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Aβ production as consequence of cellular death of a human neuroblastoma overexpressing APP

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Abstract In human brain the Aβ peptide is produced mainly by neurons and the overexpression of amyloid precursor protein (APP) that involves an increase in Aβ secretion, has been observed in some areas of the Alzheimer's disease patients brain. We have generated two stably transfected human neuroblastoma lines which overexpress APP; both of them secreted Aβ and showed morphological changes and cell death with apoptotic program characteristics. Interestingly, coculture experiments with the untransfected human neuroblastoma cell line showed that the Aβ peptide was not responsible for the death in those cell lines; additionally, we indicate that upon cell death, Aβ peptide is secreted into cell medium.

Keywords: Amyloid precursor protein; Beta amyloid; Neuronal; Cell death

1. Introduction

Alzheimer's disease (AD) is characterized by a massive neuronal death, and two typical hallmarks in the brain of the patients are neurofibrillary tangles and senile plaques. The beta amyloid (Aβ), the major constituent of the amyloid core of senile plaques, is a 39–43 amino acid peptide produced from a larger precursor, the amyloid precursor protein (APP) [1]. The APP isoform with 695 amino acids (APP695) is the most abundant in human brain and it is expressed in a high amount in post-mitotic neurons during early periods of their differentiation [2].

Several mutations in APP and presenilins segregate with inherited AD forms of early onset familial AD or FAD [3]. Most of these mutations are located within or near the Aβ sequence, result in increased Aβ production [4] and have been associated with an increased vulnerability to cell death [5].

The correlation between APP mutations, amyloid deposits and neurodegeneration led to the idea that modification of APP expression and processing in the brain is a critical event in the evolution of AD. The neurotoxic effects of Aβ have been essentially studied in vitro by treating neurons with high concentrations of synthetic Aβ peptide which result in oxidative stress and cell death [6].

Here, we report the generation of a human neuroblastoma cellular lines of cholinergic origin (SKNMC) that stably express high quantities of the APP isoform 695 and also secrete Aβ. We show evidence the APP overexpression in SKNMC induces apoptosis, but the secreted Aβ would not be responsible for the death of those lines.

2. Materials and methods

2.1. Cell culture and transfection

The SKNMC human neuroblastoma cell line (HTB10) was purchased from the American Type Culture Collection. Cells were transfected with pREP4-APP695 (kindly provided by Esperanza Morato) or with empty vector using coprecipitation with calcium phosphate [7]. Transfected APP-SKNMC cell lines (C2, C3) and the vector-transfected cell line (C1-) were cultured in MEM (Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 mU/ml penicillin G and 100 μg/ml streptomycin and 0.16 mg/ml hygromycin B at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.2. Coculture of cellular lines

Coculture experiments were performed in a Transwell® system (Costar), that allows to incubate two different cultures physically separated through a porous membrane but sharing the culture medium. Seventy two hours before each experiment, cells were seeded in multiwell 6 well (M-6) coculture plates, at a density of 50 000 cell/cm^2 for the lower and upper wells.

2.3. Conditioned medium preparation

The growth medium was replaced with fresh medium and cells were incubated for 1, 4 or 7 days after which the supernatants were harvested, centrifuged and 10× concentrated in presence of a protease inhibitors (2.5 mM EDTA, 10 μM leupeptin, 1 μM peptastin, 1 mM phenylmethylsulfonyl fluoride) cocktail using Centricon (Amicon) with a cut-off value of 3 kDa. The conditioned medium supernatants were stored at −70 °C until sandwich ELISA analysis.

2.4. Aβ detection by sandwich ELISA

We use the monoclonal antibody 6E10 (Signet), specific for amino acids 1–17 of Aβ as the capture antibody, and antibodies specific for Aβ 1–40 or 1–42 as detection antibodies (Biosource International). Standard curves of synthetic peptides Aβ 1–40 and Aβ 1–42 (Biosource International) were prepared in the same medium as samples. Under these conditions, ELISA accurately detected Aβ levels of 15 pg/ml or greater.

