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PROTEASE NEXIN-2/AMYLOID β-PROTEIN PRECURSOR IN BLOOD IS A PLATELET-SPECIFIC PROTEIN

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SUMMARY The protease inhibitor, protease nexin-2 (PN-2), is the secreted form of the amyloid β-protein precursor (APP) which contains the Kunitz protease inhibitor domain. PN-2/APP is an abundant platelet α-granule protein which is secreted upon platelet activation. PN-2/APP mRNA is present in cultured endothelial cells and the protein has been detected in plasma. In the present studies we quantitated PN-2/APP in platelets, plasma and several different cell types of the vasculature to identify the repository of the protein in the circulatory system. We report that PN-2/APP is predominantly a platelet protein in the vascular compartment. Lysates of unstimulated umbilical vein endothelial cells, granulocytes or monocytes contained little PN-2/APP based on sensitive functional protease binding and immunoblotting assays. Quantitative immunoblotting studies demonstrated that normal citrated-plasma contains ≤ 60 pM PN-2/APP. In contrast, platelets can contribute up to 30 nM PN-2/APP, indicating that they are the major source of the protein in blood.

The amyloid β-protein and its parent molecule, the amyloid β-protein precursor (APP), are constituents of senile plaques in brain and cerebrovascular deposits in individuals with Alzheimer's disease (AD) and Down's syndrome (1-5). APP can be translated from at least three alternatively spliced mRNA species to yield proteins of 695, 751 and 770 amino acids (6-8). The latter two forms contain an additional domain that is homologous to Kunitz-type serine protease inhibitors (6-8). The forms of mRNA encoding for APP-751 and 770 appear to be ubiquitous. On the other hand, mRNA encoding for APP-695, the form which lacks the Kunitz protease inhibitor domain, is found primarily in brain. The previously described protease inhibitor protease nexin-2 (PN-2)

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ABBREVIATIONS: PN-2, protease nexin-2; APP, amyloid β-protein precursor; AD, Alzheimer's disease; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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(9-11) is now known to be the secreted form of APP that contains the Kunitz protease inhibitor domain (12,13).

Several findings have suggested that circulating forms of the amyloid β-protein exist and may contribute to the pathology of certain disease states. First, these proteins are deposited in the cerebrovasculature of individuals with AD (1-4), Down's syndrome (14) and hereditary cerebral hemmorhage with amyloidosis of the Dutch-type (15). Second, amyloid β-protein deposits occur at sites of vascular malformations (16). Lastly, deposition of the amyloid β-protein has been reported in perivascular, nonneural tissues including skin, subcutaneous tissue and intestine in individuals with AD (17). Recently, we and others showed that PN-2/APP is a platelet α-granule protein which is secreted upon platelet activation (18-20). Other recent reports indicate that PN-2/APP is the most potent known physiologic inhibitor of intrinsic blood coagulation factor XIa (19,21). Together, these two findings suggest that PN-2/APP may participate in regulating blood coagulation at sites of vascular injury. Furthermore, other studies showed that APP is a potent cell adhesion molecule (22) and possesses growth supportive activity (23,24), other possible functions which may contribute to wound repair.

In addition to platelets, there may be other vascular sources of PN-2/APP in the circulatory system. Endothelial cells constitutively express APP mRNA and these levels can be increased by treatment with interleukin-I (25). APP immunoreactivity has also been detected in plasma (26). The following studies were designed to determine the major intravascular source(s) of PN-2/APP. Determining if PN-2/APP is present at significant levels in plasma, in addition to platelets, is important in understanding the physiologic, as well as possible pathophysiologic, functions of this protein. If appreciable quantities of PN-2/APP exist in plasma, it could interact with circulating cells and blood vessel walls at any time. On the other hand, if PN-2/APP circulates predominantly as an intracellular protein its delivery could be targeted, when needed, to specific sites of vascular injury and vessel wall malformations. Using functional and immunochemical assays we quantitated the amounts of PN-2/APP in plasma, platelets, granulocytes, monocytes and umbilical vein endothelial cells to determine the repositories of this protein in the vasculature. The present studies indicate that PN-2/APP circulates predominantly as a platelet protein.

