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Differential tissue expression of Annexin VIII in human

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Abstract

The expression of Annexins V and VIII by human lung, liver, kidney, skin, heart, uterus, spleen and skeletal muscle was investigated by ELISA. All investigated tissues contained Annexin V. Its level varied with the tissue from around 5 μg (skin) to approximately 120 μg (spleen) per g of wet tissue. Contradistinctionally Annexin VIII expression was less ubiquitous and less abundant. Only lung, skin, liver and kidney expressed Annexin VIII. Its levels were approximately 100-fold less then the Annexin V levels. Immunohistochemical analysis of lung sections revealed Annexin VIII presence exclusively in the endothelia. Annexin V and VIII levels of cultured human umbilical vein endothelial cells, human arterial smooth muscle cells, human lung fibroblasts and HeLa cells were measured by ELISA. All cell types expressed Annexin V whereas only HeLa cells had detectable levels of Annexin VIII. The results indicate a tissue specific expression of Annexin VIII by lung endothelium, suggesting a highly specialised function.

Key words: Annexin V; Annexin VIII; Tissue distribution; Human; ELISA; Immunohistochemistry

1. Introduction

Annexins constitute a multigene family of proteins defined by a repeated sequence motif initially termed the endonexin loop [1]. Proteins containing this motif have been identified in animals from widely separated phyla such as *Poriphera* [2], *Cnidaria* [3], *Arthropoda* [4] and *Chordata* [5–13]. Within the subphylum of vertebrates a high structural conservation of individual Annexins appears between different species. Evolution of the family is hypothesised to have occurred by duplication events of a progenitor gene containing the code for this motif [14]. The sequence motif is directly related to the functional properties of calcium and phospholipid binding [15,16]. Diversification of the family into at least 10 different Annexins in animals has probably emerged from the physiological need to diverse on the central theme of calcium and phospholipid binding. The physiological significance of the family as a whole and of the individual members in particular, however, are still not fully understood in spite of the wealth of available data (for reviews see [17–19]).

Up to now Annexin VIII is amongst the least described members of the family. It was initially discovered as a gene product by a screening procedure of a human cDNA library with a probe specific for Annexin V [11]. Recombinant Annexin VIII appeared to exhibit the Annexin typical biological properties like Ca^{2+}-dependent phospholipid binding and inhibition of phospholipase A_2 and prothrombinase activity [11]. Recently the native protein was purified from human placenta [20] where it accounts for less than 1% of the Annexins. In this respect Annexin VIII appears less abundant and less ubiquitous as other Annexins, which points to a specialised function. Recently Annexin VIII gene activation was reported in various leukemic and lymphoid cells [21,22]. It was hypothesized that Annexin VIII plays a unique role in the proliferation and/or differentiation of these cells [21].

This paper reports on an investigation of the expression of Annexin VIII protein by various human tissues and its localisation as determined by ELISA and immunohistochemistry, respectively, using polyclonal antibodies that were raised against recombinant Annexin VIII.

2. Materials and methods

2.1. Proteins

Recombinant Annexin V and VIII were obtained from *Escherichia coli* transformed with pRH291 and pRH292, respectively, as described elsewhere [11,23]. Monoclonal and polyclonal antibodies against recombinant Annexin V were produced as described elsewhere [24].

2.2. Cell cultures

Human umbilical vein endothelial cells (HUVEC) were isolated from veins of freshly obtained human umbilical cords and cultured according to Jaffe et al. [25] on fibronectin (a kind gift of Dr. J. van Mourik, Amsterdam) coated tissue culture flasks in culture medium (RPMI 1640; Flow Lab.), supplemented with 20% newborn calf serum (NCS; Gibco), heparin (50 μg/ml; Sigma H.8514), bovine brain Endothelial Cell Growth Factor Supplement (20 μg/ml; Boehringer Mannheim), L-glutamine (2 mM; Flow Lab.), streptomycin (100 μg/ml) and penicillin (100 IU/ml). Arterial smooth muscle cells (SMC) were derived from tissue explants of human umbilical cord arteries as described by Ross [26]. The SMC were further cultured on fibronectin coated tissue culture flasks in culture medium without heparin and ECGF. Human embryonic lung fibroblasts (Cell line Flow 2002) and HeLa cells (cell line ATCC CCL2) were cultured on uncoated tissue culture flasks in

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Eagle's MEM, supplemented with 1% non-essential amino acids, 10% NCS, L-glutamine (2 mM), streptomycin (100 μg/ml) and penicillin (100 IU/ml).

