDS-PAGE.

Lane 1, plasma α_2PI (2μg); Lane 2, Hep G2 α_2PI (2μg). Molecular mass markers are given along the left margin. Samples were reduced with 5% 2-mercaptoethanol and applied to 10-20% gradient gel. The proteins were stained with Coomassie Blue.

Table 1. NH_2-sequences of α_2-PI immunopurified from human plasma and conditioned media of Hep G2 cells

	Sequence A		Sequence B	
Cycle No.	Amino acid	pmol[1)]	Amino acid	pmol[1)]
	(a) Plasma α_2PI			
1	Asn	28.2	Met	16.3
2	Gln	33.3	Glu	19.1
3	Glu	20.6	Pro	12.8
4	Gln	25.2	Leu	18.1
5	Val	50.9	Gly	n.d.
6	Ser	17.2	Arg	1.1
7	Pro	30.7	Gln	20.8
8[2)]	(Leu	50.9)	(Leu	50.9)
9[2)]	(Thr	40.8)	(Thr	40.8)
10	Leu	37.0	Ser	16.8
11	Leu	29.9	Gly	13.3
12	Lys	23.9	Pro	9.3
13	Leu	7.0	Asn	6.0
14	Gly	12.1	Gln	22.4

	Sequence A		Sequence B	
Cycle No.	Amino acid	pmol[1)]	Amino acid	pmol[1)]
	(b) Hep G2 α_2PI in serum containing media			
1	Asn	4.5	Met	16.3
2	Gln	7.1	Glu	13.2
3	Glu	0.2	Pro	14.1
4	Gln	6.0	Leu	21.2
5	Val	8.3	Gly	14.7
6	Ser	0.5	Arg	1.4
7	Pro	4.4	Gln	7.8
8[2)]	(Leu	12.2)	(Leu	12.2)
9[2)]	(Thr	5.1)	(Thr	5.1)
10	Leu	2.5	Ser	1.4
11	Leu	2.2	Gly	6.8
12	Lys	1.2	Pro	5.6
13	Leu	2.2	Asn	3.6
14	Gly	2.6	Gln	4.2

α_2PI with α_2PI-deficient human plasma for 3 days, induced the complete cleavage of the "pro"-peptide (sequence A: 100%, Table 1 (d)). Incubation with PBS buffer alone did not induce the cleavage at all (sequence B: 100%, Table 1 (e)). These results altogether indicate that α_2PI is produced in liver cells and secreted as a form of α_2PI fully retaining the

Table 1 - Continued

	Sequence B	
Cycle No.	Amino Acid	pmol[1)]
(c) Hep G2 α_2PI in serum free media		
1	Met	23.5
2	Glu	12.4
3	Pro	24.2
4	Leu	23.9
5	Gly	13.5
6	Arg	1.6
7	Gln	10.0
8	Leu	30.1
	Sequence A	
Cycle No.	Amino Acid	pmol[1)]
(d) (c) incubated with α_2PI-free plasma		
1	Asn	5.8
2	Gln	6.8
3	Glu	n.d.
4	Gln	5.1
5	Val	6.4
6	Ser	1.8
7	Pro	5.3
8	Leu	10.5
	Sequence B	
Cycle No.	Amino Acid	pmol[1)]
(e) (c) incubated with PBS		
1	Met	12.8
2	Glu	5.7
3	Pro	12.6
4	Leu	12.9
5	Gly	13.5
6	Arg	3.0
7	Gln	14.6
8	Leu	20.3

[1)] Values given are the yields obtained in the individual cycle corrected for background in the previous cycles and lag in the following cycle. n.d.: not determined.
[2)] Due to identical amino acids no values could be assigned to the individual sequences.

"pro"-peptide (previously called "pro"-α_2PI (1)) and the "pro"-α_2PI is converted to "mature" α_2PI in the circulation by proteolytic cleavage. Proteases responsible for the cleavage of Pro12-Asn13 must be present in blood plasma. However, "pro"-α_2PI is not a pro-α_2PI in a true sense because processing of "pro"-α_2PI to "mature" α_2PI is not taking place within the cells.

Therefore, "pro"-α_2PI should be regarded as mature α_2PI, and previously reported "mature" α_2PI is in fact a proteolytically modified form. We have shown that "pro"-α_2PI has an inhibitory activity on plasmin similar to "mature" α_2PI but has remarkably less capacity of cross-linking to fibrin (1). Cross-linking of α_2PI to fibrin is physiologically important in inhibition of fibrinolysis (14), and the removal of the "pro"-peptide results in a more potent form of inhibitor on fibrinolysis. In conclusion, mature α_2PI is a single chain protein of 464 amino acids with NH_2-terminal Met and the NH_2-terminal 12 amino acid peptide is lost in plasma during the circulation, resulting in a proteolytically modified form of α_2PI with NH_2-terminal Asn, which possesses a more potent inhibitory activity on fibrinolysis. Identification and origin of proteases responsible for the cleavage of Pro12-Asn13 of α_2PI are intriguing subjects for further investigation.

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REFERENCES

1. Sumi, Y., Ichikawa, Y., Nakamura, Y., Miura, O. and Aoki, N. (1989) J. Biochem. 106, 703-707.
2. Bangert, K., Johnsen, A.H., Christensen, U. and Thorsen, S. (1993) Biochem. J. 291, 623-625.
3. Enghild, J.J., Valnickova, Z., Thogersen, I.B., Pizzo, S.V. and Salvesen, G. (1993) Biochem. J. 291, 933-938.
4. Saito, H., Goodnough, L.T., Knowles, B.B. and Aden, D.P. (1982) Proc. Natl. Acad. Sci. USA 79, 5684-5687.
5. Koyama, T., Koike, Y., Sugahara,Y. and Aoki, N. (1992) Thromb. Res. 66, 451-454.
6. Aoki, N., Sumi, Y., Miura, O. and Hirosawa, S. (1993) Methods. Enzymol. 223, 185-197.
7. Murakami, H., Masui, H., Sato, G.H., Sueoka, N., Chow, T.P. and Kano-Sueoka T. (1982) Proc. Natl. Acad. Sci. 79, 1158-1162.
8. Mimuro, J., Koike, Y., Sumi, Y. and Aoki, N. (1987) Blood 69, 446-453.
9. Laemmli, U.K. (1970) Nature 227, 680-685.
10. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035-10038.
11. Tone, M., Kikuno, R., Kume-Iwaki, A. and Hashimoto-Gotoh, T. (1987) J. Biochem. 102, 1033-1041.
12. Holmes, W.E., Nelles, L., Lijnen, H.R. and Collen, D. (1987) J. Biol. Chem. 262, 1659-1664.
13. Hirosawa, S., Nakamura, Y., Miura, O., Sumi, Y. and Aoki, N. (1988) Proc. Natl. Acad. Sci. USA 85, 6836-6840.
14. Aoki, N. (1993) Thromb. Haemostas. 70, 376.