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A Highly Sensitive Immunoenzymometric Assay for the Determination of Angiogenin

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Summary: A polyclonal antibody to human recombinant angiogenin was prepared in rabbits using a $Pam_3CysSerGly$ conjugate. The antibody was then used to develop the first highly sensitive enzyme-labelled immunometric assay for this vascularisation inducing and tumour associated protein. The assay was suitable for quantification of angiogenin in body fluids between 2.5 and 0.05 µg/l. The mean intra-assay imprecision was 6.0% and the inter-assay imprecision 7.9%. Angiogenin in human plasma was found to lie in the range of 0.38 to 0.11 mg/l with a mean of 0.25 ± 0.07 mg/l.

Introduction

Angiogenin is a single chain M_r 14100 protein, first isolated and characterised from a HT-29 human adenocarcinoma cell line (1). It has been shown to be an inducer of vascular growth. The protein has 35% identity with pancreatic ribonuclease (2) and has been shown to inhibit protein synthesis in vitro (3). *Saxena* et al. described angiogenin as a cytotoxic, t-RNA-specific ribonuclease in the RNase A superfamily (4). Angiogenic and ribonucleolytic activities are blocked by a tight-binding placental ribonuclease inhibitor (5). Recently, it was shown that angiogenin supports endothelial and fibroblast cell adhesion (6). *Shapiro* et al. isolated angiogenin from human plasma (7). Its mRNA was detected predominantly in the adult liver, but was also detectable at a low level in other tissues (8) and cells (9). Detailed information on the presence of the protein in body fluids and tissues is not available due to the lack of a sensitive and easily performed assay.

Here we report the production of a polyclonal antibody, which was then used to develop the first sensitive immunometric assay for the determination of this protein. The method will facilitate an understanding of the role of angiogenin in physiological and pathophysiological processes.

Materials and Methods

Materials

Nunc Immuno plates II were obtained from Nunc (Roskilde, Denmark). Horseradish peroxidase[1]) was obtained from Boehringer Mannheim (Mannheim, Germany) and 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid (ABTS) from Sigma (Deisenhofen, Germany). Human recombinant angiogenin was kindly donated by Behringwerke, Marburg. Blood samples were supplied by Prof. *Kleesiek*, Herzzentrum Bad Oeynhausen (Bad Oeynhausen, Germany). *S*-[2,3-bis(palmitoyloxy)propyl]-N-palmitoyl-cysteinyl-serinyl-glycine-succinimidyl-ester (Pam_3CysSerGlyOSu) and S-[(2*RS*)-2,3-bis(palmitoyloxy)-propyl]-N-palmitoyl-cysteinyl-lysyl-lysyl-lysyl-lysine (Pam_3Cys-Ser-Lys_4) were kindly donated by Prof. *Jung* (Tübingen, Germany).

Methods

Preparation of the S-*[2,3,bis(palmitoyloxy)propyl]-N-palmitoyl-cysteinyl-serinyl-glycine/angiogenin (Pam₃CysSerGly/angiogenin) conjugate*

Recombinant angiogenin (0.5 mg) was mixed with 5 mg $Pam_3CysSerGlyOSu$ in 500 µl freshly distilled dimethylformamide and incubated under continuous stirring for 15 h at room temperature. After removal of the dimethylformamide, the product was dissolved in *t*-butanol/water (3 + 1, by vol.) and lyophilised (10).

[1]) Enzyme
Horseradish peroxidase:
donor: hydrogen-peroxide oxidoreductase (EC 1.11.1.7)

Preparation of the antibodies

$Pam_3CysSerGly$/angiogenin conjugate (1 mg) was mixed with 0.5 mg $Pam_3CysSerLys_4$ (11), dissolved in phosphate buffered saline and injected into rabbits subcutaneously without additional adjuvants. Further booster immunisations were carried out in the same manner. The antibodies were purified according to the method of *Harboe & Ingild* (12) by salting out and ion exchange chromatography on DEAE-Sepharose. The IgG-fraction was used in the assay.

Conjugation of horseradish peroxidase to the IgG

The polyclonal IgG was reacted with horseradish peroxidase by a method described by *Tijssen & Kurstak* (13).

