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Purification and Determination of in vitro and in vivo Activities of Recombinant Human Annexin I

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I. INTRODUCTION

Annexins(also called as lipocortins) are widely distributed Ca^{2+}-dependent phospholipid binding proteins that have been implicated to play various physiological roles including phospholipase A_2 (PLA_2) inhibition, membrane fusion, anti-inflammation, anti-coagulation, cell differentiation, cell adhesion, exocytosis and interaction with cytoskeletal proteins.[1-6] However, a well-defined biological function has not been determined for any of the annexins. Recently, annexin V has been proposed to be a specific inhibitor of protein kinase C.[7]

Annexin I has been implicated as a glucocorticoid steroid induced protein which mediates the anti-inflammatory function of glucocorticoid through PLA_2 inhibition.[1,8] Annexin I is a monomeric protein of 37 kDa with in vitro PLA_2 inhibitory activity.[9] Annexin I is also known to be a major substrate for epidermal growth factor receptor kinase.[10,11]

The mechanism of PLA_2 inhibition by annexin I has not been fully understood. In vitro studies on the effect of annexins on a 14 kDa PLA_2(secretory form) from porcine pancreas have indicated that PLA_2 inhibition may be related to depletion of the substrate and not to specific interaction of the enzyme with the inhibitor.[12,13] This result is contradictory to result of in vivo test which showed anti-inflammatory effect of annexin I against carrageenin-induced rat paw edema.[8,14] Since other types of PLA_2s with various molecular sizes are present in a variety of mammalian cells,[15-18] elucidation of detailed in vitro and in vivo mechanisms of PLA_2 inhibition by annexin I need to wait further studies.

Annexin I has been isolated from various sources including human placenta, porcine lung and bovine lung[5,6,19]. The production of recombinant annexin I in E. coli has also been reported by Wallner.[9] In this report, they have indicated that the proteins were expressed in E. coli in the form of insoluble aggregates.[9]

Previously, we have shown that recombinant annexin I was expressed as a soluble protein in E. coli.[20] Needless to say, the processes for the purification of recombinant proteins largely depend upon the form of the protein in E. coli cells.

We designed a very simple process for the purification of recombinant annexin I which was produced as a soluble protein in E. coli cells. The activity of annexin I was demonstrated by the in vitro PLA_2 inhibitory activity and in vivo anti-inflammatory effect against rat paw edema.

II. MATERIALS AND METHOD

Purification of recombinant Annexin I

Recombinant plasmid pHT1 which express human annexin I in E. coli has been described.[20] E. coli C600 was used as a host strain. Recombinant annexin I was purified from E. coli C600/pHT1 by the methods analogous to the purification of annexin I from human placenta[19] with some modifications. All steps were carried out at 4℃ unless otherwise indicated.

E. coli C600/pHT1 was cultured in a benchtop fermanter (Microferm fermenter, New Braunswick Scientific Co., Inc.) under 0.5 ml/min air flow, 200 rpm agitation speed at 37℃ for 16－20 hr. Cultured cells were harvested by centrifugation at 3000 g for 15 min.

Step 1: Precipitation of annexin I with membrane

Approximately 70 g of wet cells were resuspended in 350ml of ice-cold buffer containing 25 mM Tris/HCl,pH 7.7, 1 mM DTT, 1 mM PMSF, 1 mM $CaCl_2$ and 1 ㎎/㎖ lysozyme, and disrupted by sonication with cell disruptor VirSonic 300 (The Virtris Co., Inc.). The soluble cell debris, nuclei and mitochondria were removed by centrifugation for 30 min at 20,000 g, and membraneous material was collected.

Step 2: EGTA extraction

The pellet was washed with extraction buffer(25 mM Tris/HCl,pH 7.7, 5 mM EGTA, 10 % glycerol), resuspended in the same buffer and soaked with gentle shaking for 16 hr. The solution was ultracentrifugated for 60 min at 100,000 g and supernatant was collected.

Step 3: DEAE flow through

The EGTA-extract was subjected to flow through a DEAE-cellulose (DE－52, Whatman) column previously equilibrated with 25 mM Tris/HCl,pH 7.7, 1 mM EDTA. A 5x16 cm column was used and flow rate was 0.5 ml/min. Flow-through fractions of 4 ml were collected and monitored for protein concentration. Aliquots of selected fractions were analyzed by 12 % SDS-PAGE. Fractions containing proteins of 37 kDa were pooled and concentrated by ultrafiltration (Amicon Ultrafiltration Kit, PM 10), and the concentrate was clarified by centrifugation for 15 min at 20,000 g.

Step 4: Gel filtration chromatography

The flow-through concentrates of 6 ml were fractionated by gel filtration chromatography on a Sephacryl S－300 (2.5x90 ㎝) with 25 mM Tris/HCl,pH 7.7, 1 mM EDTA, 0.15 M NaCl which was run at a flow-rate of 0.12 ml/min. Fractions of 3.0 ml were collected and analyzed by 12% SDS-PAGE. Fractions of major peak were pooled and concentrated by ultrafiltration.

