Comparative properties of human α-1-proteinase inhibitor glycosylation variants

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Variant forms of human α-1-proteinase inhibitor (α-1-PI), obtained by the treatment of human Hep G2 cells with specific inhibitors of glycosylation were tested for both inhibitory activity and heat stability. All were found to have the same second-order association rate with human neutrophil elastase, indicating a lack of importance of the carbohydrate moiety. In contrast, incompletely glycosylated forms of α-1-PI were found to be heat sensitive relative to the mature protein, suggesting a role for carbohydrate in protein stabilization.

α-1-Proteinase inhibitor; Inhibitor of glycosylation; Hep G 2 cell

1. INTRODUCTION

Proteinase inhibitors represent approximately 10% of the proteins in human plasma [1], the major component being α-1-proteinase inhibitor (α-1-PI) [2]. Decreased secretion of a variant form of this inhibitor (ZZ-phenotype) has been implicated in the development of familial emphysema [3], and it has been clearly established that this is due to a single base mutation of Glu-342 in the normal M-form of the protein to Lys-342 in the Z-protein [4]. Because of this mutation it has been suggested that a normal salt bridge between Glu-342 and Lys-290 is interrupted, thus altering the conformation of the Z-protein [5]. This, in turn, results in aberrant glycosylation and decreased secretion. More recently, however, it has been shown that replacing Lys-342 with Glu-342 in the M-protein has no effect on secretion [6]. Thus, the absence of a salt bridge is not directly responsible for the failure to adequately secrete the Z-protein.

While some of the Z-protein is found in plasma (approximately 10% of normal levels), the majority remains in the liver packaged in inclusion bodies [7]. Examination of the carbohydrate structure of both forms of this protein indicates that the secreted Z-protein is identical to that of the normal M-form, while that retained in the liver is primarily composed of high mannose side chains [8,9]. Such data would indicate that some of the Z-variant is post-translationally modified in a correct manner and that the major problem is due to an interruption in the normal rate of trimming of the high mannose forms of this protein.

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In our laboratory we have been investigating the properties of intermediate glycosylation forms of α-1-PI (M-variant) synthesized by human Hep G2 cells. These proteins were isolated from cells grown in the presence of specific inhibitors of the post-translational glycosylation process. This approach has been taken by others to compare secretion rates of plasma proteins with different carbohydrate side chain attachments in a rat hepatocyte system [10–13]. While we were interested in confirming their results using human hepatocytes, our primary goal was to determine whether carbohydrate side-chain structure had any effect on the inhibitory function of α-1-PI.

2. MATERIALS AND METHODS

Swainsonine, 1-deoxynojirimycin and 1-deoxymannojirimycin were from Calbiochem. Tunicamycin and MeO-Suc-L-Ala-L-Ala-L-Pro-L-Val-pNA were from Sigma. Antisera (goat) to α-1-PI, α-1-antichymotrypsin (α-1-Achy), and α-2-macroglobulin (α-2-M) were products of Atlantic Antibodies. MEM-cell culture medium was from Gibco. Human neutrophil elastase was prepared as previously described [14].

Cell culture and inhibitor purification: Human Hep G2 cells (kindly provided by Dr B.B. Knowles, Wistar Institute, Philadelphia, PA) were grown in Dulbecco's MEM plus 10% fetal calf serum (Flow Laboratories) with fresh medium added every 3 days. Nearly confluent human Hep G2 cells were washed twice with serum-free medium and exposed to a number of glycosylation inhibitors, including tunicamycin (3.5 μg/ml), swainsonine (3.5 μg/ml), 1-deoxynojirimycin (0.5 mM), and 1-deoxymannojirimycin (0.5 mM), all of which were dissolved in 0.1% bovine serum albumin. Cells were incubated with these inhibitors for 4 days with replacement of the medium every 24 h. Collected media were dialyzed vs 0.005 M sodium phosphate buffer, pH 6.8, and lyophilized. α-1-PI was then purified from this powder according to the procedure of Pannell et al. [15] with the addition of a final purification step on a Mono Q FPLC system. Human plasma α-1-PI (M- and Z-forms) was purified in an identical manner. The rate of secretion of α-1-PI and other plasma

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proteins by treated Hep G2 cells was followed by rocket immunoelectrophoresis [16].

The Z-form of α-1-PI was obtained from livers of ZZ homozygotes by homogenization of liver tissue in 0.05 M Tris-HCl, pH 7.5, 0.25 M sucrose. The microsomal fraction obtained by differential centrifugation was resuspended in 0.05 M glycine-NaOH buffer, pH 10.0, sonicated for one min, and shaken overnight. After lowering the pH to 7.0, the sample was centrifuged at $105\,000 \times g$ for 1 h, the supernatant dialyzed vs 0.05 M Tris-HCl, pH 8.0, and chromatographed on DEAE-cellulose, followed by Mono-Q FPLC.

Assays for inhibitory activity were performed against neutrophil elastase. Samples were incubated with excess enzyme for 1 min in 0.03 M sodium phosphate buffer, 0.15 M NaCl, pH 7.4. Residual elastase amidase activity was then measured using MeO-Suc-L-Ala-L-Ala-L-Pro-L-Val-pNA, as previously described [17]. Second order association rate constants for the interaction of individual inhibitors with elastase were performed in a similar manner, using equal activities of enzyme and inhibitor, incubating for various time periods, and measuring residual enzyme activity.