The 6E10 was coated at 10 μg/ml into 96-well immunoassay plates (Nunclon Maxisorp), overnight at 4 °C. Then, plates were blocked

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Abbreviations: APP, amyloid precursor protein; Aβ, beta amyloid; PSEN, presenilin; PARP-1, Poli (ADP-ribose) polimerase 1; AD, Alzheimer disease

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with 3% BSA in phosphate-buffered saline (PBS, pH7.4) for at least 1 h at 37 °C. They were washed with 0.05% Tween 20 in PBS (PBST). Samples and standards (50 μl/well) were added to plates and incubated at room temperature (RT) for 2 h. Plates were washed three times with PBST between each assay step.

Anti Aβ 1–40 or anti Aβ 1–42, diluted to 1 μg/ml with 1% BSA in PBST, was incubated in wells for 1 h at RT. Secondary antibody (anti-rabbit/goat horseradish peroxidase conjugate, Nordic) diluted 1/1000 with 1% BSA in PBST, was added to wells for 30 min at RT. The assay was developed with 1 mg/ml of orto-phenylendiamine (OPD) in phosphate buffer pH 6.0 and 0.1% H_2O_2. Optical densities at 450 nm were measured using a spectrophotometric plate reader (Dynatech).

2.5. Cell viability analysis

Cells were seeded on 96-multiwell plates 72 h before beginning incubations at 37 °C in an atmosphere humidified with CO_2 to 7%.

The 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay. The extent of cell death was evaluated using the 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay [8]. After culture times cells were incubated with 0.5 mg/ml MTT for 3 h at 37 °C. MTT/formazan was extracted by overnight incubation at 37 °C with 100 μl extraction buffer [20% sodium dodecyl sulphate (SDS), 50% formamide adjusted to pH 4.7 with 0.02% acetic acid and 0.025 N HCl]. Optical densities at 570 nm were measured using a spectrophotometric plate reader (Dynatech).

Release assay of the enzyme lactate dehydrogenase. The cell viability assessed by determining the lactate dehydrogenase (LDH) into the culture medium indicates loss of membrane integrity and cell death. The LDH enzyme activity was measured with a commercial kit, CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega), following the manufacture instructions. Optical densities at 490 nm were measured using a spectrophotometric plate reader (Dynatech).

2.6. Western blotting

Cell lysates were analyzed by Western blot as previously described [9]. Briefly, RIPA extracted cell lysates (20 μg determined by Bradford assay) were subjected to 8% SDS–PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes (BioRad). Non-specific reactivity was blocked by overnight incubation at 4 °C with 3% BSA/0.2% Tween-20 in PBS. Membranes were further processed by incubating with primary antibodies for 2 h at room temperature, followed by incubation with secondary antibodies (anti-mouse horseradish peroxidase conjugate, Vector Laboratories) and finally, band visualization of using the enhanced chemilumininescence Western-blotting analysis system (Pharmacia Biotech). The primary antibodies used were monoclonal anti-APP antibody, MAB348 (Chemicon International, Inc.) and monoclonal anti-PARP antibody (PharMingen International). For internal control, levels of α-tubulin (anti-alpha-tubulin, Sigma) were examined on the same blot.

2.7. Immunocytochemistry

Cells grown on coverslips were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min and permeabilized with PBS containing 0.2% Triton X-100 and 2% horse serum at 4 °C overnight. Cells were incubated at RT for 3 h with primary antibody (anti-APP, MAB348). Later, cells were incubated at RT for 1 h with biotinilated anti-mouse (Vector Laboratories) followed of the incubation with fluorescein avidin-D (Vector) at room temperature for 30 min. For nuclei staining, coverslips were incubated in PBS containing 5 μg/ml DAPI (Sigma) at for 15 min. Reactive images were observed by fluorescence microscopy (BX 51, Olympus).

2.8. Statistics

Results are expressed as means ± the standard error (S.E.) and statistical differences were determined by Student's two-tailed unpaired *t*-test.