2.3. Annexin VIII antibody preparation

Polyclonal antibodies against recombinant Annexin VIII were raised in rabbits using standard procedures. The IgG fraction of the antiserum was enriched by $(NH_4)_2SO_4$ precipitation. Immunoaffinity purified antibodies (IA-Ab_{VIII}) were obtained by chromatography of the IgG fraction using a column of Annexin VIII, that was immobilized on Affigel- 15 (BioRad). IA-Ab_{VIII} were conjugated with NHS-LC-biotin (Pierce) according to the instructions of the supplier.

2.4. Annexin V and VIII specific ELISA

The Annexin V specific ELISA is based on a polyclonal antibody as a catching antibody and a monoclonal antibody as a detecting antibody and is described elsewhere [25]. The annexin VIII specific ELISA is based on IA-Ab_{VIII} and is described as follows: 96-well maxisorp plates (Nunc) were coated with IA-Ab_{VIII} by incubating each well with 100 μl of 2.5 μg IA-Ab_{VIII}/ml of $NaHCO_3/Na_2CO_3$, pH 9.0, during 2 h at 37°C. The coated wells were further blocked for aspecific protein binding sites with PBT (phosphate buffered saline, containing 1 mg/ml bovine serum albumin and 0.05% Tween 20 (w/v)). Samples were then added to the wells and incubated for 1.5 h at ambient temperature. The wells were washed free of unbound protein with PBT before the addition of biotinylated IA-Ab_{VIII}, that was diluted in PBT. Incubation was continued for 1 h at ambient temperature. After washing with PBT, streptavidin-peroxidase (Amersham RPN 1231; 1:500 diluted in PBT) was added to each well and incubated during 1 h at room temperature. The amount of bound peroxidase per well was assessed by staining with *o*-phenylene diamine, that was diluted to 2 mg/ml in 0.1 M sodium citrate, pH 5.0, containing 0.0025% H_2O_2. The reaction was stopped with 4 N H_2SO_4 and optical density was measured at 492 nm.

2.5. Annexin VIII quantitation in human tissues and cultured cells

Human tissues were obtained after autopsy within 2 h postmortem. The tissues were solubilised to a 25% mixture (w/v) in ice-cold homogenisation buffer (50 mM Tris-HCl, pH 7.9, 4 mM EDTA, 5 mM benzamidin and 5 μM soybean trypsin inhibitor) by ultrasonication. The mixture was then centrifuged for 5 min at 10,000 × *g* and the supernatant was assayed in the Annexin V and VIII ELISAs.

Cultured human fibroblasts, human arterial smooth muscle cells, human umbilical vein endothelial cells and HeLa cells were lifted from the culture dish by mild trypsinisation and washed with PBS. The cells were finally suspended in homogenisation buffer at 10^6 cells/ml and disrupted by ultrasonication. After centrifugation for 5 min at 10,000 × *g* the supernatants were assayed in the Annexin V and VIII ELISAs.

2.6. Immunohistochemistry

Human tissue fragments were fixed in 4% neutral buffered formalin, processed and 4 μm paraffin sections were deparaffinized, rehydrated and treated with 0.6% H_2O_2 in methanol to block endogenous peroxidase activity. Endogenic biotin and avidin binding were also blocked using a commercially available Avidin/biotin blocking kit (Vector SP-2001). Diluted anti-Annexin VIII antibody was incubated with the tissue section in humidified air for 60 min at room temperature. After extensive washing with Tris-buffered saline at pH 7.5 (TBS), a diluted biotin labelled goat anti-rabbit IgG antibody (Dako, Denmark) was incubated for 60 min at room temperature. Subsequently after extensive washing with TBS diluted streptavidin-biotin-HRP solution (Dako, Denmark) was added and incubated for 30 min. Immunoreactivity was visualised by incubation with diaminobenzidine-H2 substrate.

3. Results

3.1. Annexin VIII specific ELISA

With an Annexin VIII specific polyclonal antiserum, that was used in unbiotinylated form as a catching antibody and in biotinylated form as a detecting antibody, an ELISA was constructed to quantify Annexin VIII in biological samples. The ELISA measured Annexin VIII in the range from 0.5–15 ng/ml (Fig. 1). Homology between the primary structures of the different members of the Annexin family is high in specific domains and the presence of other Annexins in the samples could give therefore false positive signals in the Annexin VIII ELISA. Annexins I through VI were purified from human placenta and identified by their respective antibodies in Western blot procedures (data not shown). When assayed by the Annexin VIII ELISA, Annexins I–VI up to 1 μg/ml did not produce signals above the blank value. When 10 ng/ml of Annexin VIII was spiked into 1 μg/ml of another Annexin solution the added amount of Annexin VIII was fully recovered by the ELISA.

3.2. Annexin V and VIII contents in human tissues

The procedure to prepare samples of the human tissues for the ELISA involved ultrasonication in buffer. In order to exclude the possibility that this procedure would affect the response in the ELISA, reference curves were prepared with purified Annexins V and VIII, that had received the same treatments. It appeared that this way of sample handling had no effect on the response in the ELISAs.