Step 5: Hydroxylapatite chromatography

The concentrate was dialyzed against 10 mM KH_2PO_4/K_2HPO_4, pH 7.7, 1 mM EDTA, and applied to hydroxylapatite column (Bio-Rad. 2.5x16 cm) previously equilibrated with the dialysis buffer. The column was washed with 160 ml of the dialysis buffer and protiens were eluted with 720 ml of linear gradients of 10 t0 200 mM KH_2PO_4/K_2HPO_4,pH 7.7, 1 mM EDTA at a flow rate of 1 ml/min. Fractions of 4 ml were collected and analyzed by SDS-PAGE. The fractions of single band on SDS-PAGE were pooled. The solution was concentrated by ultrafiltration and stored in stroage buffer (25 mM Tris/HCl,pH 7.7, 1 mM EDTA, 10 % glycerol) at -70℃.

Gel electrophoresis and protein determination

SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli.[21] After electrophoresis, proteins were visualized by staining

with 0.1% Coomassie brilliant blue R−250.

The concentration of annexin I was determined from A_{280} using calculated extinction coefficient of 18,600 $M,^{-1}$ which was determined on the basis of the deduced amino acid composition.

PLA_2 inhibition assay

PLA_2 from porcine spleen with apparent molecular weight of 100 kDa was provided by Dr. Dae Kyung Kim of Massachusetts General Hospital. Details of the purification of the 100 kDa PLA_2 has been described[18]. PLA_2 assay was performed using a method described previously[18] with a slight modification. The 10−20 nmole of L−3−phosphtidylcholine, 1−stearoyl−2−[1−^{14}C] arachidonoyl(2−AA−PC, 56.0 mCi/mmole, Amersham international plc., U.K.) was dried under nitrogen and suspended in 0.5−1.0 mℓ of distilled water by bath-type sonicatior (Ultrasonik 300, NEY) for 3x10 sec. Substrate solution containing 0.33 nmole of the phospholipid was incubated for 60 min at 37℃ with 10 ng of purified porcine spleen PLA_2 and the specified amount of the inhibitor protein in a final volume of 200$\mu\ell$ assay buffer of 75 mM Tris/HCl (pH 7.4) containing 1 μM free calcium (prepared with EGTA/$CaCl^2$ buffering system) and 1 mg/ml of fatty acid−free bovine serum albumin (Sigma Chemical Co., St. Louis, MO., U.S.A.). Reaction was stopped by adding 1.25 ml of Dole's reagent[22](2% N-H_2SO_4, 20% n-heptane, 78% isopropanol) and the hydrolysed free arachidonic acid was extracted by the method of Horigome et al.[23] In all analyses, samples were tested in triplicate and adjusted for nonspecific release by subtracting the control value in which preparations were assayed without phospholipase A_2. The release of radioactive arachidonic acid was determined by liquid scintillation counting. The results of the test samples were compared to those obtained with control preparations which were performed without annexin I. The effects of annexin I was represented by the percentage of phosphlipase A_2 activity using an equation described below,

% of PLA_2 activity = (cpm test/cpm control)x100

Anti-inflammatory effects on carrageenin-induced rat paw edema

Experiments were carried out as described elsewhere with a slight modification.[8,14] 0.1 mℓ 1% Carrageenin suspension was injected locally into the paw of male Sprague-Dawley weighing between 140-180 g. Annexin I solution was injected into the peritoneal cavity. Measurement of paw volumes were made at zero time and every hour up to 5 hr using plethysmographic techniques. Values represented were the mean of percent swelling of 5 rats in each case.

Ⅲ. RESULTS AND DISCUSSION

Purification of annexin I

In designing a simple process for purification of annexin I from E. coli, we took advantage of its property of membrane binding in a Ca^{2+} dependent manner. At first, annexin I was coprecipitated with E. coli membrane by centrifugation in the presence of 1 mM Ca^{2+}, then the membrane bound proteins were extracted by soaking the pellet in the presence with the Ca^{2+} chelator EGTA. Figure 1 shows the protein profile after each purification step. A 37 kDa band was enriched in the soluble fraction by the EGTA extraction from the cell extract of E. coli C600/pHT1(Fig. 1, lane 3). This band was not observed in the cell extract of E. coli C600(without plasmid) treated analogously(Fig. 1, lane 2). These results indicate that the 37 kDa protein is likely to be annexin I. Attempt to test activity of annexin I of these samples by the PLA_2 inhibitory effect failed since not only the cell extract from E. coli C600/pHT1 but also the one from E.

coli C600 showed the PLA_2 inhibition. This may be due to presence of membrane debris which complicates the PLA_2 assay. Therefore, annexin I was monitored by its size during the entire purification procedures.