METHODS

Plasma and cell lysate preparation. Platelet-poor plasma and platelets were prepared from freshly collected citrated-blood from normal healthy donors as described (27). Plasma samples were enriched for PN-2/APP by anion exchange chromatography (18). Human granulocytes were prepared from whole blood anticoagulated with acid-citrate-dextrose by dextran sedimentation followed by Ficoll-Hyaque centrifugation, as previously reported (28). Human monocytes were prepared from leukocyte-rich fractions by Ficoll-Hypaque and double Percoll density gradients according to published methods (29). Lysates of cultured human umbilical vein endothelial cells (passage 8-10) were prepared as previously reported (30). Lysates of all cell types were prepared with 1% Triton X-100 and the insoluble material was removed by centrifugation. Fractionated plasma samples and platelet and cell lysates were stored at -70° C.

PN-2/APP-protease complex formation. Aliquots of the platelet and cell lysates were incubated with 30 nM ^{125}I-epidermal growth factor binding protein (1.4 x 10^6 cpm/pmole) for 15 min at room temperature. An equal volume of nonreducing Laemmli SDS sample buffer was added to the samples to terminate the reaction. Without prior boiling, the samples were then analyzed on 10% nonreducing SDS-polyacrylamide minigels according to the method of Laemmli

(31). Completed gels were then stained, destained, dried and autoradiograms were prepared. ^{125}I-labeled protease:PN-2/APP complexes were identified as a 120 kDa band on the autoradiograms (11,18).

Immunoblot analysis. Aliquots of platelets, other cell lysates and fractionated plasma samples were electrophoresed on 10% SDS-polyacrylamide mini gels as described above and the proteins were electroblotted onto polyvinylidene difluoride membranes. Unoccupied sites on the membranes were blocked overnight with a solution of phosphate-buffered saline containing 0.25% gelatin. The membranes were then incubated with mAbP2-1 monoclonal hybridoma culture supernatant (18,21). The bound mouse monoclonal antibody was detected with a solution of biotinylated-sheep anti-mouse IgG (1:200) followed by a solution of biotinylated-streptavidin horseradish peroxidase conjugate (1:400). The immunoblots were then developed with a solution of 4-chloronapthol as described (21).

Quantitative immunoblotting was performed as described above except that the bound mouse monoclonal antibody mAbP2-1 was detected with a 0.5 nM solution of ^{125}I-labeled affinity purified goat anti-mouse IgG_1 (4.5×10^6 cpm/pmole). Autoradiograms were prepared from the immunoblots and the PN-2/APP bands were quantitated by a scanning laser densitometer. The scan values obtained from the fractionated plasma samples and platelet and cell lysates were compared to scans obtained from autoradiograms of standard curve immunoblots of known quantities of purified PN-2.

RESULTS

Previous studies suggested that platelets contain substantial amounts of PN-2/APP (18-20). However, since umbilical vein endothelial cells were shown to constitutively express APP mRNA (25), the major source of PN-2/APP in the vasculature is not known. Therefore, we prepared lysates of platelets, umbilical vein endothelial cells, granulocytes and monocytes, to determine the repositories of PN-2/APP in blood. Aliquots of each of these cell lysates were screened for PN-2/APP protein using the functional protease inhibition assay of SDS-stable ^{125}I-epidermal growth factor binding protein:PN-2/APP complex formation (11,18). The autoradiogram in Fig. 1 qualitatively shows that platelet lysates (lane 4) contain proportionately greater amounts of PN-2/APP than lysates of endothelial cells (lane 1), granulocytes (lane 2) or monocytes (lane 3). The findings from the PN-2/APP functional assay were supported by immunoblot analysis using the anti-PN-2 monoclonal antibody mAbP2-1. The immunoblot in Fig. 2 shows that the monoclonal antibody mAbP2-1 detected significant quantities of PN-2/APP in a platelet lysate (lane 4). In contrast, little, if any, PN-2/APP was detected in the lysates of endothelial cells (lane 1), granulocytes (lane 2) or monocytes (lane 3). Together, these findings from our qualitative functional and immunochemical assays indicate that platelet lysates contain proportionately greater quantities of PN-2/APP than other cell types in the vasculature.

