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Studies of the Blood Plasminogen Activator Induced by 1-Desamino-8-D-arginine Vasopressin with Observations in von Willebrand's Disease[1] (41407)

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Abstract. The nature of the blood plasminogen activator released in humans by infusion of the vasopressin analog, 1-desamino-8-D-arginine vasopressin (DDAVP), was studied utilizing an antibody raised against a plasminogen activator purified from the culture medium of a melanoma cell line. The melanoma activator cross-reacted immunologically with tissue plasminogen activator purified from human uterine tissue. The fibrinolytic activity of human blood elicited by DDAVP could be quenched by a melanoma activator antiserum, demonstrating that the vasopressin analog, which lacks vasopressor properties, released a tissue plasminogen activator-related substance to the blood. Urokinase-like material measured by radioimmunoassay, was not released by DDAVP. Nor was the intrinsic fibrinolytic system of plasma affected, since Cl inactivator-sensitive activity was not enhanced. In severe von Willebrand's disease, which is characterized by an endothelial defect of factor VIIIR:Ag synthesis, DDAVP did not release tissue activator into the blood, although the patients had a low resting level of this material. A defective endothelial receptor for DDAVP or some intermediary mediator is suggested in this disease.

Some physiological substances, such as adrenaline, nicotinic acid, and vasopressin are known to raise the level of blood plasminogen activator (1–4). All of them affect vascular tone, though in different ways. The fibrinolytic response to these agents does not depend on their vasoactive properties, for the analog of vasopressin, DDAVP (1-desamino-8-D-arginine vasopressin), which is almost devoid of vasopressor effect, is even more potent than vasopressin in generating plasminogen activator (3, 4). However, the origin of the DDAVP-induced activator is still unknown.

Patients with von Willebrand's disease are, to a varying extent, deficient in factor VIII-related antigen (VIIIR:Ag, von Willebrand factor), which is produced in endothelial cells. Some of the patients with severe von Willebrand's disease also seem to lack the ability to stimulate fibrinolysis in response to DDAVP in spite of a normal resting level of blood plasminogen activator (5, 6).

In the present investigation we demonstrated that the DDAVP-induced fibrinolysis in eight normal subjects is due to release of an activator related to the tissue plasminogen activator (extrinsic activator), and second, that patients with severe von Willebrand's disease have a resting level of extrinsic activator but cannot increase it further in response to DDAVP. Neither in normals nor in patients with von Willebrand's disease were urokinase-like material or the intrinsic fibrinolytic system affected by DDAVP.

Material and Methods. Two unrelated patients, a woman aged 32 years and a boy aged 13 years, with the severest form of von Willebrand's disease, were examined. The Ivy bleeding time was >30 min in both of them, VIIIR:Ag measured by immunoradiometric assay (7) was not detectable (<0.1 U/dl), VIIIR:RCF (VIII related ristocetin cofactor) also undetectable (<5 U/dl) and VIII:C very low (3 U/dl) in both. There were no other known cases of von Willebrand's disease in the families of the

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two patients suggesting that they were homozygotes for a recessive gene (8). They have earlier been shown not to respond to DDAVP by raising their VIIIR:Ag levels (9). Eight healthy volunteers (three men and five women), aged 26 to 44 years, belonged to the staff. The patients as well as the volunteers were informed about the aim of the study and gave their consent.

DDAVP (Ferring AB, Malmö, Sweden) was given in a dose of 0.4 μg/kg body weight. The drug was diluted in saline and infused iv over a period of 10 min. Blood samples were collected before and 10 min after the end of the infusion. The experiments were done in the morning with the individuals fasting whenever possible.

