kit), ATTO corporation (Tokyo, Japan).

Cell Culture Rat glioma C6 cells (JCRB9096) and human neuroblastoma SH-SY5Y cells (CRL-2266) were obtained from the Health Science Research Resources Bank (Osaka, Japan) and American Type Culture Collection (Manassas, VA, U.S.A.), respectively. Cells were maintained in continuous log phase growth in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% fetal calf serum at 37°C in a humidified incubator containing 95 % air-5 % CO_2 atmosphere.

Cytotoxicity assay To evaluate the degree of cytotoxicity, the LDH activity of the medium was measured using a commercially available kit (Cytotoxicity Detection kit (LDH), Roche Molecular Biochemicals, Indianapolis, IN, USA). Briefly, $1x10^5$ cells were plated in a 24-well culture dish and cultured for 24h and the culture medium was replaced with serum-free DMEM and incubated for 0.5-1h at 37°C. Then, Ca^{2+} ionophore (A23187) was added to the culture media and incubated for the indicated times at 37°C. After treatment, the culture medium (0.4 ml) was withdrawn and mixed with 0.2ml of substrate for LDH assay. After incubation for 30min, the reaction was stopped with 0.08ml of 2M HCl, and A_{492} was measured. The percent cytotoxicity was calculated using the maximum amount of releasable LDH activity by 1% Triton X-100 treatment according to the instruction manual.

Identification of secreted proteins from culture cells C6 cells were cultured in a 10cm-culture dish. After incubation with A23187 (0.19μM) for 24h, 5mM EGTA was added to culture sup and the sup (10ml) was collected. It was then mixed with lipid liposome (PC:PS=9:1) (100μg) and $CaCl_2$ (15mM) was added. The liposome was washed three times with 20mM Hepes (pH7.5), 0.1M NaCl and 2mM $CaCl_2$ by suspension and sedimentation with 20,000xg for 15min. Finally binding proteins were eluted with 10mM EGTA and subjected to 2D-gel. Each protein spot was hollowed and digested with porcine trypsin, and the trypsin-induced peptide fragments were extracted with formic acid and acetonitrile. The peptide mixture was analyzed with nanoscale LC-MS/MS (LCQ) and data base searching was done with Mascot Search.

Human blood plasma Human blood samples were obtained from 66 patients with dementia (47 with Alzheimer's disease (86.2±2.5 y.o.) and 19 (79.6±8.7 y.o.) with vascular dementia) and 35 controls (74.0 ± 5.8 y.o.). All patients met ICD-10 criteria for either Alzheimer's disease or vascular dementia. The patient's clinical symptoms were evaluated using the revised Hasegawa Dementia scale (HDS-R) (Hasegawa, 1983). Blood was drawn with Venoject II vacuum tubes containing EDTA (final 4mM) (Terumo, Tokyo, Japan) and plasma fraction was isolated by centrifugation at 3,000 g for 20 min. Blood was centrifuged within 3 h after sampling. Plasma fraction was centrifuged again at 3,000 g for 15 min and stored at -80°C until use.

Western blot to detect plasma annexin A5 In order to detect plasma annexin A5, lipid binding proteins were first isolated according to the procedures for recombinant annexin purification (Sohma et al., 2001a). Briefly, 1 ml of plasma was diluted with 9 ml of 10 mM phosphate buffered saline (PBS) and mixed with 100μg of multi-lamellar lipid liposome (phosphatidylcholine : phosphatidylserine=9:1). One mM $CaCl_2$ was added to the mixture and centrifuged

at 25,000xg for 30 min. The pellet was suspended with 5 ml of 20mM HEPES (pH7.0), 0.1M $NaCl_2$ and 1mM $CaCl_2$, and centrifuged again at 25,000xg for 15 min. The pellet was dissolved with SDS sample buffer (3% SDS and 50mM Tris-HCl (pH6.8)) and 10μl was subjected to polyacrylamide SDS-PAGE. The following procedures were the same as those for Western blotting described above.

