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Plasminogen activators (PAs) are serine proteases converting the inactive proenzyme, plasminogen, into the active protease, plasmin. Plasmin so formed constitutes an important source of proteolytic activity in cells, tissues and biological fluids. Two distinct types of PAs have been identified: tissue-type PA (t-PA), produced by endothelial cells but also other cell types; and urokinase-type PA (u-PA), initially isolated from urine but also produced by kidney cells and other normal or transformed cells¹³.

The regulation of PAs may occur at the level of synthesis and secretion. Their activity is also balanced by specific PA-inhibitors (PAIs). Two highly specific inhibitors are known: PAI-1, previously termed endothelial cell PA-inhibitor or fast acting PA-inhibitor²⁻¹¹⁾ and PAI-2 the placental type PA-inhibitor reviewed here. PAI-1 is synthesised by endothelial cells, and is present in many other cell types, including granulosa cells and thrombocytes^{10, 12)}. PAI-1 is probably the most important PA-inhibitor in plasma, except perhaps during pregnancy.

The focus of this review will be the biochemical characteristics and physiological role of PAI-2. The first detailed report of the presence of a urokinase inhibitor in placenta was published by Kawano et al in 1968¹³⁾. They purified the inhibitor from placental homogenate by a five step procedure including acidification, ammonium sulphate precipitation, coupling to CM-Sephadex, gel-filtration and chromatography on hydroxylapatite. Two molecular forms were partially purified, one of 43KDa, the other of 70KDa. The inhibitory effect on urokinase was more pronounced than that on t-PA from the human heart¹⁴⁾¹⁵⁾. In subsequent organ culture experiments, placental explants were found to release inhibitors capable of inhibiting the activation of plasminogen by urokinase and PAs released by kidney and fetal vessel explants¹⁶⁾. By means of reversed fibrin slide autography¹⁷⁾, sections of placental tissue were found to prevent the breakdown of a urokinase-enriched fibrin film¹⁸⁾.

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Source and purification of PAI-2

In several studies placental homogenate has been a starting material for purification of PAI-2^{19)~22)}. Immunohistochemically, PAI-2 has been localised to the trophoblastic epithelium²³⁾. Human monocyte macrophages in culture have also been found to release urokinase inhibitor of PAI-2 type^{24)~26)}. All inhibitors from monocyte macrophages that have been tested, crossreact with our monospecific antibodies against PAI-2 (unpublished data).

Recently, considerable interest has been focused on a human histiocytic cell line(U937) established by Sundström and Nilsson²⁷⁾ and a promising production of PAI-2 has been reported²⁸⁾. Stimulation of the cells with the tumour promotor, phorbol 12-myristate 13-acetate, increased the production of PAI-2 20 fold²⁹⁾. The inhibitor produced by U937 cells is immunologically identical to PAI-2 derived from placenta. The cell line U937 thus seems to be a promising source for purification of PAI-2. PAI-2 antigen has also been found in a uterine carcinoma cell line³⁰⁾, and in ascitic fluid from certain ovarian tumours³¹⁾.

The purification process outlined by Kawano et al¹⁴⁾ resulted in a preparation in which 15% of the protein consisted of PAI-2. Further work on purification procedures has been focused on the low molecular form of PAI-2. After additional chromatography on a Zn-celate affinity column and a Sephadex G-75 Superfine column, 50% of the protein concentration consisted of PAI-2²²⁾.

Thus, owing to the large amounts of contaminating proteins, several chromatographic steps are necessary for the purification of PAI-2 from placenta homogenates. We recently raised a monoclonal antibody against PAI-2 useful for purification purposes¹⁹⁾. After initial purification using CM-Sephadex, this antibody was used for affinity chromatography. The material eluted from the affinity column was finally purified in a FPLC system using a Mono-Q-column. This purification procedure yields an apparently homogenous and biologically active PAI-2 with a molecular weight of 48 kDa¹⁹⁾. With this method at least 1 mg of pure PAI-2 is obtained from 15 placentas.

Conditioned medium from the U937 cell line might have advantages as a source for PAI-2 because of its low concentration of contaminating proteins. Kruithof et al²⁹⁾ used isoelectric focusing and affinity chromatography on Cibacron Blue-Sepharose resulting in a good yield of the 47 kDa molecular form of PAI-2.

Characteristics of PAI-2

The molecular weight of the low molecular weight (LMW) form of PAI-2 has been estimated to be 43-48 kDa^{14),19),20),29)} or 46.6 kDa for the sugar free molecule according to the amino acid sequence^{32),33)}. The high molecular (HMW) form has been estimated to be about 70 kDa³⁴⁾. The molecular weight of 70 kDa was determined by molecular sieving chromatography on Sephadex G100 Superfine. With SDS-PAGE and immunoblotting against monoclonal antibodies, the molecular weight of the HMW-form was estimated to be 60 kDa³⁵⁾. This variation in values obtained with different methods is probably

due to the effect of Stokes'radius. As it has not been possible to reduce the HMW-form of PAI-2 by mercaptoethanol, it would appear to consist of a one-chain molecule. The difference in molecular weight between the LMW and HMW forms is probably due to differences in the degree of glycosylation of the respective molecules^{36,37}.