Tissue plasminogen activator. This was obtained from the culture medium of a cultivated melanoma cell line (provided by Dr. D. Collen) and is very similar or identical to human tissue plasminogen activator (10). The cells were first grown in a medium enriched with 10% fetal calf serum and then in serum-free medium through 8–16 passages for 1–2 months. The culture medium was harvested and contained about 0.25–0.5 mg activator/liter as estimated from recovery data and determined by the fibrin plate method and immunoradiometrically (see below). The activator was purified by immunosorbent chromatography utilizing antibodies against porcine tissue activator essentially as recently described for the purification of human uterine tissue activator (11). The IgG fraction from goat antiserum against highly purified porcine tissue activator was isolated and coupled to Sepharose (12). The activator was adsorbed from the culture medium on the Sepharose derivative (2 g derivative/liter medium) and eluted with KSCN. The eluted material was further purified by chromatography on arginine–Sepharose and gel filtration (11). The final product had a specific activity of 200,000–250,000 IU/mg and was electrophoretically homogeneous but contained both the one-chain and the two-chain forms of the activator (11).

Antiserum to the melanoma cell activator. This was raised in a goat by two injections, the second 3 weeks after the first, of 150 μg of purified activator, emulsified with Freund's complete adjuvant. The injections were given subcutaneously in the back at multiple injection sites. Blood was drawn 2 weeks after the second injection. IgG from the antiserum (IgG-A) and control goat serum (IgG-C) was prepared in the following way. To 50 ml serum 12.5 g ammonium sulfate was added. After 20 hr the precipitate was collected and dialyzed first against water and then 0.05 *M* sodium acetate buffer, pH 5.0, and chromatographed on DEAE-Sephadex A 50 (Pharmacia) in the same buffer. The breakthrough fraction was dialyzed against 0.1 *M* NaCl and further chromatographed on Sephadex G-200 (Pharmacia) in 0.05 *M* Tris–HCl buffer, 0.3 *M* NaCl, 0.02% sodium azide, pH 7.8.

The antiserum efficiently neutralized the fibrinolytic activity of pure melanoma activator. This was tested in the following way: 50 μl pure activator (3 μg/ml) was incubated overnight with 50 μl of various dilutions of antiserum IgG. A 1:1500 dilution of IgG completely neutralized melanoma activator activity as tested on fibrin plates (see below). The same result was obtained on short time incubation (15 min).

The antiserum and its IgG fraction gave one precipitation line with concentrated culture medium in gel diffusion (Fig. 1), but no precipitate with plasma. The melanoma activator showed an immunological reaction of complete identity with the tissue activator purified from human uterine tissue (11). Antiserum IgG (IgG-A) did not neutralize urokinase activity, tested as for melanoma activator. Extensively purified M_r 33,000 urokinase (13) did not react with the antiserum either in gel diffusion or in a more sensitive immunoradiometric assay based on radiolabeled purified antibodies from the antiserum. This assay was developed in the following way: 0.6 mg IgG-A was labeled with 37 MBq ^{125}I (14). The labeled material was incubated with pure melanoma activator that had been insolubilized to activated CH-Sepharose gel (Pharmacia), and the specific labeled antibody, which had bound to the gel, was then eluted by lowering pH to 3.0. Mela-

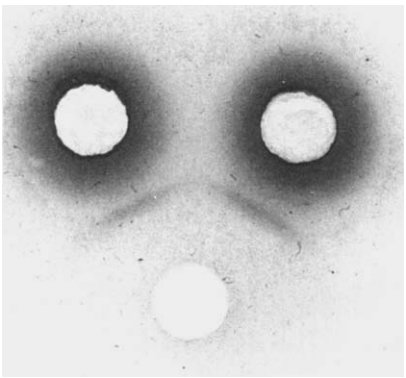

FIG. 1. Ouchterlony double diffusion plate of melanoma activator antiserum (lower well) and melanoma cell conditioned media, concentrated 200–500 times (upper wells).

noma activator-like material was measured in a two-site solid-phase system. The sample was incubated in polystyrene plastic tubes, which had been coated with IgG-A (15). In a second step the purified radiolabeled antibodies were added. After incubation and washing, radioactivity bound to the tubes was measured. The dilution curve of pure melanoma activator was linear between 20 and 1 ng/ml. The dilution curve of melanoma culture medium was parallel to that of pure activator in the linear interval of the curve. M_r 33,000 urokinase purified by *p*-aminobenzamidine–Sepharose chromatography did not react in this assay (a contamination of the urokinase 1:20,000 with tissue activator-like material would have been detected).

