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Monocyte-mediated regulation of genes by the amyloid and prion peptides in SH-SY5Y neuroblastoma cells

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ABSTRACT

Alzheimer's disease as well as prion-related encephalopathies are neurodegenerative disorders of the central nervous system, which cause mental deterioration and progressive dementia. Both pathologies appear to be primarily associated with the pathological accumulation and deposit of β-amyloid or prion peptides in the brain, and it has been even suggested that neurotoxicity induced by these peptides would be associated to essentially similar pathogenic mechanisms, in particular to those that follow the activation of microglial cells. To probe whether the neurotoxic effects induced by the β-amyloid and prion peptides are actually mediated by similar glial-associated mechanisms, we have examined the differential expression of genes in SH-SY5Y neuroblastoma cells incubated with conditioned media from β-amyloid or prion-stimulated THP-1 monocytic cells. According to microarray analysis, not many coincidences are observed and only four genes (Hint3, Psph, Daam1 and c-Jun) appear to be commonly upregulated by both peptides. Furthermore, c-Jun appears to be involved in the cell death mediated by both peptides.

1. Introduction

Alzheimer and prion pathologies are neurodegenerative diseases characterized by the progressive loss of neuronal synaptic function, an effect that in both cases appears to be secondary to the toxicity induced by key proteins, the beta-amyloid (Aβ) in Alzheimer's disease and PrP in prion pathologies, which accumulate and aggregate in the brain. At first sight, both types of pathologies exhibit different specific characteristics in relation with the mode of transmission, the etiology, or the frequency of occurrence, and they can be considered as essentially distinct pathologies (Checler and Vincent, 2002). However, despite these evident differences, these pathologies also show a number of similarities. In both cases neurodegeneration appears to be mediated by proteins that, as mentioned above, are aggregated and accumulated in the brain (Price et al., 1993). Those proteins share a complex multi-domain structure and contain toxic sequences that mediate neurotoxicity and give rise to similar damaging apoptotic phenotypes (Checler and Vincent, 2002). Moreover, they share a number of properties such as the presence of metal-binding sites that mediate the binding of metals ions, in particular copper or zinc (Barnham et al., 2006), or the existence of several repeats of a GxxxG motif in the transmembrane region, which contain a methionine residue that appears to be essential to modulate the neurotoxicity of Aβ in Alzheimer or the disease susceptibility in the prion associated pathologies (Barnham et al., 2006). Evidence reported in those articles supported the hypothesis that neurotoxicity induced by both proteins, the amyloid and prion, would be mediated by similar, if not identical, molecular pathways and opened a very attractive possibility as is the use of common therapeutic strategies to treat and prevent Alzheimer's and prion diseases.

Unfortunately, this hypothesis appears to be too simplistic, and although it is evident that both the β-amyloid and the PrP proteins are neurotoxic and induce similar processes of apoptosis and neurodegeneration, the factors and pathways that mediate these effects could be essentially different (Forloni et al., 1996; Hope et al., 1996; Brown et al., 1997). In this sense, we have also reported that both the amyloid-beta 25–35 (Aβ25–35) and the PrP106–126 fragments induce a distinct gene expression profile in the human SH-SY5Y neuroblastoma cell line. First, the number of genes significantly altered by these treatments was markedly different, 198 in the case of the prion (Martinez and Pascual, 2007b), and 67 in the case of the amyloid fragment (Martinez and Pascual, 2007a), and second, the relationship between the overexpressed and repressed genes was opposite in both cases (16/182 and 54/13, respectively).