Quantification of plasma level of annexin A5 using sandwich ELISA For quantification of the plasma level of annexin A5, sandwich ELISA was performed with monoclonal antibodies (MAbs) to native annexin A5 from huma plasenta (Romisch and Heimburger, 1990). Microtiter plates (Maxisorp, NUNC) were coated with 100μl of MAb clone No. 23 (5μg/ml), blocked with 0.1% BSA and then 50μl of 5μg/ml biotinylated-MAb clone No. 49 and 50μl of sample or standard (recombinant human annexin A5) was added to each well. After incubation and washing, 50μl of HRP-conjugated anti-annexin A5 antibody was added to each well. After incubation and washing, avidin-conjugated horseradish peroxidase (#438323 1: 2000; Zymed) was added to each well. After washing, 100μl of 3,3',5,5'-Tetramethyl Benzidine (TMB+) (DAKO, Japan) was added and incubated. The reaction was terminated with 100μl of 1M H_2SO_4 and 0.5M HCl. Both A_{450} and A_{630} were measured (A_{630} was subtracted). The detection range with this system was 0.32ng/ml - 20ng/ml.

Antibodies Polyclonal antibody against recombinant rat annexin A5 was prepared from rabbits. A specific antibody was prepared with a rat annexin A5-conjugated Sepharose 4B column.

Results

We examined Ca^{2+}-induced cytotoxicity by measuring LDH activity in culture media using rat glioma C6 cells and human neuroblastoma SH-SY5Y. Cells were incubated with various concentration of Ca^{2+}-ionophore (A23187) for 24h. Cell viability was comparable with control with 0.19μM or less of A23187 in both SH-SY5Y and C6 cells, but decreased with 0.38?μM or higher concentration of A23187 (Fig 2). To exclude leakage of intracellular ingredients from dead cells, we conducted 24h incubation with 0.19μM of A23187. We next examined the proteins secreted from culture cells following Ca^{2+}-stress, which might involve a molecular marker for dementia. As the blood-brain barrier strictly limits transport into the brain through both physical (tight junctions) and metabolic (enzymes) barriers and relatively hydrophobic materials tends to go through the barrier, we isolated the lipid liposome binding proteins from the culture media. SDS-PAGE patterns of the fractions revealed that more protein bands were observed on the SDS-gel in the fraction after A23187-treatment compared with untreated control (Fig. 3A). The sample of A23187-treatment was next subjected to 2D gel ((Fig. 3B). Each protein spot was next incubated with trypsin and identification of each protein was done with LC-MS/MS. From the Mascot search they were shown to be annexin A2, fetuin, annexin A5 and SLUG (Fig. 3C). Several lines of evidence showed that annexin A5 can be secreted in spite of the fact that it has no signal sequence (Christmas et al., 1991; Pfäffle et al., 1988; Sohma et al., 2001b).

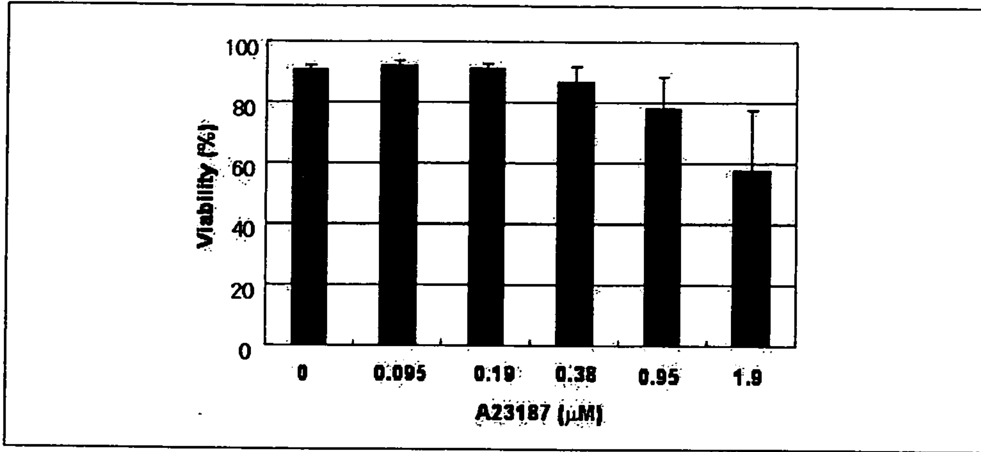
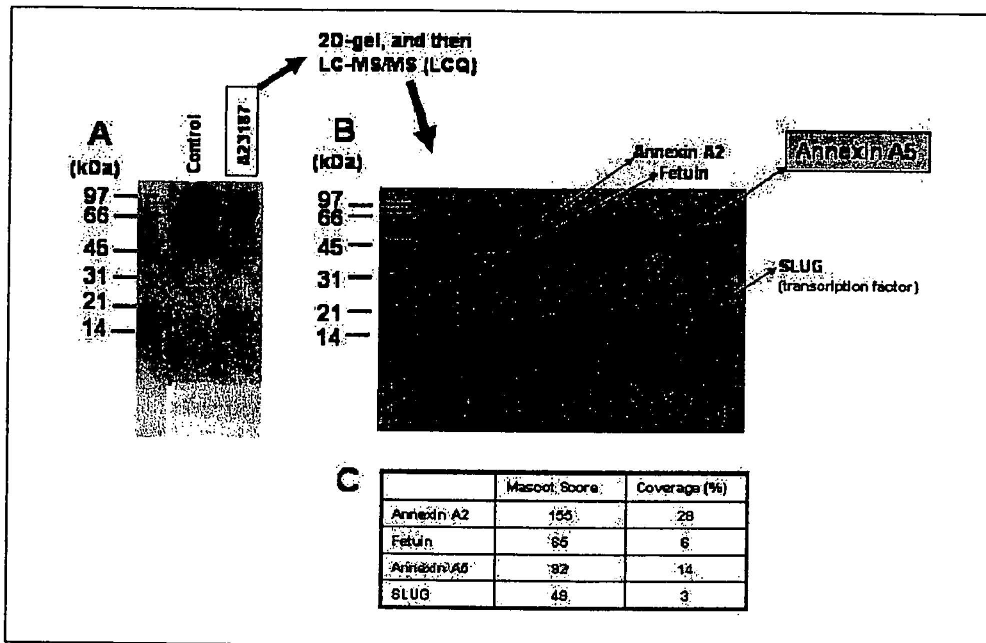