C1 inactivator, purified to homogeneity, was kindly provided by Professor A.-B. Laurell, Department of Microbiology, University of Lund, Sweden.

Blood collection. Citrated platelet-poor plasma was prepared as previously described (6). Plasma samples were not frozen but immediately tested for fibrinolytic activity.

Assay of plasminogen activators. These were determined by measuring fibrinolytic activity on plasminogen-rich human fibrin plates (16). The plates were prepared by clotting a 0.15% fibrinogen solution (Kabi, Stockholm) with thrombin on plastic plates to create a thin fibrin film as described previously (16). Thirty-microliter drops were placed on the fibrin film and the plates incubated at 37° for 20 hr. The results were expressed as square millimeters of lyzed area. The assay was performed on citrated plasma, euglobulin precipitates, and dextran sulfate precipitates in the following way: *Plasma* (200 μl) was incubated with 5 μl IgG-A at room temperature for 10 min. Thirty microliters of the mixture was applied to the fibrin plate. Plasma samples mixed with either IgG-C or NaCl served as controls. The *euglobulin precipitates* from 1 ml plasma were dissolved in 0.5 ml Owren's buffer (16). Two hundred microliters was incubated with 5 μl IgG-A, IgG-C, or NaCl for 10 min and the mixtures tested for fibrinolytic activity on fibrin plates as above. *Dextran sulfate precipitates* were prepared according to Kluft (17). The precipitates were dissolved, either in 1 ml EDTA buffer (17) or in 1 ml EDTA buffer with 2 m*M* flufenamate (Aldrich). To the solution of precipitate in only EDTA buffer, Cl inactivator was added. The dextran sulfate precipitate solutions contained, as a rule, 30% of the Cl inactivator of the plasma, determined with quantitative electroimmunoassay (Laurell). A standard amount of Cl inactivator was added to the precipitates, and it was afterward checked that the concentration in the solutions fell within 80–120% of that in plasma. One hundred microliters of the solutions was incubated with 20 μl IgG-A, IgG-C, or NaCl and tested on fibrin plates in the way described. The use of a larger amount of antiserum IgG with these precipitates was to ensure maximal neutralization with the antiserum.

Urokinase in plasma and precipitates was determined immunologically with a radioimmunoassay, utilizing an antiserum raised in a goat against purified M_r 33,000 urokinase as previously described (18). The antiserum gave a reaction of complete identity between M_r 33,000 and M_r 54,000 urokinase in double immunodiffusion test. The assay sensitivity in plasma was 2 ng/ml.

Results. Figure 2 shows the results with plasma in the normal subjects. Unfrozen

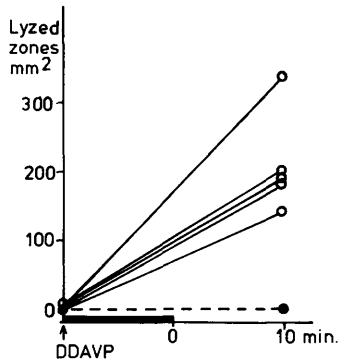

FIG. 2. The effect of 1-desamino-8-D-arginine vasopressin on plasminogen activator activity of plasma in normal subjects. Samples were obtained before and 10 min after the end of the infusion. Activator activity was measured on plasminogen-containing fibrin plates and expressed in mm² lyzed zones. ○, Plasma + control serum IgG (IgG-C); ●, plasma + melanoma activator antiserum (IgG-A).