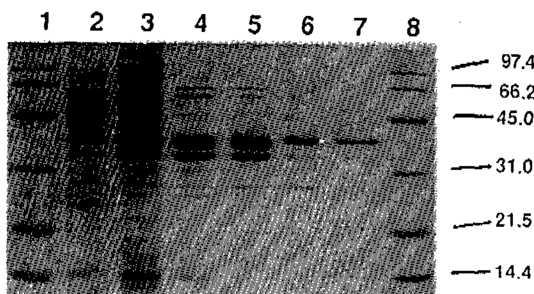

Fig 1. Analysis of samples of each step in purification procedure by SDS-PAGE. Lane 1,8, molecular weight marker; 2, EGTA-extract from E. coli C600;3, EGTA-extract from E. coli C600/pHT1;4, DE-52 flow-through;5, Sephacryl S-300 major peak;6, Hydroxylapatite major peak;7, Storage pool of purified sample;

Annexin I was further enriched by application of the EGTA extract onto a DE-52 ion exchange column. Annexin I was not bound to the resin and eluted from the column under the experimental conditions(Fig. 1, lane 4). The flow-through fractions were pooled, concentrated, and loaded onto a gel filtration column. Fig. 2 shows

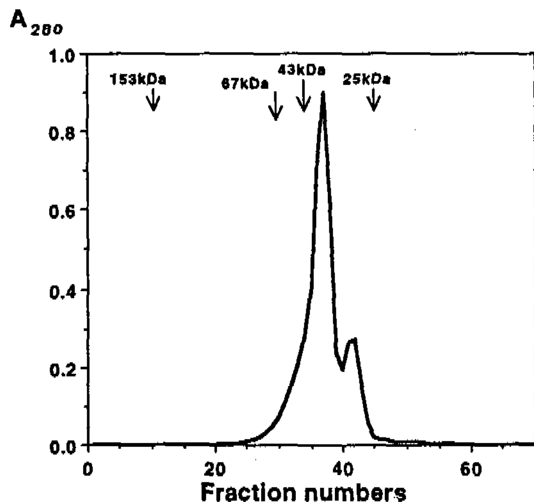

Fig 2. Protein profile from Sephacryl S-300 column chromatography. The DE-52 flow-through was concentrated to 6 ml by ultrafiltration and loaded onto the column equilibrated with 25 mM Tris/HCl,pH 7,7, 1 mM EDTA, 0.15 M NaCl.

the result from the Sephacryl S-300 chromatorgaphy. Annexin I was eluted in the major peak, and a protein of smaller size assumed to be a degradation product of annexin I was also present in the same peak. To remove the smaller protein, hydroxylapatite column chromatography was performed. Proteins were eluted with a linear gradient of potassium phosphate from 10 mM to 200 mM. As shown in Fig. 3, three peaks were resolved. Analysis of the protein profile of each peak by SDS-PAGE revealed that the 37 kDa protein was eluted at 100 mM phosphate as major peak(Fig. 1, lane 6). To asses its purity, every fraction of the major peak was analyzed by SDS-PAGE(data not shown). Fractions containing a single band were pooled and concentrated(Fig. 1, lane 7). This was used for in vitro and in vivo activity tests.

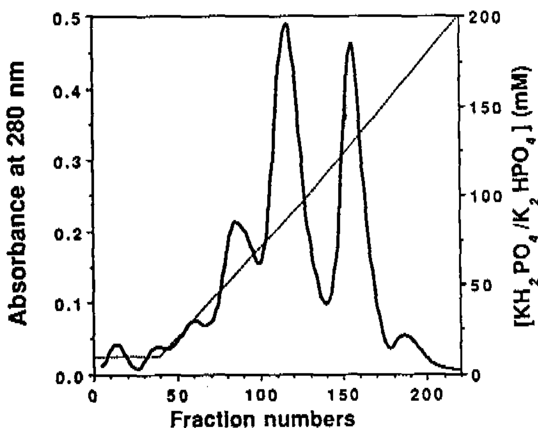

Fig 3. Result of hydroxylapatite column chromatography. The fraction of 30-40 in Fig. 2 was pooled, concentrated by ultrafiltration, dialized against 10 mM KH_2PO_4/K_2HPO_4,pH 7.7, 1 mM EDTA, and loaded onto a column equilibrated with the same buffer. Elution was performed with the linear gradients of 10 to 200 mM of

the same buffer.

Although recombinant annexin I was successfully purified by DE-52 flow-through and gel filtration chromatography, problems of self-aggregation and fragmentation of annexin I were often occurred. Self-aggregation was reduced by the addition of glycerol to 10% in the buffer, however, fragmentation was somewhat increased under the same conditions. The aggregated and fragmented forms of annexin I were removed by repeated chromatography on a hydroxylapatite column.

