Plasminogen activator inhibitor (type-1) in rat adrenal medulla

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Summary. Plasminogen activator inhibitor type-1 (PAI-1) was identified in extracts of rat adrenal medulla, and its immunohistochemical localization was studied together with that of tissue-type plasminogen activator (t-PA). By staining of adjacent sections and by doublestaining of the same section we demonstrate that the same cells of the adrenal medulla contain both PAI-1 and t-PA immunoreactivity in the cytoplasm. In addition a few ganglion cells of the adrenal medulla were found to contain PAI-1 but not t-PA. Neither of the components were found in the adrenal cortex. Analysis of extracts from isolated adrenal medulla using reverse zymography showed the presence of a plasminogen activator inhibitor with $M_r \sim 46 000$. The inhibitory activity disappeared when the extract was passed through a column with sepharose-coupled anti-PAI-1 IgG, while the run-through from a similar column coupled with preimmune IgG still contained the inhibitor. The present findings suggest that PAI-1 could play a role in the regulation of t-PA activity in the rat adrenal gland medullary cells.

Introduction

Plasminogen activators are serine proteases which convert the proenzyme plasminogen to the active protease plasmin by limited proteolysis. Plasmin is a broad spectrum protease which is capable of degrading fibrin and a number of other proteins. At least two types of plasminogen activators control this proteolytic cascade: urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA). The molecular structure of these two proteins is well described (for review see refs. Astrup 1978; Reich 1978; Rohrlich and Rifkin 1979; Markus 1983; Daño et al. 1985; Blasi et al. 1987). The regulation of plasminogen activating activity is possible at different levels of the cascade reaction: biosynthesis, secretion, activation of proenzymes and by specific plasminogen activator inhibitors. Two types of plasminogen activator inhibitors, PAI-1 and PAI-2, have so far been identified. The two inhibitors are both glycoproteins with $M_r$ of approx. 50 000 and are SERPINs (serin protease inhibitors). Their primary structure has been investigated by cDNA cloning and amino acid sequencing, and it has been found that PAI-1 is an Arg-SERPIN having an arginine residue at the reactive center (Loskutoff and Edgington 1981; Andreasen et al. 1986a–c, 1987; Coleman et al. 1986; Ginsburg et al. 1986; Loskutoff et al. 1986; Nielsen et al. 1986; Ny et al. 1986; Pannekoek et al. 1986; Ye et al. 1987).

In the intact organism PAI-1 has until now been found in plasma and thrombocytes (Stalder et al. 1985; Thorsen and Philips 1984; Kruithof et al. 1986). It has recently been shown that PAI-1 is present in Lewis lung carcinoma, and it has been proposed that PAI-1 plays a part in the control of the net plasminogen activation in this tumor (Kristensen et al. 1988).

t-PA is found in endothelial cells (Kristensen et al. 1984) and is thought to play a role in thrombolysis. In addition t-PA has been found in several different peptide hormone producing cells, namely somatostatin cells in the hypothalamus and islets of Langerhans (Virji et al. 1980; Kristensen et al. 1987), pituitary gland (growth hormone producing cells) (Ganellin-Piperno and Reich 1983; Kristensen et al. 1985), and adrenal medullary cells (noradrenaline containing cells) (Kristensen et al. 1986). In addition t-PA has been found in pachytyene spermatocytes of the rat seminiferous epithelium (Vihko et al. 1988).

Here we describe the immunohistochemical staining for PAI-1 in the rat adrenal medulla. We show that PAI-1 immunoreactivity is present in the endocrine cells that contain t-PA immunoreactivity and demonstrate the presence of PAI-1 inhibitory activity in extracts of adrenal medullary glands.

Materials and methods

Materials. The following materials were obtained from the indicated sources: nitro blue tetrazolium (grade III; NBT), 5-bromo-4-chloro-3-indoly1 phosphate (BCIP) and levamisole (Sigma, St. Louis, MO); N,N-dimethylformamide, 1,4-diazabicyclo(2,2,2)-octane (DABCO), methylgreen and eriochrome black (Merck, Darmstadt, West Germany); biotinylated swine anti-rabbit IgG; horse antimouse IgG; anti-PAI-1 IgG (Amersham, England). All other materials were obtained from the best commercially available grade.