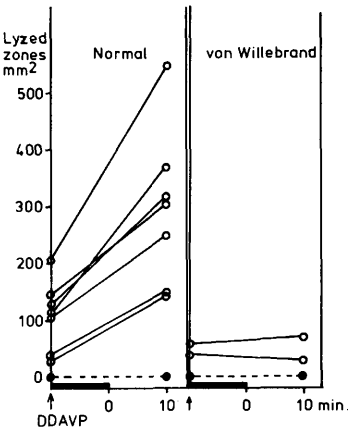

FIG. 3. The effect of 1-desamino-8-D-arginine vasopressin on plasminogen activator activity of resuspended euglobulin precipitates in seven normal subjects and two patients with severe von Willebrand's disease. Samples were obtained and activities expressed as in Fig. 2. ○, Euglobulin precipitates + IgG-C; ●, euglobulin precipitates + IgG-A.

plasma was tested on fibrin plates immediately after sampling. Most of the normal subjects had no detectable spontaneous fibrinolytic activity in their plasma before the infusion of DDAVP while the plasma from a few gave small lyzed zones. This activity was quenched by the specific anti-tissue activator antiserum (IgG-A). After the infusion the plasminogen activator invariably rose markedly in plasma from all the normals (Fig. 2). The activity could be completely quenched by IgG-A. Urokinase determined radioimmunologically was undetectable (<2 ng/ml) in the plasma of the six normals tested before as well as after DDAVP infusion.

Working with the corresponding ordinary euglobulin precipitates resting levels of plasminogen activator were regularly recorded in all the normals although they varied much (Fig. 3). The activator was invariably neutralized by the IgG-A. After administration of DDAVP the plasminogen activator rose to high levels, but here again, all the activity could be quenched by the antibodies. Urokinase was undetectable in the euglobulin precipitates from the three normals tested both before and after DDAVP infusion (≤2 ng/ml).

The dextran sulfate precipitates, in which the intrinsic activator system(s) is activated, were dissolved either in EDTA buffer with flufenamate or EDTA buffer without flufenamate, but added Cl inactivator. Flufenamate is said to inhibit coprecipitated Cl inactivator, thereby allowing intrinsic activators to express themselves in addition to extrinsic activator. When extra Cl inactivator is added, intrinsic activators should be neutralized and only extrinsic activator determined. In the system without Cl inactivator, IgG-A quenched only part of the activity in the samples taken before the infusion of DDAVP (Fig. 4). The part that could be neutralized by IgG-A increased after DDAVP infusion, while the part that could not be quenched remained unchanged (=intrinsic activator). In the system with added Cl inactivator some activity was present in the precipitates obtained before the infusion and it increased markedly after the infusion. IgG-A completely neutralized the Cl inactivator-resistant activities both before and after administration of DDAVP. Urokinase determined radioimmunologically was undetectable in the dextran sulfate precipitates (dissolved in flufenamate-

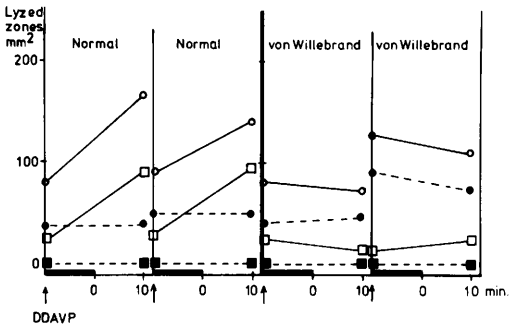

FIG. 4. The effect of 1-desamino-8-D-arginine vasopressin on plasminogen activator activity of resuspended dextran sulfate precipitates in two normal subjects and two patients with severe von Willebrand's disease. Samples were obtained and activities expressed as in Fig. 2. ○ dextran sulfate precipitates + IgG-C; ● dextran sulfate precipitates + IgG-A; □ dextran sulfate precipitates + IgG-C + Cl inactivator; ■ dextran sulfate precipitates + IgG-A + Cl inactivator.

containing EDTA buffer) before (<2 ng/ml) and certainly also after (≤2 ng/ml) DDAVP infusion (four normals tested).