Complex formation between u-PA and PAI-2 was demonstrated for the first time by means of agarose gel electrophoresis and autoradiography using ¹²⁵I labelled u-PA²¹. The two u-PA forms of 31 and 54 kDa were incubated with the LMW-form of PAI-2. In electrophoresis the LMW-form of u-PA in complex with PAI-2 moved to the β_1 -position, and the HMW u-PA in complex with PAI-2 to the β_2 -gamma position. As u-PA inactivated with the artificial specific serine protease inhibitor, diisopropylfluorophosphate, did not bind to PAI-2, the complex formation must be dependent on the active site serine of u-PA. The u-PA/PAI-2 complex binding was irreversible. This complex formation has since been confirmed^{19,20,29,38}. The molecular weights of the complexes were about 15 kDa lower than expected, indicating that part of the inhibitor molecule had been split off at complex formation^{19,34}.

t-PA occurs in both a one and a two chain form³⁹, and a noteworthy finding has been that these two forms differ in the extent of their inhibition by PAI-2²². In a further kinetic study⁴⁰, it was shown that both the one and two chain forms of t-PA form 1: 1 complexes with the inhibitor, and that the two-chain form binds the inhibitor very strongly whereas the one-chain form forms a much weaker complex. This difference in inhibition and the lesser efficiency in complex formation of the one chain form of t-PA have since been confirmed^{20,29}. Thus, PAI-2 inhibits u-PA about ten times better than it does two chain t-PA, though the latter also binds the inhibitor strongly. PAI-2 is a poor inhibitor of one-chain t-PA and forms a much weaker complex with it.

The properties of PAI-2 differ from those of PAI-1 in many respects. The inhibitory activity of PAI-2 decreases in SDS⁴¹, in contrast to that of PAI-1 which is enhanced in SDS^{11,41,43}. PAI-2 is an α_2 -globulin^{14,21} while PAI-1 is a β -globulin⁴⁴. Neither monoclonal nor polyclonal antibodies against PAI-2 react with PAI-1^{41,45}. Like PAI-2, PAI-1 also effectively inhibits u-PA^{2,4,6,46}, but in contrast to PAI-2 it neutralises the one-chain and two-chain molecular forms of t-PA in plasma at very similar rates^{47,48}. The isoelectric point for PAI-2 is about 5.0-5.2, and for PAI-1 4.5-5.0⁷.

Additional information about the structure and function of PAI-2 has been obtained from the sequence analysis of cDNA coding for PAI-2. The deduced amino acid sequence consists of 415 amino acids, corresponding to a protein with the molecular weight of 46.6 kDa. Sequence comparisons indicate PAI-2 to be a member of the SERPIN (serine protease inhibitor) gene family. It is particularly similar to ovalbumin, but no more similar to endothelial cell PAI-1 than it is to any other mammalian serpin³².

There are three potential N-glycosylation sites in the deduced protein sequence of PAI-2. Alignment of the amino acid sequence of PAI-2 predicts that the active site is located between Arg 380 (P1) and Thr 381 (P1')⁴⁹. Thus the alignment is consistent with the known Arg-specificity of PAs. A noteworthy feature is that, like ovalbumin,

PAI-2 lacks a NH_2 -terminal signal peptide.

Determination of PAI-2

In pure preparations of PAI-2, the inhibitory activity can be measured in clot lysis systems or assays using synthetic substrates; for samples containing both PAI-2 and PAI-1, however, such assays are difficult to design despite the differences in the inhibition of the one-chain form of t-PA by PAI-2 and PAI-1. Thus no specific activity assays are available for the determination of the PAI-2 content in clinical samples. An approximate estimation can be made by using u-PA or two-chain t-PA in the assay with short incubation times, thereby measuring the activity of both PAI-1 and PAI-2. The use of one-chain t-PA, then, measures only the activity of PAI-1^{50),51)}.

For determination of the concentration of PAI-2 antigen a solid phase double sandwich ELISA has been designed for use with both monoclonal and polyclonal PAI-2 antibodies. Microtitration plates are coated with a monoclonal antibody, and a polyclonal goat anti-PAI-2 is used as a second antibody. Peroxidase conjugated rabbit-anti-goat Ig and 5-amino-salicylic acid H_2O_2 is used to quantify bound goat-antibody. The method has been used to measure concentrations between 3 and 200 $\mu\text{g}/\text{l}$ ^{34),35)}. Kruithof⁵⁰⁾ used a radioimmunoassay with a detection limit of 10 $\mu\text{g}/\text{l}$, in which purified PAI-2 was labeled with ^{125}I to a specific radioactivity of 20 $\mu\text{Ci}/\mu\text{g}$ PAI-2.

