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α2-Macroglobulin and other proteinase inhibitors do not interfere with the secretion of amyloid precursor protein in mouse neuroblastoma cells

Bart De Strooper, Fred Van Leuven and Herman Van Den Berghe

Center for Human Genetics, University of Leuven, Leuven, Belgium

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A series of proteinase inhibitors active against proteinases of all four major classes, including highly purified and well-characterized α2-macroglobulin, added to the cell culture medium of murine Neuro 2a neuroblastoma cells did not interfere with APP secretase activity. We therefore advance the hypothesis that APP secretase activity is localized in an intracellular compartment.

Amyloid precursor protein; Mouse; α2-Macroglobulin; Proteinase inhibitor; Alzheimer's disease

1. INTRODUCTION

The dementia in Alzheimer's disease is probably a consequence of progressive neural loss, particularly in the temporal lobe of the neocortex. This neural death is accompanied by amyloid plaques and neural tangles. The main component of the plaques is the amyloid peptide, a 39–43 amino acid fragment of the amyloid precursor protein (APP). This peptide is neurotoxic [1,2]. Transgenic mice [3,4] over-expressing the complete APP develop amyloid deposits [3], but neural cell death in the brains of these mice was not observed [5]. Therefore the strongest evidence for the central role of APP in the pathogenesis of Alzheimer's disease remains the co-segregation of point mutations in the APP gene with the disease in a few families [6–10].

APP is synthesized as an integral membrane protein [11]. An ill-understood cleavage step separates the integral membrane domain from the extracellular domain, which is then secreted into the medium [11]. Since this proteolytic cleavage occurs in the amyloid sequence itself [12–14], APP secretion and formation of the amyloid peptide are mutually exclusive processes. Inhibition of the cleavage step would allow one to explore possible alternative pathways for the metabolism of APP. Prime candidates in that regard are α1-antichymotrypsin and α2-macroglobulin (α2M), both proteinase inhibitors that are present in neural plaques [15,16]. Recently it was reported that simple addition of α2M to the culture medium of neural cells inhibits the normal cleavage of APP [17]. Moreover, addition of interleukin-6 (IL-6) to the cell cultures induced the production of α2M [17]. The hypothesis was advanced that local production of IL-6 in the brain of Alzheimer patients would induce local production of α2M which then would interfere with normal APP metabolism [17,18]. Before exploring this hypothesis by over-expression of α2M in the brain of transgenic mice, we first attempted to confirm these potentially interesting observations in a murine system. The results reported here demonstrate that none of the observations summarized above were confirmed in our system. The results are suggestive of an intracellular localization of the APP secretase activity.

Correspondence address: F. Van Leuven, Center for Human Genetics, University of Leuven, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium. Fax: (32) (16) 215 997.

2. MATERIAL AND METHODS

Neuro 2a neuroblastoma cells were obtained from the American Type Culture Collection and cultured in DME/F12 (Gibco) with 10% FCS. The human neuroblastoma cell line, SK-N-SH (also from ATCC), was cultured in DME/F12 with 2% Ultroser (Gibco). For metabolic labelling experiments of the Neuro 2a line, 400,000 cells were seeded into a T25 flask (Falcon) and after 24 h the medium was replaced by DME/F12 without serum. After 24 or 48 h of culture in serum-free conditions, which resulted in neural differentiation of the cells, pulse-labelling was done with 150 μCi per ml [^{35}S]methionine (NEN) in methionine-free DME for 20 min. After a chase period performed in DME/F12, APP was isolated by double-immune precipitation as described [19]. For the metabolic labelling of SK-N-SH cells, 1,500,000 cells were seeded into a T75 flask, cultured for 48 h in DME/F12 with 10% FCS, 0.5 mM dbCAMP (Sigma), 10 μM retinoic acid (Janssen Chimica) and 20 ng/ml NGF-β (Boehringer). These drugs have been shown to result in neurodifferentiation of human neuroblastoma cells [17]. Further processing of the cells was done as for the Neuro 2a cells.