Two-site immunoenzymometric assay

Microtitre plates were coated with 100 µl antibody at 4 °C overnight at a concentration of 2 mg/l in coating buffer (50 mmol/l sodium carbonate, pH 9.6). After twice washing with phosphate buffered saline/Tween (0.2 mol/l sodium phosphate, pH 7.4, 0.15 mol/l NaCl, 0.5 g/l Tween 20) standards and samples were diluted in phosphate buffered saline/Tween containing 10 g/l bovine serum albumin and incubated overnight at 4 °C or for 2 h at room temperature. Both incubation times gave similar results. After 4 washes with phosphate buffered saline/Tween, 100 µl of the conjugate solution (IgG/peroxidase-conjugate 1 : 2000 in phosphate buffered saline/Tween containing 10 g/l bovine serum albumin) were placed in each well and incubated for another 2 h at room temperature. After 6 washes with phosphate buffered saline/Tween, the substrate reaction was performed by incubation with ABTS-solution (1 g/l in 0.1 mol/l citric acid, adjusted to pH 4.2 with Na_2HPO_4, 0.5 g/l Tween 20, 1.3 mmol H_2O_2). Absorbance was measured using a Dynatech Microreader MR 4000 (Denkendorf, Germany) at 405 nm. We always used the same antibody for immobilisation and for peroxidase-labelling.

Recovery experiment

Recovery of angiogenin in plasma. The antigen standards, diluted in 0.2 mol/l phosphate-buffered saline/Tween/bovine serum albumin, were added to the prediluted plasma samples. This mixture was serially diluted in 8 steps and analysed by immunoenzymometric assay. Means from those dilutions giving response of $A_{405\,nm}$ between 0.2 and 1.5 were selected.

Plasma analysis

Plasma samples of healthy donors were stored at −75 °C until required. A predilution of 1 : 100 was chosen for measurement. All tests, performed twice on two different days, resulted in deviations not above the inter-assay coefficients given in table 1.

Results

Production of antibodies

The $Pam_3CysSerGly$/angiogenin conjugate in combination with $Pam_3CysSerLys_4$ as adjuvant was very effective in the production of antibodies in rabbits. Remarkably high titres remained constant for over one year, and more than 0.5 g of antibody could be gained from one rabbit.

Sensitivity and precision

The standard curve for angiogenin was used to optimize reaction conditions (fig. 1). The conditions which gave a good change in colour with the change in concentration are given above (fig. 2). Intra-(within)-assay run and inter-(between)-run standard deviations (SDs) and coefficients of variation (CVs) are summarised in table 1. The working range was calculated according to the method of *Porstmann & Kiesig* (14). Angiogenin can be assayed in the concentration range between 2.5 and 0.05 µg/l. Using plasma from several

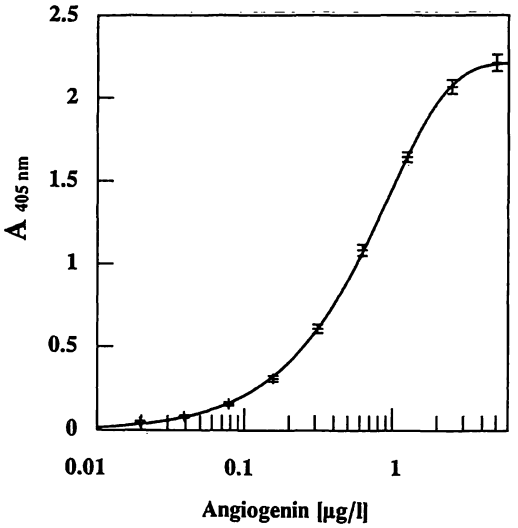

Fig. 1. Standard curve for the sandwich ELISA of angiogenin. The standard curve was determined under optimised assay conditions (see Methods). Standard deviations were calculated from the absorbence values.

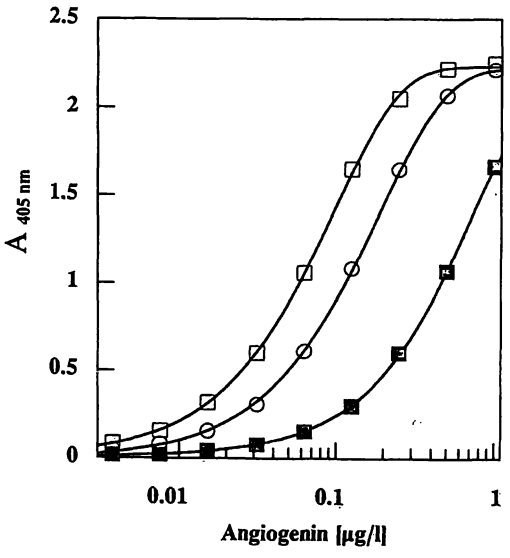

Fig. 2. Standard (○) and sample curves (■ □) for the sandwich ELISA of angiogenin. All samples were serially diluted in 0.2 mol/l phosphate buffered saline/Tween/bovine serum albumin.

Tab. 1. Precision of the sandwich ELISA for the determination of angiogenin in phosphatase-buffered saline/Tween/bovine serum albumin. In order to ascertain the precision, profile concentrations were determined from the absorbance values using prediluted plasma samples at different concentrations. These concentrations were used to calculate the standard deviations (SD) and coefficients of variation (CV).