Table 1 lists the human tissues, that were investigated by the ELISAs, together with the measured contents of Annexins V and VIII. All tissues contained Annexin V, whereas Annexin VIII was detectable only in lung, skin, liver and kidney. When Annexin VIII was spiked into the tissue extracts to a level of 1 ng/ml this amount was fully recovered by the ELISA, which indicates once more the specificity of the Annexin VIII ELISA.

In the positive tissues Annexin VIII levels were approximately 70–100 times less than Annexin V levels. The Annexin VIII contents was the highest in lung tissue as was the Annexin VIII over Annexin V ratio of this

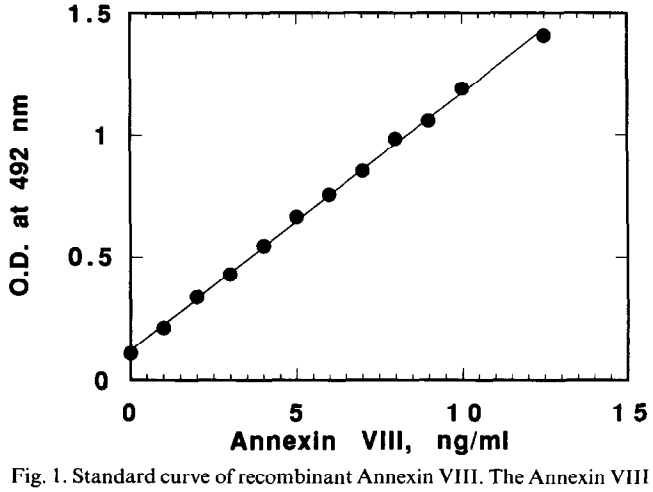

Fig. 1. Standard curve of recombinant Annexin VIII. The Annexin VIII specific ELISA was performed with purified recombinant Annexin VIII as described in section 2.

Fig. 2. Immunohistochemical staining of a frozen section of human lung. A frozen section of human lung was incubated with anti-Annexin VIII antibodies. Specifically bound antibodies were visualised with anti-rabbit antibodies conjugated to horseradish peroxidase as described in section 2.

tissue. Western blot analysis revealed that the lung tissue contained an anti-Annexin VIII positive band of 35 kDa, that comigrated with recombinant Annexin VIII (not shown). No other bands were visible.

3.3. *Immunohistochemical localisation of Annexin VIII in human lung tissue*

The human lung was further investigated for its expression of Annexin VIII. Using immunohistochemical techniques on 4 μm sections of human lung it was shown that the endothelium of venous as well as arterial vessels stained with IA-Ab_{VIII} (Fig. 2). The other cell types appeared negative for IA-Ab_{VIII}. Staining of the lung endothelium was obliterated by preincubation of the primary antibody with purified Annexin VIII.

3.4. *Annexin VIII expression by cultured cells*

The expression of Annexin VIII appeared to be restricted to a few tissues and, within the lung tissue, to the endothelial cell. We investigated a number of cultured cell types by the ELISA procedures. In agreement with the tissue distribution profile Annexin V was expressed by all cell types investigated (Table 2). On the other hand, Annexin VIII was not detectable in smooth muscle cells and lung fibroblasts as expected but was also not detectable in HUVEC. This finding suggests that endothelial expression of Annexin VIII is determined by the tissue environment. HeLa cells, a human epitheloid carcinoma cell line, express Annexin VIII at a level which is about 14 times lower than the Annexin V level. Western blot analysis with anti-Annexin VIII antibodies revealed a 35 kDa band, that comigrated with recombinant Annexin VIII (not shown). No other bands were visible.

4. Discussion

The genetic information of Annexin VIII, a four domain structured member of the Annexin family, has been discovered in a human placenta cDNA library by a

Table 1
Annexin V and VIII contents in human tissues as measured by ELISA

Tissue sample	Annexin V[a]	Annexin VIII[a]	VIII/V
Lung	16.1	0.23	0.014
Skin	5.3	0.07	0.014
Liver	19.3	0.17	0.009
Kidney	18.2	0.09	0.005
Heart	11.5	N.D.[b]	–
Skeletal muscle	5.9	N.D.	–
Uterus	38.8	N.D.	–
Spleen	122	N.D.	–

The numbers presented are the mean of duplo's of two separate determinations.
[a] Numbers are expressed in μg Annexin per g of wet tissue.
[b] Not detectable.

screening procedure that employed a probe designed to pick up Annexin V cDNA [11]. Prior to discovery of the native Annexin VIII protein its recombinant counterpart was expressed in *Escherichia coli* and investigated for several biological properties. These studies show that the Annexin VIII message encodes a protein, that displays biological activities, which are typical for Annexins, e.g. the Ca^{2+}-dependent phospholipid binding and the inhibition of phospholipase A_2- and prothrombinase activity. Whether these properties reflect the physiological function of Annexin VIII has not yet been subject of investigations since the native Annexin VIII escaped detection in tissues until recently.

