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Apoptosis is directly related to intracellular amyloid accumulation in a cell line derived from the cerebral cortex of a trisomy 16 mouse, an animal model of Down syndrome

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ABSTRACT

Human Down syndrome (DS) represents the most frequent cause of mental retardation associated to a genetic condition. DS also exhibits a characteristic early onset of neuropathology indistinguishable from that observed in Alzheimer's disease (AD), namely the deposition of the β-amyloid peptide. Early endosomal dysfunction has been described in individuals with DS and AD, suggesting an important role of this subcellular compartment in the onset and progression of the pathology. On the other hand, cholesterol activates the amyloidogenic processing pathway for the amyloid precursor protein, and the lipoprotein receptor-related peptide interacts with the β-amyloid peptide. In the present work, using cell lines derived from the cortex of both normal and trisomy 16 mice (Ts16), an animal model of DS, we showed that the application of exogenous β-amyloid has cytotoxic effects, expressed in decreased viability and increased apoptosis. Supplementation of the culture media with cholesterol associated to lipoprotein increased cell viability in both cell lines, but apoptosis decreased only in the normal cell line. Further, intracellular β-amyloid content was elevated in trisomic cells following cholesterol treatment, with higher values in the trisomic cell line. Immunocytochemical detection showed intracellular accumulation of exogenous β-amyloid in Rab4-positive compartments, which are known to be associated to endosomal recycling. The results suggest that the intracellular β-amyloid pool plays a central role in apoptosis-mediated cell death in the trisomic condition.

Down syndrome (DS) patients exhibit neuropathology that is indistinguishable from that observed in AD upon reaching 30–40 years of age, possibly due to the overexpression of APP [14]. An animal model of DS is trisomy of chromosome 16 in the mouse (Ts16), as most genes present in the murine autosome can be mapped to human chromosome 21. As Ts16 animals die in utero, partial trisomic mouse models, such as the Ts65Dn, have been generated. In this sense, the Ts65Dn mouse model exhibits enlarged endosomes, similar to those observed in early stages of AD, and their morphology is normalized when APP gene expression is reduced [9]. Regretfully, Ts16 is lethal in the mouse, which prompted our task of establishing immortalized cell lines from neural tissue of Ts16. The cell lines established from the cerebral cortex of a Ts16 animal, named CTb, retain properties and cellular dysfunctions observed in primary cultures of aneuploid neurons [2,7], when compared to a similar cell line, CNh, obtained from a normal littermate. Namely, the CTb line exhibits altered glutamatergic and cholinergic function which are normalized when APP expression is lowered to levels comparable to the normal CNh cell line [22,25]. Further, we have also showed that it exhibits endosomal abnormalities related to APP processing when treated with cholesterol [4].

Amyloid toxicity is induced according to the aggregation state of the peptide. In this regard, several lines of evidence show that oligomers and fibrils are responsible for such phenomena [15,24]. For fibril formation, oligomers must acquire a beta-pleated sheet conformation, which can be favored by other factors such as acidic pH [28] or presence of heavy metals [13]. Under these conditions, aggregated amyloid peptide induces oxidative stress, generating mitochondrial dysfunction [1,18], increased lipoperoxidation [10,20], and finally cell death by acute effects or apoptosis. Cholesterol induces the amyloidogenic processing pathway of the amyloid precursor protein (APP) [3,30], and cholesterol associ-

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ated to lipoproteins interacts with aggregated amyloid peptides inducing their internalization [23] and subsequent localization to degradation compartments. Several studies, which include the genetic condition associated to the apolipoprotein $\varepsilon 4$, and the use of statins, show a role of cholesterol in the pathogenesis of intracellular βA4 peptide accumulation [5,11]. Intracellular amyloid accumulation has been observed in compartments related to recycling and degradation pathways such as recycling, early and late endosomes [8] and macroautophagic vesicles [33,35]. Further, lysosomal compartments have also been implicated in amyloid pathology, and in this regard, an association of lysosomal hydrolases to amyloid peptides processing has been described [16,19].