3. Results

We have generated cellular lines of SKNMC human neuroblastoma that stably overexpress the APP protein with the purpose of analizing, in a human neuronal model, the correlation between Aβ production and the neuronal death. We stably transfected the SKNMC cell line with an expression vector (pREP4) which contains the APP695 cDNA, and we have established two cell lines (C2 and C3) with APP overexpression; the same neuroblastoma transfected with pREP4, without the insert, was used as control culture (C1-). We have characterized the APP overexpression of C2 and C3 lines as well as the integrity of the overexpressed protein, using Western blot assay (Fig. 1(A)) with the anti-APP MAB348 antibody. The quantification by densitometry revealed overexpression levels of 6 and 1.5 times for C2 and C3, respectively, with respect to SKNMC (Fig. 1(B)). The cytoplasmic pattern for APP observed by immunofluorescence for C2 and C3 lines (Fig. 1(C)), is according with its physiologic localization [10]; SKNMC and C1- cultures, that only express the endogenous APP protein, hardly show label of APP.

We subsequently quantified the two main species of amyloid peptide, Aβ 1–40 and Aβ 1–42, using a "sandwich" ELISA assay. As it is observed in the Fig. 2 (panels A and B) the C2 cell line that express higher APP levels, is also the line that secretes more Aβ peptide. Aβ concentration was of 2500 pg/ml (600 pM) for Aβ 1–40 at 24 h after platting the cells, reaching to a plateau of 4100 pg/ml (1000 pM) at 72 h; Aβ 1–42 is not detectable until 48 h, and it reaches a concentration of

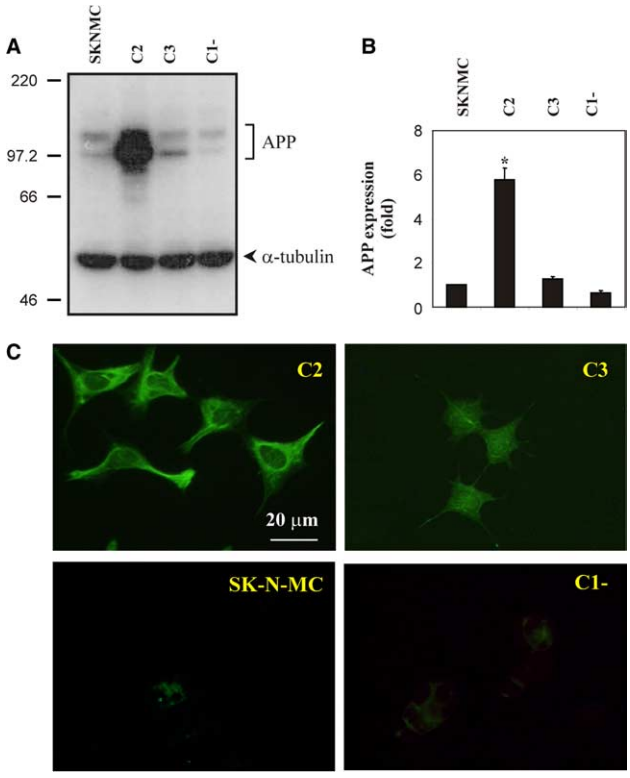

Fig. 1. Overexpression of human APP in SKNMC cell lines (C2, C3) stably transfected with pREP4-APP695. (A) Western blot showing APP expression in C2 and C3 (SKNMC cell lines stably transfected with pREP4-695), C1- (SKNMC cell line stably transfected with pREP4 vector) and SKNMC. (B) Quantification by densitometry of APP expression. Bars indicate fold expression referred to the APP expression in SKNMC and normalized with α-tubulin. Values are given as means ± S.E. from three independent experiments (*, $P < 0.05$). (C) Immunofluorescence for APP of C2, C3, C1- and SKNMC cell lines.