Despite this, it has been also well established that in addition to those effects directly induced on neurons, neurotoxicity of both peptides can be largely mediated by microglia activation, and the

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consequent release of cytotoxic molecules such as proinflammatory cytokines or reactive oxygen intermediates. In fact, an increasing body of evidence indicates that neuronal death induced by the β-amyloid and the prion peptides in the brain is mainly associated to processes that are promoted by activation of the surrounding microglial cells (Dheen et al., 2007). To analyze possible coincidences between the neurotoxic effects induced by both peptides through activation of microglia, we have now analyzed the gene expression profile in SH-SY5Y neuroblastoma cells exposed to conditioned media obtained from Aβ25–35 or PrP106–126 stimulated THP-1 human monocytic cells, which were used as a surrogate model of human microglia.

2. Materials and methods

2.1. Cell culture and treatments

Human monocytic THP-1 cells and SH-SY5Y human neuroblastoma cells were cultured as previously described (Combs et al., 1999; Villa et al., 2002) in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco.Life Technologies Ltd., Paisley, Scotland, UK), and maintained at 37 °C in a 5% CO_2 atmosphere.

Synthetic Aβ25–35 obtained from AnaSpec and synthetic PrP106–126 obtained from Neosystem, were prepared as follows: briefly, Aβ25–35 was dissolved in deionized-distilled water at a concentration of 2.5 mM and stored at −80 °C. Previous to the experiments, the stock solution was diluted to the desired concentrations and then added to the culture medium. The prion peptide 106–126 was dissolved in PBS at a concentration of 2 mM and maintained for 48 h at room temperature to allow polymerization, before being added to the cells.

For experiments, THP-1 monocytes were incubated for 48 h with or without 10 μM Aβ25–35 or 10μM PrP106–126, and the conditioned media collected and centrifuged to eliminate cell residues. The cell-free supernatants were then used to replace the culture media of SH-SY5Y cells previously plated in 6-well plates and grown for 24 h in RPMI/10%FBS media. Six hours after addition of conditioned media a set of cells was harvested for posterior RNA extraction and determination of gene expression. The remaining cells were incubated in the presence or in the absence of conditioned media for 24 h and then photographed and fixed for histological analysis or collected and saved for subsequent protein or RNA extraction.

2.2. Fluorescence microscopy

SH-SY5Y cells were incubated for different time periods with the supernatants of amyloid- or prion-treated THP-1 cells or RPMI fresh medium, and then fixed with 4% paraformaldehyde at pH = 7.4 and room temperature for 1 h, permeabilized with 0.1% Triton-X-100, and then subjected to TUNEL assay to evaluate the cellular death by using the in situ cell death detection kit Fluorescein (Roche). To further confirm the findings of TUNEL assay, cells were washed with PBS, stained with DAPI, and analyzed by fluorescence microscopy, using a Zeiss Axiophot inverted fluorescence microscope (515–565 nm for TUNEL, and 450–480 nm for DAPI). Images of cells were then digitized using an Olympus DP70 color camera.

2.3. MTT cell viability assay

Cell viability was evaluated in 96-well culture plates using a colorimetric assay based on the reduction of tetrazolium dye (MTT) to a blue formazan product. After incubation for 4 h with MTT (0.5 mg/ml) at 37 °C, isopropanol/HCl was added to each 96-well and the absorbance of solubilized MTT formazan products was measured spectrophotometrically at 570 nm.

2.4. RNA interference

Expression of c-Jun in the SH-SY5Y cells was inhibited by using the Santa Cruz Biotechnology c-Jun siRNA (h2) (catalog number sc-44201). Cells were grown to 60% confluence and the c-Jun siRNA or a control siRNA (catalog number sc-37007) were transfected (using the transfection reagent sc-29528) according to the manufacturer's instructions. At 24 h after transfection, the medium was replaced and the cells were further incubated for 48 h. Next, media were removed and the cells were exposed to the THP-1 conditioned supernatants for 24 h.

2.5. RNA preparation and microarray analysis

For RNA isolation cell lysates were homogenized and the RNA purified by using the QIAshredder and RNeasy Mini kits of Qiagen, according to the manufacturer's recommendations. The final amount of isolated RNA was determined in each sample by spectrophotometry and its quality assessed by electrophoresis on agarose gels.