Occurrence of PAI-2 in the organism and its physiological significance

Besides its presence in the placenta, PAI-2 has been found to occur in pregnancy plasma^{34),35),50)}. The increase during pregnancy is almost linear, and term levels have been reported to be 100–300 $\mu\text{g}/\text{l}$ with a decrease to non-detectable levels within about a week post partum^{34),50)}. The HMW form of PAI-2 was found to be almost exclusively predominant in maternal plasma^{24),35)}. PAI-2 has also been found to occur in amniotic fluid and umbilical cord plasma. In maternal plasma, amniotic fluid and cord plasma from the same deliveries, the median values of the concentrations were reported to be 75, 89, and 13 $\mu\text{g}/\text{l}$, respectively. The HMW and LMW forms of PAI-2 were found at about the same concentrations in amniotic fluid but the LMW-form predominated in cord blood³⁵⁾. The physiological significance of the occurrence of two molecular forms of PAI-2 might consist in the regulation of placental passage and its distribution to mother and child.

PAI-2 has also been detected in males. In a recent study of 120 male blood donors, PAI-2 was detected in 5 of them at concentrations ranging from 9–60% of that in term plasma. In an apparently healthy non-pregnant woman, levels of PAI-2 fluctuated between 30 and 90% of the level found in term plasma over a 6 month period. An 80 KDa immunoreactive but non-active form of PAI-2 was isolated from this woman⁵²⁾. Occurrence of PAI-2 in plasma of apparently healthy men and non-pregnant women may result from activated macrophages^{24)~26)} or stimulation of release from epidermal cells, which recently have been shown to contain PAI-2⁵³⁾.

Fibrinolytic activity in plasma successively decreases during pregnancy^{54)~61)}, even after

stimulation by venous occlusion⁵⁸⁾⁶¹⁾, and it remains reduced until separation of the placenta, after which it rapidly returns to non-pregnant levels⁵⁹⁾. It was early shown that pregnancy plasma has a urokinase inhibitory capacity which could explain this reduction of fibrinolytic activity during pregnancy⁶²⁾⁶³⁾.

Not only PAI-2, but also the fast acting PA-inhibitor, PAI-1, has been reported to increase during pregnancy⁵⁰⁾⁶⁴⁾⁶⁵⁾. The question has been raised of whether PAI-1 and/or PAI-2 is responsible for the depressed fibrinolytic activity during pregnancy. Immunoaffinity absorption of PAI-2 was followed by loss of about half of the inhibitory capacity⁶⁶⁾. Both types of PAI are thus responsible for the inhibitory capacity of plasma during pregnancy^{50)61)64)~66)}.

Although fibrinolytic activity is absent in homogenates of placenta, immunoreactive material of 20–25 ng/g placental tissue has been detected with a u-PA radioimmunoassay, which also measures u-PA in complex¹⁹⁾. This might be of interest with regard to the aetiology of premature separation of the placenta, as activation of the fibrinolytic system is a common finding in such cases. In a study on local fibrinolysis in placenta at spontaneous abortion, Ikeuchi⁶⁷⁾ demonstrated a decrease in u-PA inhibitory activity in the placental villi, as compared with values in normal pregnancies. Threatening premature separation of placenta has also been successfully treated with tranexamic acid, an artificial inhibitor of plasminogen activation⁶⁸⁾. These observations argue for the importance of a well-functioning balance between activator and inhibitor. The purpose of the increase in concentration of PAI-2 antigen in the placenta is probably to prevent abruptio placenta, and to secure haemostasis at separation of the placenta in the third stage of labour.

PAI-2 might also be of physiological importance to the growing fetus. Fetal organs in culture have been shown to release u-PA⁶⁹⁾. A function of PAI-2 might be to balance the u-PA activity during the growth and remodelling of tissue⁷⁰⁾. As far as the child is concerned, the function of PAI-2 is probably to prevent bleedings such as intraventricular haemorrhagia, a not uncommon complication in cases of premature birth⁷¹⁾⁷²⁾.

Therapeutic implications

PAI-2 might also be useful clinically, to inhibit excessive effect of t-PA or u-PA in thrombolytic therapy, for instance, or in cancer therapy. Although increased concentrations of PAI-1 in plasma are commonly found in cases of recurrent deep vein thrombosis^{73)~76)}, there is no evidence of PAI-2 having any thrombogenic effect. This lack of thrombogenic effect may be due to the difference in inhibition of the one- and two-chain forms of t-PA by these inhibitors. The efficacy of the two forms of t-PA in a fibrinolytic process is apparently much the same³⁹⁾⁷⁷⁾⁷⁸⁾. PAI-2 ensures a fibrinolytic potential by leaving the one-chain form of t-PA essentially uninhibited; thus PAI-2 might prove both safe and useful in regulating excessive effects of thrombolytic therapy, and in the specific treatment of PA-induced haemorrhagic diathesis.

Fibrin is a necessary matrix for the proliferation of tumour vessels. Residual fibrin has continuously to be removed by u-PA produced by the tumour cells¹⁾. Inhibition of this

process with antifibrinolytic agents, resulting in a corresponding inhibition of tumour growth, has been shown both in experimental tumours^{79)~81)}, and in human tumours^{82~84)}. The effect of PAI-2 on tumour u-PA is intriguing and might prove useful for inhibiting the fibrinolytic process which is a prerequisite for the proliferation of tumour vessels.

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