Pepinsky and Hauptmann have detected Annexin VIII protein in placental tissue [20]. In contrast to the other members Annexin VIII mRNA [11] and protein [20] are expressed at low levels in this tissue. Previously Tsao et al. reported a partial sequence of a rabbit lung protein (33000 PBLP), which shares 88% homology with the first repeat of Annexin VIII [27]. On basis of the observed high interspecies conservation of several Annexins [7] this would indicate that 33000 PBLP is the rabbit homologue of human Annexin VIII. However, the presented purification data [28] allow the estimation that one New Zealand rabbit lung contains at the least 0.5 mg of 33000 PBLP, a level which is beyond that of Annexin VIII in placenta [20] and human lung (this report). Hence, in spite of the high sequence similarity the 33000 PBLP is probably distinct from Annexin VIII.

This paper reports the expression of Annexin VIII protein in various human tissues and compares this with the expression of Annexin V, which is known to occur widely spread and abundantly in the organism [29]. To this end an Annexin VIII specific ELISA was constructed with rabbit polyclonal antibodies raised against the recombinant Annexin VIII. With the ELISA technique it is shown that Annexin VIII expression is limited both in the sense of tissue distribution and concentration per tissue. In these respects Annexin VIII is discerned from most other members, which are widely spread and abundantly expressed in the organism. In comparison to Annexin V, Annexin VIII is expressed at levels, which are around two orders of magnitude lower. The abundant and ubiquitous presence of the first suggests its participation in the cellular basic machinery. Along this line of reasoning Annexin VIII would be a candidate for a highly specialised function accomodating tissue and cell-type specific requirements. This notion is strengthened by the fact that Annexin VIII is not expressed by every cell-type and for a given cell-type not in every tissue. The endothelial cells of the lung contain Annexin VIII, whereas it appears to be absent in other cell-types of the lung. Moreover, Annexin VIII appears not to be a common property of the endothelial cell since endothelial rich tissues like heart and spleen, and cultured HUVEC lack this protein [this report,30]. Tissue and cell-type specific expression has been recognised in vertebrates [31] and in lower organisms [32] for some other members of the Annexin family. The physiological roles of these Annexins are also not yet defined but are sought in the region of highly specialised functions.

Table 2
Annexin V and VIII contents in cultured cells as measured by ELISA

Tissue sample	Annexin V[a]	Annexin VIII[a]	VIII/V
HUVEC	2.08	N.D.[b]	–[b]
Smooth muscle cell	2.24	N.D.	–
Fibroblast	4.44	N.D.	–
Hela cell	0.94	0.069	0.073

The numbers presented are the mean of duplo's of three separate determinations.
[a] Numbers are expressed in μg Annexin per mg of total cellular protein.
[b] Not detectable.

No biological activities of the native Annexin VIII have been reported so far. The recombinant product exhibits Ca^{2+}-dependent phospholipid binding and based hereon anti-phospholipase A_2 and prothrombinase inhibitory activity and phospholipid vesicle aggregating capacity (CPMR; unpublished results). These activities are also expressed by other Annexins and have led to postulation of physiological roles in the areas of inflammation [33], haemostasis [34,35] and exocytosis [36].

Activation of the Annexin VIII gene has recently been reported for a primary leukemic cell [21] and several leukemic and lymphoid cell lines [22]. Coagulopathien are mostly observed in patients suffering from acute promyelocytic leukemia type M3. As these myeloid cells express Annexin VIII gene transcripts it has been suggested that the Annexin VIII protein is directly associated with the bleeding disorder of these patients. These myeloid cells shut-down the Annexin VIII gene when they are directed towards maturation by agents like all trans retinoic acid and phorbol ester [21,22]. This suggests that Annexin VIII is linked to the machineries that determine proliferation and differentiation. Whether Annexin VIII in these cells is a marker of proliferation and differentiation or a factor driving or regulating one of these processes is unknown. HeLa cells with epithelial like morphology, that proliferate continuously, and the lung endothelial cells, which likely have a very low proliferative index, both express Annexin VIII. Hence, Annexin VIII expression may occur in differentiated as well as undifferentiated cells and in proliferating as well as non-proliferating cells. The direction in which the search for the function(s) of Annexin VIII should be pursued is not yet clear. Elucidation of the biochemical characteristics and the subcellular localisation of the native protein would mean a step further.

In conclusion, Annexin VIII displays a unique tissue and cellular distribution in human. When expressed it occurs at relatively low levels. All together these data

indicate a highly specialised function(s) accomodating specific tissue and cell-type dependent requirements.

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