Cell lines derived from cerebral cortex of normal (CNh) and Ts16 mice (CTb), an animal model of Down syndrome, were used. Cells were maintained in DMEM/F-12 1:1 culture medium supplemented with 4% (v/v) fetal bovine serum, 2 mM L-glutamine, and 1% penicillin/streptomycin/fungizone solution (Invitrogen). Cultures were kept in an incubator at 37 °C, in an atmosphere of 100% relative humidity and 5% CO_2. Cholesterol supplementation was carried out using cholesterol associated to lipoproteins from bovine serum (Sigma, cat n° L4646) at a final concentration of 150 μg/ml. Cultures in both conditions were kept for 3 days prior to experiments. Amyloid β-peptide 1–42 ($\beta A4_{1-42}$) and biotinylated amyloid β-peptide 1–42 (biot-$\beta A4_{1-42}$) were purchased from AnaSpec. Lyophilized peptides were reconstituted in sterile NH_3OH 0.1%, to a final concentration of 500 μM. The amyloid solution was aliquoted in volumes of 10 μl and stored at −20 °C. Prior to use, peptides were dissolved in DPBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 0.1 mM, KH_2PO_4 1.2 mM, pH 7.4) at a final concentration of 100 μM and stored at 37 °C for 2 days to promote aggregation. To test cell viability, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed to test amyloid cytotoxic effects. Briefly, cells were exposed to aggregated $\beta A4_{1-42}$ 5 μM for 2 days, after which the culture medium was replaced by DMEM/F-12 1:1 without phenol red and MTT (Sigma) 1.5 mg/ml. Cells were incubated for 4 h and the formazan generated was dissolved in isopropanol. The absorbance was then read in an ELISA plate reader at 540 nm. To calculate the viability percentage, control plates not exposed to βA4 were included in every condition. $\beta A4_{1-42}$-mediated toxicity is presented in Fig. 1A. Incubation with $\beta A4_{1-42}$ resulted in a comparable decrease in cell viability in both cell line, with values of 58 ± 4.5 and 62.7 ± 2.3% for CNh and CTb cell lines, respectively, compared to sister cultures kept in absence of βA4. When the media was supplemented with cholesterol, a protective effect could be observed on βA4-mediated cell toxicity. Indeed, cholesterol supplementation resulted in increased viability, with values of 72.8 ± 3.8 and 87.8 ± 1.7% for CNh and CTb cell lines, respectively (Fig. 1A). Interestingly, the protective effect of cholesterol on cell toxicity was greater for CTb cells.

To test apoptosis, TUNEL assays were performed and detection of incorporated dUTP was carried out with the ABC Elite detection system (VectorLabs) and visualization by the DAB substrate kit (VectorLabs). The percentage of apoptosis was estimated by counting the total number of TUNEL positive cells under the microscope in every cell sample population. Basal values of apoptosis were similar among the different treatments, ranging from 1 to 2% total cell population (data not shown). $\beta A4_{1-42}$-induced apoptosis was calculated as the percentage of apoptosis minus the basal value for every culture condition. When both cell lines were treated with the amyloid peptide, apoptosis levels were 8.4 ± 0.6 and 15.3 ± 0.7% for CNh and CTb cell lines, respectively (Fig. 1B), suggesting that the trisomic cell line presents greater susceptibility to amyloid peptide-mediated apoptosis than the CNh cell line. When the media was supplemented with cholesterol, $\beta A4_{1-42}$-mediated apoptosis was reduced significantly to 2.3 ± 0.7 in the normal derived CNh cell line. However, the CTb cell line showed comparable levels of apoptosis as compared to cultures kept in the absence of cholesterol (14.3 ± 0.8%, non-significant) (Fig. 1B).