In order to confirm that platelets are the major source of PN-2/APP in the circulatory system, we determined the concentration of PN-2/APP in normal citrated-plasma, platelets, and the other cell types, by quantitative immunoblotting using monoclonal antibody mAbP2-1 and a ^{125}I-labeled secondary antibody. Fig. 3 shows the standard curve that was generated from laser densitometric scanning of an immunoblot of known quantities of purified PN-2. Table 1 summarizes the results obtained from the quantitative immunoblotting studies. Human platelets (1×10^8 platelets) contain 1.1 ± 0.3 μg of PN-2/APP ($n = 5$). Assuming a normal level of 3×10^8 platelets per ml of blood, upon activation and release, platelets could contribute up to 30 nM PN-

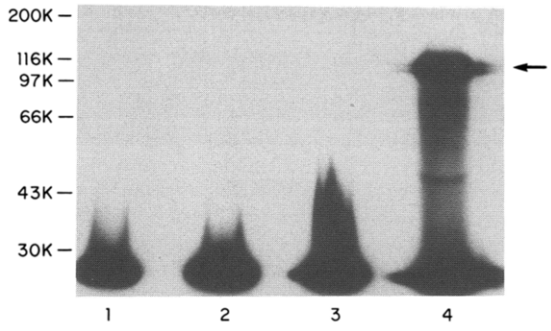

Fig. 1. ^{125}I-labeled epidermal growth factor binding protein-PN-2/APP complex formation in cell and platelet lysates. Cell and platelet lysates were prepared as described in "Methods" and cell lysates were adjusted to final concentrations of 3.5-5 x 10^7 cells/ml. The platelet lysate was 1 x 10^8/ml. Twenty five µl aliquots of each lysate were incubated with 30 nM ^{125}I-labeled epidermal growth factor binding protein and subjected to SDS-PAGE and autoradiography as described in "Methods." Lane 1, human umbilical vein endothelial cell lysate; lane 2, human granulocyte lysate; lane 3, human monocyte lysate; and lane 4, human platelet lysate.

2/APP in blood. Even higher concentrations could be achieved in the immediate microenvironment of the activated platelet surface. In contrast, normal citrated-plasma contains 6.7 ± 2.6 ng of PN-2/APP/ml (n = 5). This concentration of ≤ 60 pM of free PN-2/APP in plasma and is consistent with the levels described in a recent preliminary report (32). In the lysates of the endothelial cells, granulocytes or monocytes very little, if any, PN-2/APP was detected by the quantitative immunoblotting assay indicating that the levels of protein associated with these cells is < 10 pM. These results indicate that platelets are the major intravascular source of PN-2/APP.

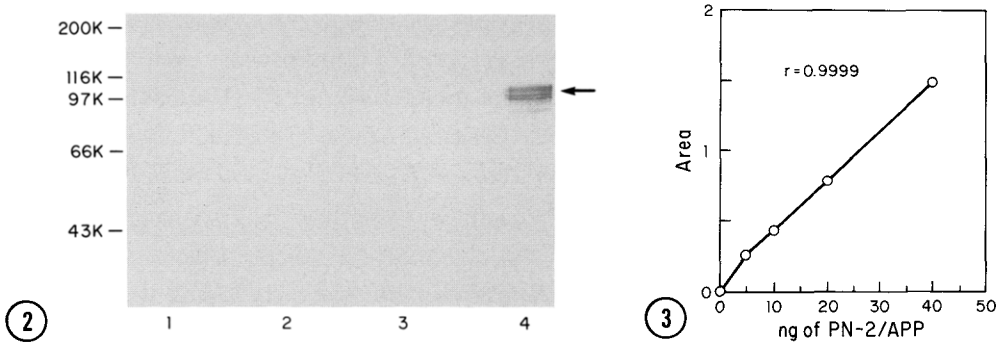

Fig. 2. Immunoblot of PN-2/APP in cell and platelet lysates. Cell and platelet lysates were prepared as described in "Methods" and the cell lysates were adjusted to final concentrations of 3.5-5 x 10^7 cells/ml. The platelet lysate was 1 x10^8/ml. Twenty five µl aliquots of each lysate were subjected to SDS-PAGE and immunoblotted using the monoclonal antibody mAbP2-1 as described in "Methods." Lane 1, human umbilical vein endothelial cell lysate; lane 2, human granulocyte lysate; lane 3, human monocyte lysate; and lane 4, human platelet lysate.

Fig. 3. Quantitative immunoblot standard curve. Known quantities of purified PN-2/APP were subjected to SDS-PAGE and immunoblot analysis was conducted with monoclonal antibody mAbP2-1 and ^{125}I-labeled goat anti-mouse IgG. An autoradiogram was prepared and quantitated by a scanning laser densitometer.