*Fig. 2. Ca^{2+}-induced cell damage in culture cells. Cell viability (rat glioma C6 cells) was measured by LDH assay (see "Materials and Methods") after incubation with various concentration of A23187 for 24h. Data represent mean±S.D. of three experiments. *, significant difference when compared with untreated control (P<0.05).*



	Mascot Score	Coverage (%)
Annexin A2	155	28
Fetuin	65	6
Annexin A5	82	14
SLUG	49	3

Fig. 3. Identification of proteins secreted from C6 cells by Ca^{2+}-stress. Culture medium of C6 cells after incubation with or with A23187 was collected and after incubation with 0.19μM of A23187 for 24h, culture media were collected. Phospholipid binding fractions (see "Materials and Methods") were subjected to SDS-PAGE (15% gel) (A). The A23187-treatment sample was subjected to 2D-gel and stained with silver nitrate (B). Protein identification of each spot was done as described in "Materials and Methods". Mascot scores are shown (C).

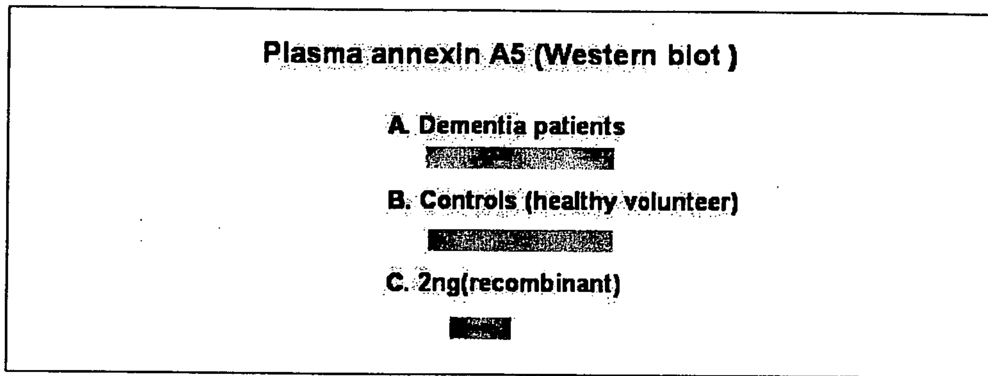


Fig. 4. Plasma annexin A5 in Alzheimer's disease patients (Western blot). Lipid liposome binding fraction of blood plasma from either dementia patients (A) or healthy volunteers (B) was subjected to SDS-PAGE and Western blot was performed with polyclonal specific antibodies to annexins A5. Blot with 2ng recombinant annexin A5 is shown in (C).

These results led us to speculate that annexin A5 might be secreted in pathological states of suffering from Ca^{2+}-stress such as in dementia.