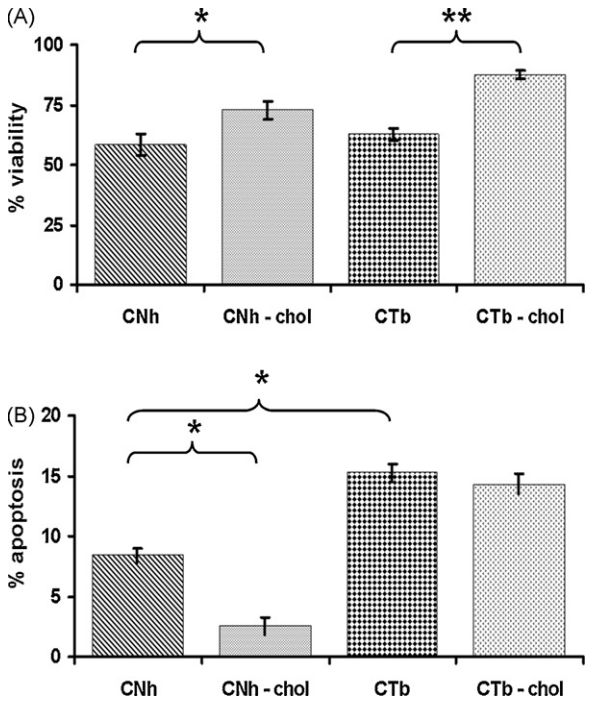

Fig. 1. βA4 [1–42] mediated toxicity and apoptosis in CNh and CTb cell lines in the presence and absence of cholesterol. Cells were exposed to βA4 [1–42] 5 μM for 2 days. (A) A decrease in βA4 [1–42]-mediated toxicity is evident when both cell lines were supplemented with cholesterol, as expressed in increased in cell viability. The latter effect was stronger in the Ts16-derived cell line. (B) A decrease in βA4 [1–42]-mediated apoptosis is evident in the normal CNh cell line supplemented with cholesterol associated to lipoproteins. Both cell lines present differential apoptotic responses when exposed to amyloid peptides, reflected as an increase in apoptosis labeling in the trisomic condition. $^*p < 0.05$, $^{**}p < 0.01$, $n = 4$. CNh-chol and CTb-chol represent cell lines supplemented with cholesterol associated to lipoproteins (150 μg/ml).

To test the internalization of $\beta A4_{1-42}$, biotinylated form of the peptide was used (biot-$\beta A4_{1-42}$). Aggregation was attained by the same procedure described above for the non-biotinylated peptide. The aggregated peptide was added to the culture medium at a final concentration of 1 μM, and the cells were incubated for 6 h at 37 °C. After incubation with biot-$\beta A4_{1-42}$, the cells were washed with cold DBPS and fixed with freshly prepared 4% paraformaldehyde. Detection of biotinylated amyloid peptide was carried out using avidin conjugated horseradish peroxidase (Av-HRP) (BioGenex), and the reaction was later visualized using the colorimetric peroxidase substrate, TMB. The colorimetric reaction was stopped by addition of a HCl 0.1% solution to each plate, which was later read in an ELISA plate reader at 450 nm. To determine total biot-$\beta A4_{1-42}$, cells were permeabilized with ethanol 70% and biotin was then detected with Av-HRP. To evaluate intracellular amyloid, the extracellular biotinylated amyloid was blocked by incubation with avidin 0.1% solution (Dako), permeabilized with ethanol 70%, and detection of biotinylated intracellular amyloid peptide with Av-HRP. Intracellular biot-$\beta A4_{1-42}$ was estimated as the percentage of the total measured biot-$\beta A4_{1-42}$. In this sense, when cells were supplemented with cholesterol, CNh cells showed a statistically significant decrease in the intracellular content of biot-$\beta A4_{1-42}$. Conversely, CTb cells showed no significant variation between both conditions (Fig. 2). To test if the recycling endosome machinery is involved in this event, we performed immunocytochemistry against the recycling endosome protein Rab4, and evaluated the colocalization of both proteins. Fig. 3 shows CNh and CTb cell

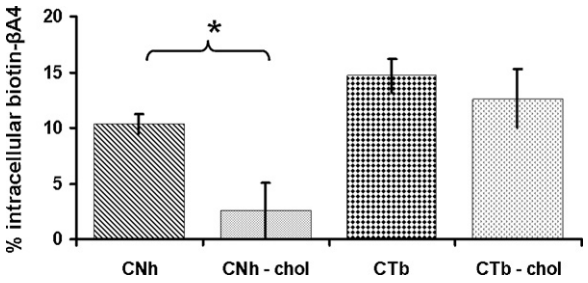

Fig. 2. Intracellular βA4 [1–42] in CNh and CTb cell lines in the presence and absence of cholesterol. CNh and CTb cells were incubated with biotinylated βA4 [1–42] 1 μM for 6 h at 37 °C. Cells were fixed with cold formaldehyde 4% and intracellular biot-βA4 [1–42] was detected. Only the CNh cell line supplemented with cholesterol associated to lipoproteins (150 μg/ml) exhibited a decrease in the intracellular biot-βA4 [1–42] content. $^{*}p < 0.05$, $n = 3$. CNh-chol and CTb-chol represent cell lines supplemented with cholesterol associated to lipoproteins.

lines supplemented with cholesterol associated to lipoproteins that present Rab4 immunoreactivity. The results indicate a persistent colocalization in the trisomic condition after 6 h of biot-$\beta A4_{1-42}$ incubation. At this same time period, biot-$\beta A4_{1-42}$ was undetectable in the normal derived CNh cell line.