TABLE 1. Quantitation of vascular PN-2/APP

Source	Immunoblot Quantitation	Vascular Concentration	% Contribution
Platelets	1.1 ± 0.3 μg/10^8 platelets	≥30 nM	>99
Plasma	6.7 ± 2.1 ng/ml plasma	≤60 pM	<0.5
Endothelial cells	<20 ng/10^7 cells	<10 pM	<0.1
Granulocytes	<10 ng/10^7 cells	<10 pM	<0.1
Monocytes	<20 ng/10^7 cells	<10 pM	<0.1

DISCUSSION

Although a previous study described the immunochemical identification of secreted forms of APP (equivalent to PN-2) in plasma (26) and APP mRNA has been shown to be present in cultured endothelial cells (25), no specific comparative information has been available to determine the major intravascular source of PN-2/APP. The finding that the majority of intravascular PN-2/APP is contained within platelets indicates that this protein is a platelet-specific protein. The quantity of platelet PN-2/APP (1.1 μg/10^8 platelets) suggests that it is a major platelet component comprising 0.5% of total platelet protein. The present finding that platelets are the major circulating repository of PN-2/APP also provides important insights about its potential physiologic interactions. Since PN-2/APP is primarily contained in platelet α-granules and is secreted upon platelet activation along with other granule constituents (18-20), platelets can provide effective targeting and release of large amounts of PN-2/APP at specific injury sites in the vasculature. At these sites, PN-2/APP may participate in one or more of the numerous events in wound repair. For example, PN-2/APP has been reported to possess growth supportive activity (23,24) and is a potent cell adhesion molecule (22), two properties which occur during the repair process. More striking are the protease inhibitory properties of PN-2/APP. PN-2/APP is the most potent inhibitor known of intrinsic blood coagulation factor XIa (19,21). Since factor XIa binds to the surface of activated platelets to convert factor IX to IXa (33,34), platelet PN-2/APP may play an important role in regulating factor XIa activity near and at the surface of activated platelets. Since PN-2/APP is present in platelets at high concentrations (Table 1), regulation of intrinsic blood coagulation by this inhibitor may be targeted uniquely to the microenvironment of the generating thrombus. In contrast, other plasma-born protease inhibitors of factor XIa such as α_1 protease inhibitor and antithrombin III (35) may function in plasma.

Several studies have suggested the possibility that deposits of the amyloid β-protein in senile plaques, cerebrovascular deposits and in peripheral nonneural tissues might partly originate from the circulation (1,2,16,17). PN-2/APP has also been identified in some of these deposits (12,36). The present results suggest that some of these deposits might originate from platelets. It is noteworthy that recent studies showed that PN-2/APP secreted from transfected cells contains only the first fifteen amino acids of the amyloid β-protein at its carboxy terminus (37). It has yet to

be determined if PN-2 from normal or Alzheimer's disease platelets contains the entire amyloid β-protein domain and thus could be a potential source of the amyloidogenic fragment.