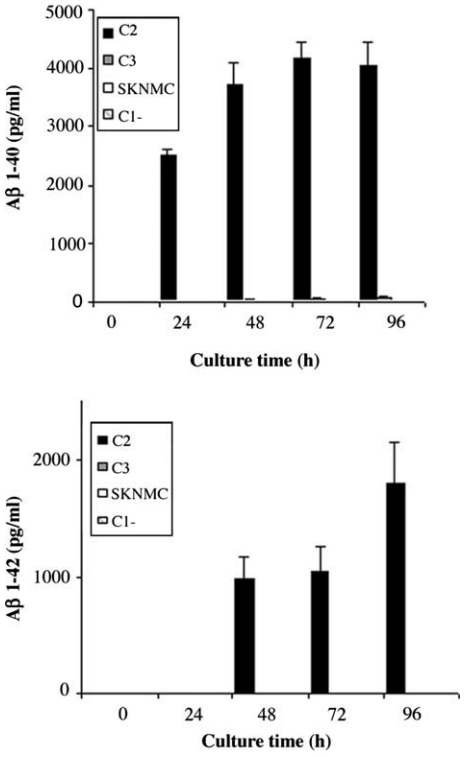

Fig. 2. Kinetics of Aβ secretion in SK-N-MC, C1-, C2 and C3 cellular lines. Quantification by sandwich ELISA specific for each one of Aβ species (Aβ 1–40 and Aβ 1–42) present in the culture medium (mean ± S.E., $n = 3$), at the indicated culture times.

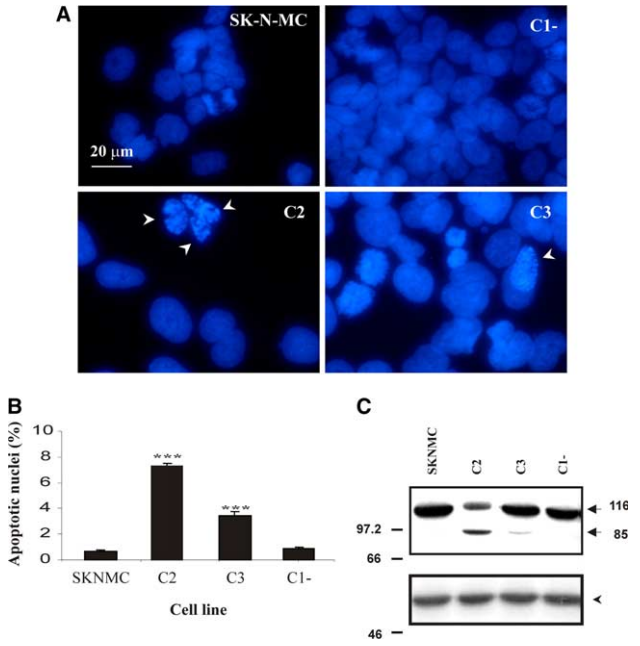

Fig. 3. Apoptosis in the C2 and C3 cellular lines. (A) Representative images of nuclear morphology analysis (DAPI) staining of the different cellular lines. The nuclear shape of apoptotic cells is indicated. Scale bar = 20 μm. (B) Quantification of the morphological analysis. For each cell culture, 6 fields of 3 independent cultures were analyzed. Results are given as the percentage of apoptotic nuclei per field (***$P < 0.001$). (C) Representative Western blot shows the degradation of the PARP-1. The arrows indicate the bands corresponding to the protein PARP-1 without degrading (116 kDa) and to the fragment of 85 kDa resulting of its proteolysis. In the inferior panel, the arrow indicates the band corresponding to the α-tubulina.

1800 pg/ml (400 pM) at 96 h. On the other hand, the C3 cell line produces levels of secreted Aβ 1–40 much lower (100 pg/ml: 20 pM), than the C2 cell line; and Aβ 1–42 is not detectable at none of the assayed times. SKNMC and C1- lines did not produce detectable levels of secreted Aβ 1–40 or Aβ 1–42.

During the cellular lines characterization we found C2 cell line presented a morphological changes series (data not shown), among those a greater neurite extension, greater cellular volume and greater adhesion to the culture plate, as well as a slower growth than SKNMC, C3 and C1- cell lines. Likewise, C2 and C3 presented detectable cellular death that did not present neither the SKNMC cellular line, nor the C1- control line.