In vitro PLA_2 inhibitory activity

The PLA_2 inhibitory effect of annexin I was performed using the 100 kDa porcine spleen PLA_2 as described in 'Materials and Methods'. PLA_2 activity was measured in the presence of various amount of annexin I between 5 ng and 100 ng. As shown in Fig. 4, PLA_2 activity was

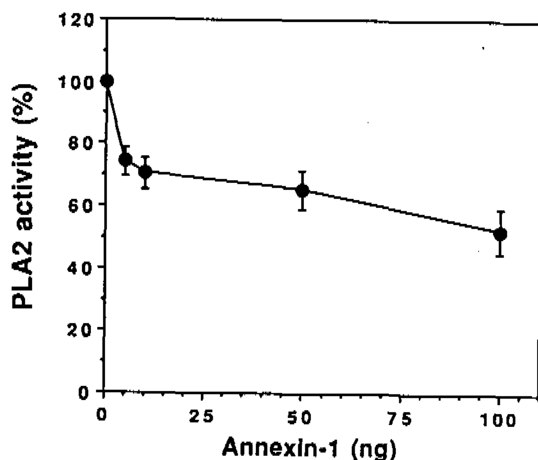

Fig 4 PLA_2 inhibitory activity of annexin I. Various amount of purified annexin I was incubated with 0.33 nmole 2-AA-PC for 1hr at 37℃ in the buffer containing 1 μM free calcium.

decreased to 75% of the original in the presence of 5 ng of annexin I. When annexin I was increased to 100 ng, the activity was further decreased to 53% of the original PLA_2 activity. The range of annexin I concentration used in this study was as small as 1/500 compared to that of prior studies which suggested inhibition of PLA_2 by annexin I was due to the depletion of substrate.[12, 13] Our results indicates that the mechanism of PLA_2 inhibition may be different from what has been suggested. A series of experiments are in progress for investigation of a detailed mechanism.

In vivo anti-inflammatory effect

To test the inhibitory activity of annexin I on carrageenin-induced edema, the protein was peritonically injected at doses of 0-500 g/kg as soon as local injection of carrageenin into the rat paw. The result is shown in Fig. 5. When carrageenin was injected, maximal edema occurred after 3 hrs of injection. The edema was decreased with injection of annexin I in a dose dependent manner. Injection of annexin I did not change the time needed for maximal edema. This results are similar to those that have been reported previously.[8]

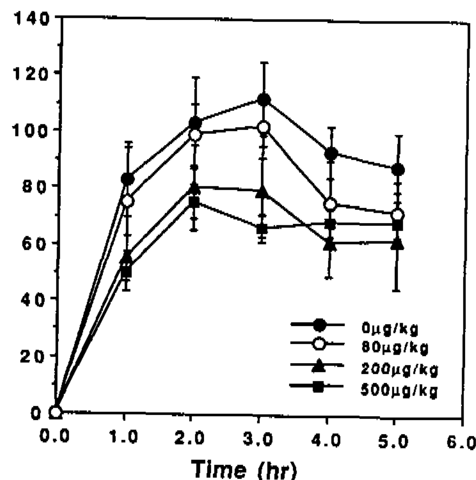

Fig 5. Anti-inflammatory effects of annexin I on carrageenin-induced rat paw edema. Each point repre-

sents the mean of the values from 5 rats.

In summary, we designed a very simple process for the purification of recombinant annexin I which was expressed as a soluble protein in E. coli. This purification process could be used to purify a large amount of protein. The purified annexin I showed PLA_2 inhibitory activity against 100 kDa porcine spleen PLA_2 and anti-inflammatory activity on carrageenin-induced rat paw edema.

Ⅳ. ACKNOWLEDGEMENT

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=국문 초록=

재조합 인간 아넥신 Ⅰ의 정제와 생체내 및 시험관내 활성 측정

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아넥신 Ⅰ은 칼슘의존적으로 인지질막에 결합하는 단백질군의 하나인 아넥신류에 속하며, 상피성 성장 인자 수용체 키나제의 기질로 알려져있다. 그러나, 생체내 역할과 그 기전에 대하여는 밝혀지지 않고있다. 아넥신 Ⅰ의 생물학적 기능에 대한 자세한 연구를 하기위해 재조합 아넥신 Ⅰ을 수용성 상태로 생산하였고, 대장균에서부터 EGTA추출한 후, DE-52 이온 교환, gel filtration, hydroxylapatite 칼럼을 통과시켜 대량으로 정제하였다. 정제된 아넥신 Ⅰ은 시험관내에서 PLA_2를 억제하였고, 생체내에서 쥐 발바닥 부종에 대한 소염 효과를 나타내었다.

Key words: Annexin Ⅰ, Purification, Anti-inflammation, Phospholipase A_2