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REFERENCES

1. Glenner, G. G., and Wong, C. W. (1984) *Biochem. Biophys. Res. Commun.* **120,** 885-890
2. Glenner, G. G., and Wong, C. W. (1984) *Biochem. Biophys. Res. Commun.* **122,** 1131-1135
3. Masters, C. L., Multhaup, G., Simms, G., Pottgiesser, J., Martins, R. N., and Beyreuther, K. (1985) *EMBO J.* **4,** 2757-2763
4. Masters, C. L., Simms, G., Weinmann, N. A., Multhaup, G., McDonald, B. L., and Beyreuther, K. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82,** 4245-4249
5. Masters, C. L., and Beyreuther, K. (1988) In *Aging of the Brain,* Volume **32,** pp. 183-204, Raven Press, New York
6. Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F., and Cordell, B. (1988) *Nature* **331,** 525-527
7. Tanzi, R. E., McClatchey, A. I., Lamperti, E. D., Villa-Komaroff, L., Gusella, J. F., and Neve, R. L. (1988) *Nature* **331,** 528-530
8. Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S., and Ito, H. (1988) *Nature* **331,** 530-532
9. Knauer, D J., and Cunningham, D. D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2310-2314
10. Knauer, D. J., Thompson, J. A., and Cunningham, D. D. (1983) *J. Cell. Physiol.* **117,** 385-396
11. Van Nostrand, W. E., and Cunningham, D. D. (1987) *J. Biol. Chem.* **262,** 8508-8514
12. Van Nostrand, W. E., Wagner, S. L., Suzuki, M., Choi, B. H., Farrow, J. S., Geddes, J. W., Cotman, C. W., and Cunningham, D. D. (1989) *Nature* **341,** 546-549
13. Oltersdorf, T., Fritz, L. C., Schenk, D. B., Lieberburg, I., Johnson-Wood, K. L., Beattie, E. C., Ward, P. J., Blacher, R. W., Dovey, H. F., and Sinha, S. (1989) *Nature* **341,** 144-147
14. Malmund, N. (1972) In: *Aging and the Brain, Third Edition* (C. M. Gaitz, ed.), pp. 63-87. Plenum Publishing, New York
15. VanDuinen, S. G., Castano, E. M., Prelli, F., Bots, G. T. A. B., Luyendijk, W., and Frangione, B. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84,** 5991-5994
16. Hart, M. N., Merz, P., Bennett-Gray, J., Menezes, A. H., Goeken, J. A., Schelper, R. L., and Wisniewski, H. K. (1988) *Am. J. Pathol.* **132,** 167-172
17. Joachim, C. L., Mori, H., and Selkoe, D. J. (1989) *Nature* **341,** 226-230
18. Van Nostrand. W. E., Schmaier, A. H., Farrow, J. S., and Cunningham, D. D. (1990) *Science* **248,** 745-748
19. Smith, R. P., Higuchi, D. A., and Broze, G. J. (1990) *Science* **248,** 1126-1128
20. Bush, A. I., Martins, R. N., Rumble, B., Moir, R., Fuller, S., Milward, E., Currie, J., Ames, D., Weidemann, A., Fischer, P., Multhaup, G., Beyreuther, K., and Masters, C. L. (1990) *J. Biol. Chem.* **265,** 15977-15983
21. Van Nostrand, W. E., Wagner, S. L., Farrow, J. S., and Cunningham, D. D. (1990) *J. Biol. Chem.* **265,** 9591-9594
22. Schubert, D., Jin, L-W., Saitoh, T., and Cole, G. (1989) *Neuron* **3,** 689-694

23. Schubert, D., Cole, G. Saitoh, T., and Oltersdorf, T. (1989) *Biochem. Biophys. Res. Commun.* **162,** 83-88
24. Saitoh, T., Sundsmo, M., Roch, J-M., Kimura, N., Cole, G., Schubert, D., Oltersdorf, T., and Schenk, D. B. (1989) *Cell* **58,** 615-622
25. Goldgaber, D., Harris, H. W., Hla, T., Maciag, T., Donnelly, R. J., Jacobsen, J. S., Vitek, M. P., and Gajdusek, D. C. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86,** 7606-7610
26. Podlisny, M. B., Mammen, A. L., Sclossmacher, M. G., Palmert, M. R., Younkin, S. G., and Selkoe, D. J. (1990) *Biochem. Biophys. Res. Commun.* **167,** 1094-1101
27. Schmaier, A. H., Smith, P. M., Purdon, A. D., White, J. G., and Colman, R. W. (1986) *Blood* **67,** 119-130
28. Gustafson, E. J., Schmaier, A. H., Wachtfogel, Y. T., Kaufman, N., Kucich, U., and Colman, R. W. (1989) *J. Clin. Invest.* **84,** 28-35
29. Leung, L., Saigo, K., and Grant, D. (1989) *Blood* **73,** 177-184
30. Schmaier, A. H., Murray, S. C., Heda, G. D., Farber, A., Kno, A., McCrae, K., and Cines, D. B. (1989) *J. Biol. Chem.* **264,** 18173-18179
31. Laemmli, U. K. (1970) *Nature* **227,** 680-685
32. Barbour, R., Sherard, S., Sclossmacher, M., Podlisny, M., Selkoe, D., and Schenk, D. (1990) *Neurobiol. Aging* **11,** 307 (abstr.)
33. Walsh, P. N., Sinha, D., Koshy, A., Seaman, F. S., and Bradford, H. (1986) *Blood* **68,** 225-230
34. Walsh, P. N., Sinha, D., Kueppers, F., Seaman, F. S., and Blankstein, K. B. (1987) *J. Clin. Invest.* **80,** 1578-1586
35. Scott, C. F., Schapira, M., James, H. L., Cohen, A. B., and Colman, R. W., (1982) *J. Clin. Invest.* **69,** 844-852
36. Tagliavini, F., Ghiso, J., Timmers, W. F., Giaccone, G., Bugiani, O., and Frangione, B. (1990) *Lab. Invest.* **62,** 761-767
37. Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D., and Ward, P. J. (1990) *Science* **248,** 1122-1124