When we examined the nuclear morphology of SKNMC, C1-, C2 and C3 lines using DAPI staining (Fig. 3, panels A and B) we found C2 and, in smaller proportion, C3, presents a significant percentage of nuclei with condensed chromatin (C2: 7.28%, C3: 3.4%) compared with SKNMC and C1- (0.63% and 0.89% of apoptotic nuclei, respectively). The apoptotic program involves a specialized cellular machinery which includes the activation of caspases [11], and when we analized the degradation of the caspase sustrate, poly (ADP-ribose) polymerase 1 (PARP-1), (Fig. 3(C)) both in C2 and C3 lines the anti-PARP antibody revealed besides the band corresponding to the whole PARP-1 protein (116 kDa), a band corresponding to 85 kDa fragment resulting from PARP-1 degradation as consequence of the activation of the apoptotic program. In contrast, in SKNMC and C1- lines the proteolytic degradation of PARP-1 was not detected. These results indicated a positive correlation among APP overexpression and the apoptotic program activation of cellular death.

The synthetic Aβ concentration necessary to produce cytotoxicity in SKNMC neuroblastoma (10 μM) (data not shown) was several orders of magnitude higher than the Aβ secreted concentration by C2 (Aβ 1–40: 1 nM; Aβ 1–42: 400 pM) and C3 (Aβ 1–40: 20 pM). This result suggest that the lack of action of Aβ on untransfected SKNMC cells is not due to a problem at the cell receptor level, but simply, of Aβ concentration. On the other hand, Aβ levels secreted by C2 line were the same order of magnitude than the physiological Aβ concentration in cerebrospinal liquid [12]. With the purpose of studying if the Aβ secreted by C2 cells produced cell death in SKNMC we performed coculture experiments after checking the "Transwell®" system did not affect Aβ levels secreted by C2 (data not shown). When we analyzed the viability of lines C2, C1- and SKNMC in the different cocultures, although the loss of viability of C2 was 30% approximately respect to untransfected cells (Fig. 4(A)), we observed that the viability of SKNMC was not affected by coculturing it with C2. Additionally, the proteolytic degradation of PARP-1 was observed in C2 line, but not in SKNMC line cocultured in presence of C2 or C1- (Fig. 4(B)).

Therefore, our results indicated that Aβ peptide secreted by the stable cellular line overexpressing APP (C2) does not seem to be the responsible of the basal death which presents this neuroblastoma, and the death of C2 and C3 lines could take place as consequence of APP overexpression.

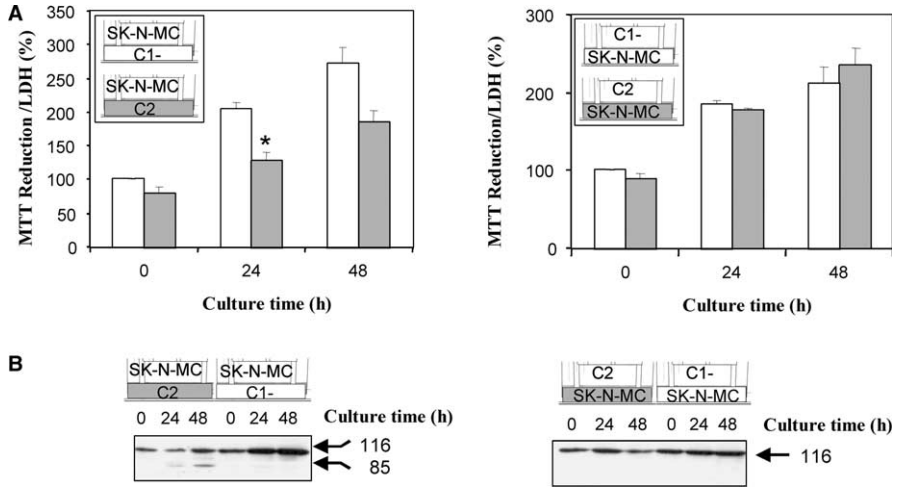

Fig. 4. The Aβ secreted by the C2 cell line does not produce cell death in the SKNMC neuroblastoma. The SK-N-MC, C2 and C1- cell lines were cocultured in the system "Transwell" and seeded in the upper or lower well. (A) Cell viability measured by MTT assay and normalized with the LDH activity. Results (mean ± S.E.) are given as the percentage of viability as compared with the SKNMC control culture ($*P < 0.05$, $n = 3$). (B) Degradation of the PARP-1 analyzed by Western blot.