We next performed Western blot analysis to identify annexin A5 in blood plasma. As a good deal of plasma proteins such as albumin and globulin interfere with separation of smaller amount of protein contents on SDS-PAGE, to concentrate annexin proteins in the blood we isolated Ca^{2+}-dependent lipid liposome binding fraction. With this fraction an immunostained band with anti-annexin A5 IgG with a molecular mass of 33kDa was detected in the samples from AD patients but not in control (Fig. 4), suggesting that AD patients possess a high level of annexin A5 in the blood. To quantify the plasma level of annexin A5, we established sandwich ELISA with two different clones of monoclonal antibodies. The plasma level of annexin A5 was significantly

*Table 1. Plasma level of annexin A5 in Alzheimer's disease patients (sandwich ELISA). Plasma level of annexin A5 was measured with sandwich ELISA with two clones of monoclonal antibodies to human annexin A5 (see "Materials and Methods"). Data represent mean±SD. B, Plasma level of human samples. AD, Alzheimer's disease patients; VD, vascular dementia patients; C, control (healthy volunteers). *, significant difference when compared with control ($P<0.01$).*

Subject	No. of subject	Age (y.o.±SD)	Plasma annexin A5 (ng/ml±SD)
Control	35	74.0±5.8	0.87±0.21
AD	47	86.2±2.5	3.57±2.49
VD	19	79.6±8.7	2.69±2.27

higher in the AD patients compared with controls (Table 1). The level was also higher in the vascular dementia patients compared with controls (Table 1). The level was higher in the AD patients compared with in the vascular dementia patients, but not significantly. Further study with a great number of samples is needed to clarify whether the different is significant or not. Next, to examine if there is any relation between plasma level of annexin A5 and the pathological stage of dementia, we examined the relation between plasma annexin A5 concentrations and HDS-R scores. There was a tendency toward an increasing plasma level of annexin A5 with a decreasing score of HDS-R (data not shown). Further study is needed to clarify this matter.

Conclusions

In this study we demonstrated that annexin A5 is elevated due to Ca^{2+}-stress in culture cells and is secreted and that the plasma level of annexin A5 in dementia patients was significantly higher than in controls.

References

Christmas, P., Callaway, J., Fallon, J., Jones, J. and Haigler, H. T. Selective secretion of annexin 1, a protein without a signal sequence, by the human prostate gland. *J. Biol. Chem.* 266, 2499-2507 (1991).

Dickson, D. W. Apoptotic mechanisms in Alzheimer neurofibrillary degeneration: cause or effect? *J. Clin. Invest.* 114, 23-27 (2004).

Hasegawa, K. The clinical assessment of dementia in the aged: a dementia screening scale for grading the cognitive state of patients for clinician. In *Aging in the Eighties and Beyond*, (ed. M. e. a. Bergener), pp. 207-218. New York: Springer (1983).

LaFerla, F. M. Calcium Dyshomeostasis and Intracellular Signalling in Alzheimer's Disease. *Nature rev. Neurosci.* 3, 862-872 (2002).

Mattson, M. P. Pathways towards and away from Alzheimer's disease. *Nature* 430, 631-639 (2004).

Pfäffle, M., Ruggiero, F., Hofmann, H., Fernández, M. P., Selmin, O., Yamada, Y., Garrone, R. and von der Mark, K. Biosynthesis, secretion and extracellular localization of anchorin CII, a collagen-binding protein of the calpactin family. *EMBO J.* 7, 2335-2342 (1988).

Romisch, J. and Heimburger, N. Purification and characterization of six annexins from human placenta. *Biol. Chem. Hoppe-Seyler* 371, 383-388 (1990).

Saito, K., Elce, J. S., Hamos, J. E. and Nixon, R. A. Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease:A potential molecular basis for neuronal degeneration. *Proc. Natl. Acad. Sci. USA* 90, 2628-2632 (1993).

Sohma, H., Creutz, C. E., Gasa, S., Ohkawa, H., Akino, T. and Kuroki, Y. Differential lipid specificities of the repeated domains of annexin IV. *Biochim. Biophys. Acta* 1546, 205-215 (2001a).

Sohma, H., Ohkawa, H., Akino, T. and Kuroki, Y. Binding of annexins to lung lamellar bodies and the PMA-stimulated secretion of annexin V from alveolar type II cells. *J. Biochem.* 130, 449-455 (2001b).