Protein interaction between βA4 or APP C-terminal fragments (CTFs) and the lipoprotein receptor-related protein (LRP) increases internalization of amyloid precursors and subsequently results in βA4 accumulation in intracellular compartments related to degradation mechanisms, such as the endosomal/lysosomal pathway [32]. These cholesterol-mediated effects could be related to increased cell viability and decreased apoptosis related to βA4 exposure [29], but the protective role appears to be controversial [12,27]. Nevertheless, in the present study, cholesterol-associated neuroprotection exhibited a differential response between the normal and trisomic condition. Indeed, both cell lines studied were sensitive to βA4 exposure, exhibiting approximately a 40% toxicity for CNh and CTb cell lines when aggregated amyloid peptides were included in the culture medium. When cholesterol associated to lipoproteins was added externally, there was a significantly stronger neuroprotective effect in the Ts16-derived cell line, which exhibited around 10% of toxicity as evaluated by the MTT reduction assay, whereas the CNh cell line exhibited approximately 25% of toxicity. In the CTb cell line treated with cholesterol associated to lipoproteins, βA4-induced toxicity appears to be mainly produced by apoptosis. Indeed, the values obtained in the CTb cell line in the aforementioned conditions are quite comparable (12% of toxicity measured by MTT assay vs. 13% of apoptosis measured by TUNEL assay). Hence, it is plausible to infer that cell death in the trisomic condition operates primarily via an apoptotic pathway. Conversely, in the normal CNh cell line, cholesterol supplementation induced a statistically significant decrease in toxicity and apoptosis, an effect comparable to that previously observed in embryonic cerebral cortex cultures [27]. In this cell line, cell toxicity under cholesterol supplementation conditions was around 25%, and it could be mostly explained by acute toxic events related to amyloid peptides, and not by apoptotic mechanisms which represent only 3% of total cell death observed. According to the above, in the Ts16 condition, apoptotic mechanisms would be the main responsible for βA4-mediated neuronal damage, whereas direct toxic mechanisms would have a stronger contribution in CNh cell death.

According to our results, a statistically significant decrease in intracellular βA4 levels occurs when the CNh cell line is treated with cholesterol associated to lipoproteins. However, in the trisomic CTb cell line, the intracellular content of βA4 remains unchanged up to 6 h post-exposure to the peptide, when compared with cells kept in the absence of cholesterol. When cholesterol is associated to lipoproteins, an interaction exists between the cholesterol carrier and the βA4 peptide, which is linked to an internalization event of peptides derived from APP processing by way of the lipoprotein related peptide (LRP) [21,34]. The CTb cell line exhibits endosomal dysfunctions similar to those observed in individuals with DS and AD, which in turn are not found in the normal CNh cell line [4]. It is then plausible to attribute βA4 accumulation in intracellular compartments to dysfunction of degradative pathways such as lysosomal degradation or macroautophagy. In this sense, alkalinization of lysosomes or inhibition