Concentration (µg/l)	Intra-assay imprecision (n = 8)		Inter-assay imprecision (n = 12)	
	SD (µg/l)	CV (%)	SD (µg/l)	CV (%)
1.700	0.135	7.9	0.145	8.5
0.850	0.040	4.7	0.066	7.9
0.425	0.023	5.4	0.037	8.6
0.213	0.013	6.1	0.014	6.5

donors, it was confirmed that the results always showed the same proportional response to dilution in donor plasma and the reference material. Thus, it is acceptable to record results from the reference curve (15). The mean analytical recovery tested for concentrations between 2.5 and 0.16 µg/l was 99%, which was very good for this method.

Determination of angiogenin concentrations in plasma

In order to determine normal ranges for angiogenin 65 plasma samples from healthy donors were analysed. The measurement was unaffected by repeated freezing of the sample.

Angiogenin in the plasma was found in the range of 0.38 to 0.11 mg/l with a mean of 0.25 ± 0.07 mg/l (fig. 3). There was no significant difference in the concentrations determined in the plasma of female or male donors.

Discussion

In this paper, we present the first highly sensitive assay for angiogenin. The antibodies prepared by the use of a novel adjuvant were of good affinity and titre. The sensitivity of the assay was about 300 times higher than that of a previously reported assay, which was based on the binding of angiogenin to a placental ribonuclease inhibitor and measurement of its ribonucleolytic activity (16). The ELISA procedure described here is much easier and faster to perform. Angiogenin has 35% identity with human pancreatic ribonuclease (2). Since this value is not very high, cross-reactivity with this ribunuclease is not to be expected.

Shapiro et al. (7) reported the isolation of angiogenin from human plasma with a yield of 60–150 µg/l. From our studies, there is no doubt that they must have lost some protein during their purification procedure. In another paper *Bicknell* et al. (17) reported a value of 400 µg/l in human plasma without giving the assay procedure or number of samples. Our ELISA procedure represents the first highly reliable routine determination of angiogenin in plasma samples and other body fluids.

Angiogenin is a constituent of normal plasma from healthy donors (7). Its participation as a regulatory component in vascular growth and blood vessel formation is still unresolved, since vascular growth is rapid in the developing rat fetus though angiogenin mRNA levels are low or undetectable (8). Angiogenin may, however, be involved in such processes in adults or during wound healing, since the chick chorioallantoic assay responds to as little as 35 fmol per egg. New blood vessels are formed in the rabbit cornea following application of 3.5 pmol angiogenin (2). Angiogenin may also be a serum tRNase and it may hydrolyse tRNA-like molecules outside cells, and perhaps also inside cells when such molecules gain entry

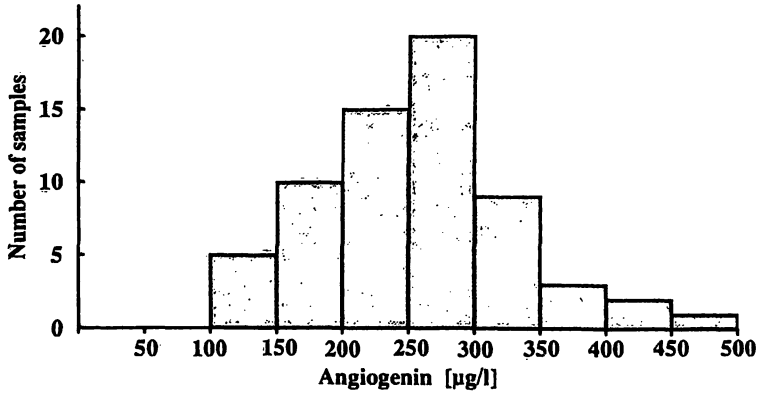

Fig. 3. Distribution of angiogenin in human plasma from healthy donors (n = 65).

during certain physiological or pathological processes. A cytotoxic effect was reported for angiogenin when injected into *Xenopus* oocytes, and this was related to its cellular tRNase activity (4). Angiogenin activates phospholipase C, elicits a rapid incorporation of fatty acids into cholesterol esters in vascular smooth muscle cells (18), and depresses the concentration of cAMP in aortic smooth muscle cells by a mechanism that is sensitive to pertussis toxin (19).

Since angiogenin is a tumour-derived protein with in vivo angiogenic activity, first isolated from an established human adenocarcinoma cell line (1), it may also serve as a tumour marker for certain malignant carcinomas. The ELISA established here will provide an important tool for assaying the angiogenin concentrations of plasma, tissues and tissue fluids, in order to correlate its concentration with pathological events.

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