Tissue material. Male Wistar rats weighing 250–350 g were anesthetized with diethyl ether. Animals to be used for immunohistochemistry were perfusion fixed intracardially – by 60 ml cold (4°C) 0.01 $M$ sodium phosphate, pH 7.4 containing 0.15 $M$ NaCl (PBS)
followed by 100 ml cold (4°C) 1% (wt/vol) paraformaldehyde in 50 mM phosphate buffer, pH 7.4.

Adrenals were fixed for 14–16 h at 4°C, followed by 4–6 h rinsing in 0.1 M sodium phosphate buffer, pH 7.4 containing 20% (wt/vol) sucrose. The glands were frozen in melting Frigen-22. Cryostat sections were cut at -18°C and collected on chrome-alum-gelatine coated slides (Kristensen et al. 1985).

Alternatively, for zymographic analysis, fresh PBS-perfused adrenal glands were removed and washed in PBS. The adrenal marrow was isolated from the cortex using a stereomicroscope. Adrenal marrow from 6 rats was gently dried on filter paper, weighed (30–35 mg) and homogenized in 0.1 M Tris, pH 8.1 containing 0.5% (wt/vol) Triton X-100, 5 mM EDTA, 30 μM p-nitrophenyl-p’-guanidino benzoate (NPGB) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (10 μl per mg tissue). The extracts were centrifuged 10000 x g for 10 min at 4°C, and supernatants were collected into new tubes.

**Antibodies.** Human PAI-1 was purified by affinity chromatography using a monoclonal antibody (Nielsen et al. 1986), and used for immunization of rabbits, following a previously described scheme (Dane et al. 1980). The rabbit anti-human PAI-1 IgG was purified using affinity chromatography with protein-A Sepharose and the IgG was shown to react with rat PAI-1 and in addition to react weakly with mouse IgG (leaking from the affinity column) and fibronectin. The anti-PAI-1 IgG was therefore absorbed by passage of a 1 ml Sepharose column as described (Kristensen et al. 1985) coupled with 1 mg human fibronectin purified from human plasma as described by Vuento and Vaheri (1979) and 1 mg of mouse monoclonal anti-PAI-1 IgG clone 2 (Nielsen et al. 1986). The removal of antibodies reacting with mouse IgG and human fibronectin was monitored using ELISA (Kristensen et al. 1985) and after this absorption the anti-human PAI-1 antibodies were shown to react with rat PAI-1 using immunoblotting, ELISA, inhibitor neutralization in reverse zymography (not shown) by and purification of rat PAI-1 from conditioned culture fluid of HTC cells (see below). Anti-PAI-1 IgG was absorbed on purified PAI-1 by passing twice through a column with either Sepharose-coupled purified human PAI-1 (used in the present study) or purified rat PAI-1 (results not shown).

The anti-PAI-1 IgG was tested for reactivity with human t-PA using ELISA and for control staining experiments, a preparation of anti-PAI-1 IgG was passed through a column of Sepharose-coupled purified human t-PA. The ability of this column to remove anti-t-PA IgG from a preparation of anti-human t-PA IgG was confirmed in a separate experiment using ELISA and immunohistochemical staining of adrenal tissue.

Antibodies against human t-PA and capable of immunohistochromically detecting rat t-PA were described previously (Kristensen et al. 1984, 1985, 1986, 1987).

**Rat PAI-1.** HTC rat hepatoma cells were grown in monolayer cultures in Dulbecco-modified Eagle’s medium containing 10% fetal calf serum. Cell were washed twice with 20 ml PBS, pH 7.4 and maintained in medium without serum for 4 days before incubation with medium containing 10–6 M desmethylam (Andreasen et al. 1986a). Conditioned medium was harvested after 2 or 3 days of incubation and replaced with new medium containing desmethylase, 10 μg of rabbit anti-human PAI-1 was coupled to 3 ml CNBr-activated Sepharose 4B following the manufacturer’s recommendations. After packing, the column was equilibrated with 0.1 M Tris-HCl, pH 8.1, and approximately 500 ml of conditioned medium was loaded on the column overnight. After washing with 10 ml of 0.1 M Tris-HCl, pH 8.1, and 70 ml 0.1 M Tris-HCl, pH 8.1, with 1.0 M NaCl, the column was eluted with 0.1 M glycine-HCl, pH 2.5 with 0.5 M NaCl, into vials containing 1/10 of the eluted volume of 1.0 M Tris-HCl, pH 9.0. Purification was done at 4°C. SDS-PAGE analysis and Comassie-blue staining showed that the eluate contained one major band with a Mr of ~50000 and a weaker band around 45000. A very weak band with Mr ~100000 was occasionally seen. The two bands with Mr ~50000 are the typical pattern seen with purified rat PAI-1 (Zehnp et al. 1987).