A polyclonal antibody against APP was raised in rabbits by immunization with a fusion protein from the T7 gene 10 protein (pGEMEX, Promega) and the C-terminal part of mouse APP, starting from amino acid 321 [20]. IL-6 and monoclonal antibodies (mAb's) against N-CAM and APP were obtained from Boehringer-Mannheim. α2M was isolated from citrated human plasma by polyethylene glycol 6000 precipitation, gel filtration on Ultrogel AcA 34 (LKB) and affinity chromatography on Cibacron blue Sepharose (Pharmacia), as detailed previously [21]. A final purification of native α2M was accomplished

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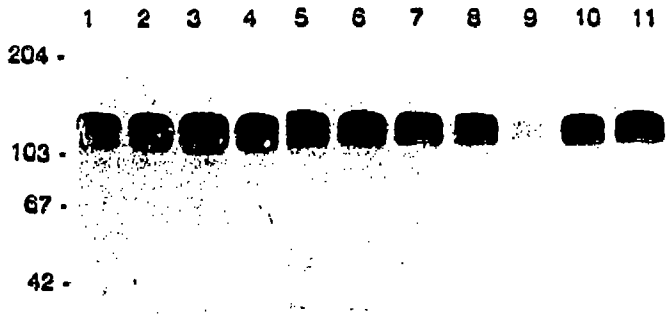

Fig. 1. Effect of proteinase inhibitors on the secretion of APP into the culture medium by Neuro 2a cells. Cells were pulse labelled for 20 min and chased for 2 h in the presence of (lane 1) no proteinase inhibitor; (lane 2) pepstatin (1 μg/ml) and bestatin (50 μg/ml); (lane 3) pepstatin (1 μg/ml); (lane 4) bestatin (50 μg/ml); (lane 5) α1-antichymotrypsin (50 μg/ml); (lane 6) α2M (100 μg/ml); (lane 7) trypsin inhibitor (100 μg/ml), aprotinin (10 μg/ml) and TLCK (100 μg/ml); (lane 8) E64 (10 μg/ml) and leupeptin (1 μg/ml); (lane 9) iodoacetamide (200 μM); (lane 10) phenantroline (1 mM); (lane 11) no proteinase inhibitor. All experiments were done on at least two different occasions with fresh proteinase inhibitor preparations. Medium was collected from the cells and double-immune precipitation was performed with a polyclonal anti-APP antibody. Immune precipitates were analyzed on 7% polyacrylamide gel. Molecular weight markers are indicated on the left. The apparent inhibition by iodoacetamide (lane 9) is due to cell death.

by hydrophobic interaction chromatography on a TSK Phenyl-5PW column (LKB) [21]. Other proteinase inhibitors (see Fig. 1) were obtained from Calbiochem, Sigma and Boehringer.

Total RNA was isolated by acid guanidinium thiocyanate extraction [22]. Northern blotting was performed using standard protocols. After hybridization overnight at 42°C, washes in 0.3% SSPE, 0.5% SDS at 42°C were performed and autoradiography was carried out for 48 h at −70°C.

3. RESULTS

After 24 h in serum-free medium, almost all Neuro 2a neuroblastoma cells in the culture flask were differentiated and had developed neurites. Addition of NGF-β (20 ng/ml) or dbCAMP (1 mM) did not remarkably influence the time-course of this process. Retinoic acid (10 μM) proved toxic to the cells. The drugs were therefore not used in further experiments. Cell extracts of the differentiated cells stained in Western blotting with a mAb to N-CAM which confirmed their neural phenotype.

After metabolic pulse labelling with [^{35}S]methionine, protein bands of about 120 kDa were immuno-precipitated with a polyclonal antibody to murine amyloid precursor protein. Without chase the protein was associated with the cells, while after 2–4 h of chase most of the labelled APP proteins were present in the culture medium (Figs. 1 and 2).