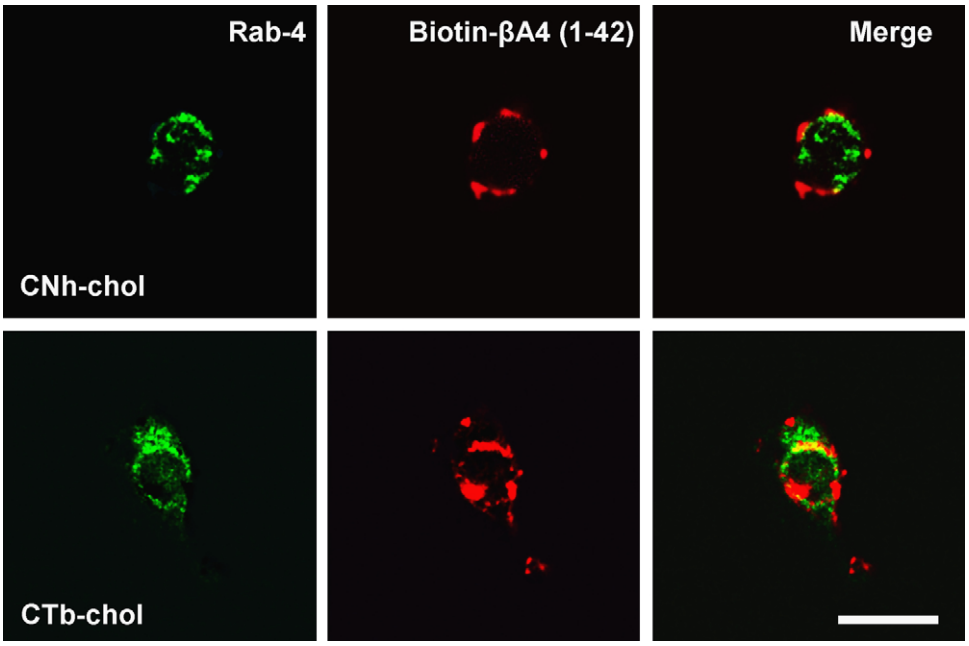

Fig. 3. Internalized βA4 [1–42] colocalize with Rab4 in the CTb cell line treated with cholesterol. Immunocytochemistry against the endosomal recycling peptide Rab4 (FITC labeling) and fluorescence detection of biot-βA4 [1–42] (Texas Red labeling). Colocalization of both peptides in the trisomic CTb cell line treated with cholesterol associated to lipoproteins (150 μg/ml) is evident after 6 h of exposure. Scale bar: 25 μm.

of lysosomal hydrolases could increase the effect observed in the trisomic cell line. On the other hand, avoiding amyloid internalization or increasing the recycling machinery function would decrease intracellular amyloid accumulation and the apoptotic cell death related to intracellular amyloid in the CTb cell line. In the CNh cell line, the presence of intracellular biotinylated βA4 was reduced when culture medium was supplemented with cholesterol, whereas in the CTb cell line, the intracellular biotinylated βA4 level remained unchanged. We hypothesize that, in both cell lines, cholesterol promotes βA4 internalization and the degradation/recycling process. However, in CTb cell line, this process appears to be operating ineffectively, thus generating intracellular accumulation of this kind of peptides due to the endosomal abnormalities described.

Increasing evidence postulates a role of cholesterol in the neuropathology of AD [17,26]. In this work, we have shown that cholesterol associated to lipoproteins presents a neuroprotective effect on cell lines derived from cerebral cortex of normal (CNh) and Ts16 (CTb) mice, mainly against apoptotic cell death in the CNh cell line. However, in the Ts16 cell line this protection is observed only against the acute toxic effects of amyloid peptides, not related to apoptosis. Indeed, the apoptosis levels in the CTb cell line remained unchanged between control and cholesterol supplementation conditions. Similar events were observed when intracellular amyloid levels were studied, suggesting a role of intracellular amyloid accumulation in apoptotic cell death in the Ts16 condition. Meanwhile, cell damage observed by cytotoxic and acute amyloid effects are decreased in the CTb cell line supplemented with cholesterol, probably by redistribution of plasma membrane domains and the decreased or null recycling to cell surface of the internalized amyloid peptide from early endosomes [4], being accumulated in an intracellular form and promoting apoptotic cell death. When cholesterol supplementation is not performed, the effect of intracellular accumulation is evident as apoptotic cell death and the cytotoxic effects observed could be related to cell surface associated amyloid. This event is not present into the CNh cell line where intracellular accumulation is not observed, probably due to the normal functioning of the recycling machinery.

As previously discussed, cholesterol associated to lipoproteins presents a protective effect on both cell lines by differential mechanisms, probably related to intracellular amyloid accumulation. Cholesterol associated to lipoproteins increases βA4 internalization, where this peptide would localize in compartments related to recycling machinery in the Ts16-derived cell line, but not in the CNh cell line, suggesting an intracellular accumulation in such compartments and dysfunction in the recycling machinery in Ts16 cell line as proposed previously [4], and that could be also operating in DS [8,9].

Increasing evidence exists on the role of degradation pathways in the neuropathology of AD. In this regard, macroautophagy appears to play an important role in these events. This pathway is important in subcellular degradation mechanisms that operate under specific stimuli or conditions [6,31]. When we studied the intracellular localization of biotinylated amyloid, only a fraction of the total peptide was accumulated in Rab4-positive recycling endosomal vesicles. It is then possible that some of the intracellular biotinylated amyloid peptide observed may not be associated to this kind of compartments, and in turn could be directly related to macroautophagy or another degradative pathway [34].

In conclusion, the cell lines evaluated present: (a) apoptotic events related to amyloid peptide exposure, (b) cholesterol associated to lipoproteins induces neuroprotective effects and (c) the neuroprotective effects are associated to intracellular accumulation of amyloid peptides in Ts16, an event that has been observed in individuals with DS.

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