4. Discussion

All mutations in APP, PSEN1 and PSEN2 genes associated to familial forms of AD increase the production of Aβ [13]. These observations and the broadly reported neurotoxic Aβ peptide effect [14] led to formulate the already classic amyloidogenic theory of AD [15], according to which, Aβ peptide plays an early and central role in the pathogenesis of AD.

Although the APP amyloidogenic processing is majority in neurons [16], most of studies have been carried out in non-neuronal cultures [17] and, in murine neuronal cultures [18]; possibly due to the difficulty to obtain stably transfected human neuroblastoma cellular lines [19]. In this context, we decided to generate lines of SKNMC human neuroblastoma which overexpress in a stable way the 695 isoform of APP protein (C2 and C3 lines) and to analyze Aβ production in these human neuronal lines. Although both lines produced Aβ 1–40, we found only the line which expressed high APP levels (C2) secreted also Aβ 1–42. These results were coherent with those published indicating the majoritary species (90%) are 40 amino acids, both in healthy individuals and in AD patients [12]. Aβ concentration secreted by our C2 line (Aβ 1–40: 1000 pM, Aβ 1–42: 400 pM) is greater than the ones reported for other neuronal models which express human APP695 (Aβ 1–40: 7–25 pM, Aβ 1–42: non-detectable; [17,20]) and similar to that observed in cultures of hamster CHO cellular line stably transfected with APP695 (Aβ 1–40: 807 pM, Aβ 1–42: 11.8 pM; [17]). Likewise, Aβ concentration secreted by our C2 line is in the same magnitude order than in the cerebrospinal fluid of healthy individuals and AD patients [12].

The morphological changes observed, during the characterization of our cellular lines of SKNMC human, are consistent with some of the functions assigned to APP, like the cell–cell [21] and cell–extracellular matrix adhesion [22], as well as the role in the regulation of the neurite growth and extension [21]. Likewise, C2 and C3 lines presented some cellular death levels that do not present neither SKNMC, nor the C1- control line. We found proteolytic degradation of PARP-1 protein in C2 and C3 cellular lines that demonstrated a positive correlation among APP overexpression, Aβ production and cellular death. Our results are consistent with the involvement of caspases in Aβ peptide formation [23], and with data published showing that APP overexpression in rat hippocampal neurons produce their apoptotic degeneration in vitro and in vivo [24,25], in a process that implies caspase 3 activation.

With the purpose of checking the secreted Aβ peptide role in the death of our cellular lines, we carried out coculture experiments, and we found Aβ secreted by C2 line did not affect the cellular viability of SKNMC line. In this sense, it has been described the rat neuronal culture treatment with culture medium – that contains different quantities of Aβ 1–40/Aβ 1–42 – coming from cells that overexpress APP does not affect the viability of the cultures, although these authors attribute the death observed in the rat neuronal cultures that overexpress APP to the production of intracelular Aβ [17,20]. However, we did not detect intracellular Aβ in C2 and C3 lines (data not shown) making improbable the death observed in our cellular lines are due to accumulation of intracellular Aβ.

Along this work, we have described Aβ peptide secreted by SKNMC human neuroblastoma cell lines that overexpress APP in a stable way would not be responsible for the death that these cells present, but however APP overexpression could produce the death, and rises the concentration of secreted Aβ as a consequence.

In summary, our results indicate, in a human neural cell model, that APP overexpression induces the apoptotic death of neurons accompanied by the secretion of Aβ 1–40 and Aβ 1–42 species; that in turn seem not to be able of inducing the death of SKNMC cells.

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