A series of inhibitors of all known classes of proteinases, including the broad spectrum proteinase inhibitor α2M and α1-antichymotrypsin, were added to the

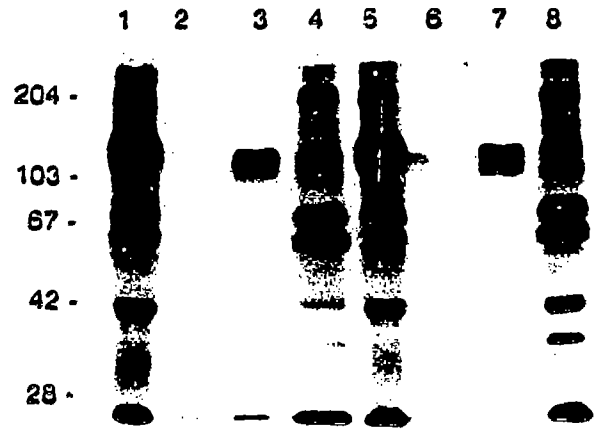

Fig. 2. Effect of α2M on APP synthesis. A pulse-chase experiment on Neuro 2a cells cultured in serum-free medium for 24 h in the absence (lanes 1–4) or presence (lanes 5–8) of α2M (1 mg/ml). Fresh inhibitor was added during the pulse and again during the chase period. Double-immune precipitated APP was applied on a 7% SDS-polyacrylamide gel. (Lanes 1,2,5,6) Immune precipitation was performed on cell extracts (lanes 1,5) or culture medium (lanes 2,6) immediately after the pulse with 150 μCi/ml [^{35}S]methionine for 20 min. (Lanes 3,4,7,8) Immune precipitation was performed after a chase of 4 h in complete medium: (lanes 3,7) culture medium, (lanes 4,8) cell extracts. Notice the slightly higher mobility of the secreted APP compared to the cell-associated APP. We observed no effect on the incorporation of label in the cells by α2M. Total precipitable label in the cell extracts used in this experiment was 29×10^6 cpm and 27×10^6 cpm in lanes 1 and 5, respectively, and 24×10^6 cpm and 25×10^6 cpm in lanes 4 and 8, respectively. Gels were exposed for different time periods to ascertain linearity of the signals.

culture medium during the chase period. None influenced secretion of APP (Fig. 1).

Previous publications [17,18] have suggested that α2M inhibits APP secretase activity. Therefore the possible effect of α2M was investigated in detail.

Addition of highly purified α2M from two different batches at high concentrations (100 μg/ml and 1,000 μg/ml), pre-incubation of the cells for 24 h, and addition of fresh α2M to the medium during the pulse and again during the chase period, were all without influence on the cleavage of APP (Fig. 2). It is therefore clear that α2M does not affect the metabolism of APP in mouse Neuro 2a cells under these conditions. We extended our observations to a human neuroblastoma cell line, SK-N-SH. These cells were cultured for 48 h in medium with neurodifferentiators and supplemented with 100 μg/ml α2M. They were then pulse labelled for 20 min in the presence of 100 μg/ml freshly added α2M and finally chased in the presence of 500 μg/ml α2M (Fig. 3). Double-immune precipitation of APP from the culture medium again did not reveal any difference in secretase activity between the control and the test sample (Fig. 3).

We further tested the effect of IL-6 (200 U/ml) on the

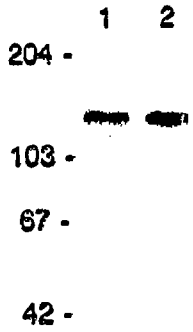

Fig. 3. Effect of α2M on APP secretion in SK-N-SH human neuroblastoma cells. A pulse-chase experiment on SK-N-SH cells was performed as in Fig. 2. Double-immune precipitation was performed on the culture supernatants after 2 h of chase in the absence (lane 1) or in the presence of 0.5 mg native α2M (lane 2). Under the culture conditions used, apparently only the APP form containing the Kunitz inhibitor domain is synthesized in the SK-N-SH cell line used.

induction of α2M in Neuro 2a cells. We were unable to detect α2M mRNA either in non-stimulated or in IL-6-stimulated Neuro 2a cells, cultured in either serum-free or in serum-containing medium: APP mRNA was detected in all experiments (not shown). Mouse liver mRNA was used as a positive control for α2M-mRNA (Fig. 4).