Heat inactivation of α-1-PI preparations was obtained by incubating in 0.03 M sodium phosphate, pH 6.8 at 60°C for varying time periods, removing aliquots, and measuring residual inhibitory activity.

3. RESULTS AND DISCUSSION

Hep G2 cells are known to synthesize at least twenty serum proteins [18], including several proteinase inhibitors [19]. Using control cells we were easily able to demonstrate the production of α-1-PI, α-1-Achy, and α-2-M by rocket immunoelectrophoresis (Table I).

In the presence of tunicamycin the secretion of α-1-PI was diminished by over 45%, a result in line with the data obtained by Gross et al. [13] while using a rat hepatocyte system. 1-deoxynojirimycin was less effective, while both swainsonine and 1-deoxymannojirimycin did not influence the secretion of α-1-PI. These results suggest that the lack of carbohydrate on α-1-PI in tunicamycin-treated cells reduces its secretion dramatically, while the presence of glucose residues on uncompleted side chains (1-deoxynojirimycin treatment) also retards secretion. In contrast, the presence of high mannose side chains (swainsonine and deoxymannojirimycin treatments) does not apparently affect secretion of α-1-PI from Hep G2 cells, as previously shown [13] in the rat system. Similar data were found for both α-1-Achy and α-2-M, although the latter appeared to be less effected by treatment of cells with 1-deoxynojirimycin.

Studies on the inhibition of both human neutrophil elastase and porcine pancreatic trypsin by the various purified, partially glycosylated forms of α-1-PI indicated that each had essentially the same k_{ass} for the individual enzymes (Table II).

This indicates that in the human system, at least, carbohydrate is not at all important for inhibitory activity. As reported previously [20], we also found that the Z-plasma form of α-1-PI had a k_{ass} value with elastase which was about 40% lower than that of the normal M-form. Significantly, we found the same result with the Z-liver protein. Since each carries different carbohydrate side chains due to the incomplete glycosylation of the Z-liver protein [8,9], it is clear that the reason for the lower k_{ass} is due to differences in protein conformation provided by the amino acid mutation at residue 342 and not due to aberrations in carbohydrate

Table I

Effect of inhibitors of glycosylation on serpin secretion in human Hep G2 cells

Treatment	Inhibitor synthesis (%)		
	α-1-PI	α-1-Achy	α-2-M
Control	100	100	100
+ Tunicamycin	51	nd	58
+ Swainsonine	116	106	109
+ 1-Deoxynojirimycin	68	89	72
+ 1-Deoxymannojirimycin	103	95	83

Media from cultured Hep G2 cells (10^6 cells) were harvested after 24 h incubation in the presence or absence of inhibitors and tested for individual proteins by quantitative rocket immunoelectrophoresis. nd, not determined.

Table II

Second-order association rate constants for the inhibition of neutrophil elastase by human α-1-PI glycosylation variants

Inhibitor variant	k_{ass} ($M^{-1} \cdot s^{-1}$)
MM-Plasma	2.4×10^7
ZZ-Plasma	1.1×10^7
ZZ-Liver	1.1×10^7
Hep G2 cells	
Control	2.7×10^7
+ Swainsonine	2.8×10^7
+ Tunicamycin	2.6×10^7
+ 1-Deoxynojirimycin	2.2×10^7
+ 1-Deoxymannojirimycin	2.3×10^7

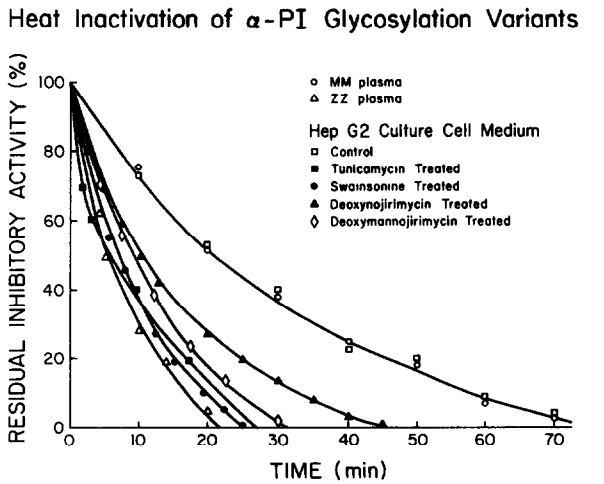

Fig. 1. Effect of heat on the stability of α-1-PI glycosylation variants. Samples were heated at 60°C and residual activity against human neutrophil elastase measured.

structure. Comparable results were obtained when porcine pancreatic trypsin was tested with the k_{ass} being two orders of magnitude lower, as previously reported [21].

Although carbohydrate does not appear to have any influence on the inhibitory activity of α-1-PI, it is important in the maintenance of stability. As shown in Fig. 1 both the M-form of α-1-PI isolated from plasma and that obtained from control Hep G2 cell media showed similar labilities in heat inactivation studies. In contrast, all of the partially glycosylated inhibitors isolated from treated cells had markedly reduced stability. Included in this latter list was the plasma Z-form of α-1-PI despite the fact that it is known to contain completed complex carbohydrate side chains [8]. Presumably, this is also due to the altered protein conformation of this mutant form of inhibitor [4].

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