**Immunohistochemistry.** Cryostat sections were thawed in 0.05 M Tris-HCl pH 7.4, containing 0.15 M NaCl (TBS) for 5 min, and fixed in 1% (wt/vol) paraformaldehyde in 50 mM phosphate buffer, pH 7.4 for 10 min. After washing 3 x 10 min in TBS with 1% (wt/vol) Triton X-100 (TBS-Triton) the sections were incubated in 30% (wt/vol) normal swine serum for 30 min and washed briefly in TBS-Triton. Incubation for 16–18 h at 4°C with primary rabbit antibodies (10 μg/ml) diluted in 10% (wt/vol) normal swine serum, was followed by 1 h at room temperature. Sections were washed 3 x 10 min in TBS-Triton and antibody bound was detected by incubation with swine anti rabbit IgG alkaline phosphatase conjugated, diluted 1:20 in 10% normal swine serum. Sections were then incubated 3 x 10 min in TBS-Triton and 10 min in 0.1 M Tris, pH 9.5 with 1 M NaCl and 5 M MgCl2. Alkaline phosphatase reactivity was demonstrated with the NBT-BCIP detection system (McGrady 1970; Kristensen et al. 1987). Sections were counterstained in nuclear fast red (0.05%). Staining controls included deletion of the various antibody layers and substitution of the primary antibody with a similar amount of preimmun IgG from the same rabbit or with IgG absorbed with purified preparations of the corresponding antigen (Larsson 1981; Kristensen et al. 1985, 1987).

**Doublestaining.** Sections were pretreated as described above and incubated with rabbit anti human t-PA overnight at 4°C and 1 h at room temperature. After washing for 3 x 10 min in TBS-Triton, sections were incubated for 30 min with biotinylated swine anti-rabbit IgG 1:200 in 10% normal swine serum, washed for 3 x 10 min in TBS-Triton, incubated for 30 min with FITC conjugated streptavidin and washed 3 x 10 min in TBS-Triton. Then the sections were dehydrated using graded ethanol and placed in a tightly sealed 0.41 jar containing 0.75 g paraformaldehyde powder (Wang and Larsson 1985). The closed jar was placed in an oven at 55°C for 1 h. The sections were rehydrated, washed for 4 x 10 min in TBS-Triton and incubated with second primary antibody (rabbit anti-PAI-1) (Wang and Larsson 1985). Reaction of the second antibody with the antigen was demonstrated with alkaline phosphatase methods described above. Sections were counterstained in methyl green (0.01%) and eriochrome black (1.65%) (Schenk and Churukian 1974) and mounted in glycerol-TBS 9:1 containing 30 mg/ml DABCO (Johnson et al. 1982). Control experiments included omission of second primary antibody and substitution of second primary antibody with preimmune or PAI-1 absorbed immune IgG (see discussion).

**Biochemical analysis.** Preimmune and anti-PAI-1 IgG was coupled to Sepharose following the manufacturer’s recommendations and columns were equilibrated with TBS. PBS (pH 7.4) was added to adrenal gland marrow extract supernatants to reach a total volume of 1.4 ml. To each column, 700 μl of the diluted extract was applied, and columns were washed with 6 ml of TBS. The first 3 ml of the run-through was collected, 60 μl 5% (wt/vol) SDS was added and the samples were dialysed against 2 x 2 L 0.1% SDS for ~16 h at 4°C and freeze-dried. The samples were reconstituted in 60 μl of sample buffer (Laemmli 1970) without SDS and SDS-PAGE was performed using 6%–10% acrylamide separating gels and a 4% stacking gel (Laemmli 1970). The SDS-polyacrylamide gels were washed in 0.5% Triton X-100 and layered on a fibrin-agarose gel containing plasmogen and urokinase as described (Andreasen et al. 1986a and b). Marker proteins were as described in Larsson et al. (1984).

**Results**

Immunohistochemical staining of the rat adrenal gland showed PAI-1 immunoreactivity in a subpopulation of adrenal medullary cells (Fig. 1a). These cells are seen as separate cell clusters. Staining of an adjacent section showed