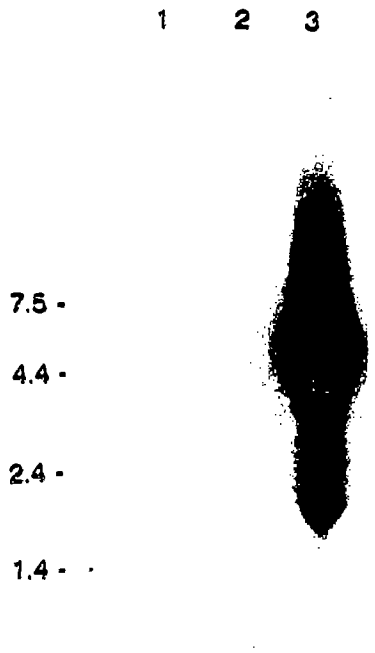

Fig. 4. Northern blot of total RNA extracts with an α2M-specific probe. Neuro 2a cells were cultured in serum-free medium for 24 h in the absence (lane 1) or presence of 200 U/ml interleukin 6 (lane 2). (Lane 3) Mouse liver RNA. In every lane 10 μg total mRNA was applied. The probe was a 3.1 kb cDNA *Eco*RI fragment of mouse α2M (Van Leuven, unpublished result). Markers are indicated on the left.

4. DISCUSSION

Since APP is an integral membrane protein, secretion must involve a cleavage step separating the integral membrane domain from the secreted part of the protein. Given the highly conserved primary structure of APP in mouse and human (97.6% identity at the amino acid level [20]), we expected and also observed similar kinetics for the secretion of APP in a mouse neural cell line relative to human cell lines ([11], and our observations).

In this system, however, we did not observe any interference of the secretion process by α2M (in concentrations up to 1 mg/ml). This was confirmed in the human neuroblastoma cell line, SK-N-SH. Moreover, co-transfection of COS cells with mouse α2M and APP also did not influence the secretion of APP (our unpublished results). It is therefore less likely that differences in the cell system used can explain the discrepancies between our observations and those previously published [17]. Since the α2M preparation we used was purified following well-established protocols [21], and has been thoroughly characterized as being the active form of α2M, we suggest that the inhibitory effect of the α2M observed by Ganter et al. [17] could have been caused by other substances in the preparation of α2M. This would explain the fact that very high concentrations were needed to observe the effect on APP processing. It remains to be seen whether the addition of that preparation, which also diminished the total incorporation of [^{35}S]methionine (see the legend to Fig. 3 in [17]), would have other effects on the cells unrelated to the proteinase inhibitor capacity of α2M. The induction of α2M in neural cells by IL-6 was previously investigated at the protein level using a polyclonal α2M antiserum [17]. We were not able to detect mouse α2M mRNA, which is a more sensitive and specific assay. The hypothesis that IL-6 would induce synthesis of α2M in neural cells, and thereby would interfere with the normal metabolism of APP [16,18], is thus not consistently confirmed in all experimental systems.

As well as α2M, inhibitors of all major classes of proteinases, including α1-antichymotrypsin which is a specific marker for the plaques in Alzheimer's disease [15], also had no effect on the cleavage of APP. A possible interpretation of these data is that the hypothetical APP secretase is a highly specific proteinase with which none of the tested proteinase inhibitors interact. On the other hand, however, major structural alterations of APP at the cleavage site [23] do not alter its secretion into the medium, which suggests that the specificity of APP secretase is relatively low. We advance, therefore, the alternative possibility that the secretase activity is localized intracellularly and that the proteinase inhibitors added to the culture medium do not attain the secretase under the experimental conditions used. Cathepsin B, a lysosomal proteinase, has been ad-

vanced as a possible APP secretase based on its activity on synthetic peptides mimicking the cleavage site in APP [24]. Others [25,26], however, found no effect of lysosomal proteinase inhibitors on APP secretase activity. Although our results do not contradict the possibility of lysosomal processing of APP, other cellular compartments (e.g. endosomes, Golgi-apparatus) remain possible as well.

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