Two patients with severe von Willebrand's disease were tested in the same way. They had no measurable level of plasminogen activator in plasma before or after DDAVP infusion. In the ordinary euglobulin precipitates a resting level of plasminogen activator was recorded. DDAVP did not induce any increase in this activator. IgG-A neutralized all the activator both before and after infusion of DDAVP (Fig. 3). In the dextran sulfate precipitates the total activators did not change after infusion of DDAVP, and the part that could not be quenched by IgG-A (=intrinsic activator) remained constant as did also the Cl inactivator-resistant activity (Fig. 4).

Discussion. Venous occlusion (19) and physical exercise (20, 21) normally raise the level of plasminogen activator in plasma. The activity induced by these stimuli is resistant to Cl inactivator indicating that it is of extrinsic vascular origin (17, 22). Rijken *et al.* (23) directly demonstrated that this activator is related to the tissue plasminogen activator purified from uterine tissue and present also in the vessel wall.

Our studies in the healthy volunteers showed that the fibrinolytic response to DDAVP infusion is due to release of the so-called extrinsic activator. In plasma and ordinary euglobulin precipitates, where the intrinsic fibrinolytic system is not appreciably activated, all the induced activity could be quenched by an antiserum against tissue activator, which is known to cross-react with the extrinsic vascular activator (23). In the dextran sulfate precipitates, which also include intrinsic activators, the specific antiserum quenched part of the activity before, and a larger part after, the DDAVP infusion while the remaining Cl inactivator-sensitive activity did not change. This shows that DDAVP does not affect the intrinsic fibrinolytic system. Furthermore, DDAVP did not release any urokinase, which was undetectable with a radioimmunoassay (sensitivity 2 ng/ml) before as well as after the infusions.

The effect of DDAVP thus resembles that of venous occlusion and physical exercise. Among pharmacological and physiological agents known to elicit a fibrinolytic response, DDAVP is especially interesting as it more or less completely lacks vasoactive properties. The release of extrinsic activator from the vessels must therefore be controlled by some other mechanism. The vascular activator is believed to be produced in endothelial cells (23, 24), and a direct effect of DDAVP on these cells may therefore be postulated.

DDAVP has also another interesting effect, i.e., a factor VIII-enhancing effect in normal subjects and in mild von Willebrand's disease (4–6, 25). This has been utilized therapeutically. In severe von Willebrand's disease, however, no such effect is apparent. VIIIR:Ag is normally synthesized in endothelial cells (26), but not in patients with severe von Willebrand's disease (27).

Recently it has been demonstrated that some patients with severe von Willebrand's disease are deficient in their fibrinolytic response to DDAVP as well as in factor VIII. This abnormality of fibrinolysis is seen in only some patients with severe von Willebrand's disease (probably the homozygotes of the autosomal recessive type) and not in

the heterozygotes or milder forms (6). In the present work we have studied only DDAVP-unresponsive patients and found that also such patients have a resting level of blood plasminogen activator that can be quenched by tissue activator antiserum as in normal subjects, and thus identical with the extrinsic activator. They also have an intrinsic activator system sensitive to Cl inactivator inhibition. DDAVP did not induce any increase either in the extrinsic activator or the intrinsic activators in the two patients tested. The intrinsic activators in von Willebrand's disease thus do not differ from those in normals. On the contrary, although patients with severe von Willebrand's disease can synthesize the extrinsic activator, they cannot increase it further in response to DDAVP, possibly owing to a defective receptor site or some other abnormality of the endothelial cell in addition to the factor VIII defect. The extrinsic activator responsible for the resting level in plasma in these patients may perhaps be synthesized in cells other than endothelial.

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