Preparation of cDNA, cRNA, hybridization and scanning of microarrays was performed following manufacturer's protocols. cDNA, and biotinylated cRNAs were synthesized from 5 μg RNA samples with the GeneChip expression 3′ amplification reagents (one-cycle cDNA synthesis, and IVT labelling) kits of Affymetrix, and biotinylated probes were hybridized to an Affymetrix gene chip human genome U133A Plus 2.0 array, a microarray that contains more than 54,000 probe sets and allows an accurate analysis of the quantitative expression of over 47,000 transcripts, including 38,500 well characterized human genes. Analysis for differential expression was performed using the R platform for statistical analysis (R Foundation for Statistical Computing, Vienna) and several packages from the Bioconductor project (Gentleman et al., 2004; Carey et al., 2005). The raw data were imported into R and pre-processed using the *affy* package and the robust multichip average method (Irizarry et al., 2003). Genes were selected based on fold change. For this task an absolute fold change of 1.6 was used.

2.6. RT-PCR amplification of mRNA

RT-PCR was used to validate the differential expression of several Aβ- or PrP-responsive genes detected in the microarray analysis. Total RNA was extracted from cell cultures as mentioned above and cDNA was prepared from 250 ng of RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). For quantitative PCR, a cDNA aliquot corresponding to 5 ng of the starting RNA was used, with Taqman Assay-on-Demand primers and the Taqman universal PCR master mix, No Amp Erase UNG (Applied Biosystems) on a 7900HT fast real-time PCR system (Applied Biosystems). The PCR program consisted in a hot start of 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. PCRs were performed in triplicates, using the 18S gene as internal standard and the 2-cycle threshold method for analysis (Livak and Schmittgen, 2001).

2.7. Statistical analysis

When appropriate, the significance of differences was calculated with the Student's *t*-test, and it was indicated in the corresponding figure by the following symbol: $^{*}p < 0.05$ and $^{**}p < 0.01$.

3. Results

3.1. Effects of the THP-1 conditioned medium on neuronal morphology and viability

It is widely accepted that both the β-amyloid and prion peptides induce microglial activation and the subsequent secretion of cytokines and neurotoxic reactive oxygen species (Forloni et al., 1993; Klegeris et al., 1997), which in turn may increase neuronal apoptosis (Dheen et al., 2007).

To further analyze and compare how the β-amyloid- and prion-stimulated microglia may affect neurons, we have exposed SH-SY5Y neuroblastoma cells to conditioned media obtained from THP-1 cells, a monocytic cell line that exhibits responses to stimuli similar to those of microglia (Combs et al., 1999). THP-1 cells were incubated with or without 1, 2, 5 or 10 μM Aβ25–35 or PrP106–126 for a 48 h time period and the recovered conditioned media was added to SH-SY5Y neuroblastoma cells that were then incubated for an additional 24 h time period. The morphology and viability of cells were estimated by optical microscopy, TUNEL and DAPI staining (Fig. 1), and by MTT assay and Western blot analysis of caspase-3 (Fig. 2).

As illustrated in Fig. 1, morphology and viability of cells were not affected by conditioned medium from THP-1 cells treated with scrambled sequences, with 5 μM Aβ25–35 or PrP106–126 (panel A), or with lower doses of those peptides. However, the cell morphology as well as the viability appeared to be severely affected after 24 h of exposition to conditioned media from 10 μM Aβ25–35 or PrP106–126 treated THP-1 cells (panel B). As shown in this panel, a large amount of neuroblastoma cells become apoptotic after 24 h incubation with conditioned media from 10 μM Aβ25–35 or PrP106–126 treated cells. The number of cells was clearly reduced by both treatments and the residual cells became more rounded in appearance. Fig. 2 includes the results obtained in a representative MTT assay (panel A), which confirms the reduced viability of cells, and the levels of activated caspase-3 detected by Western blot (panel B), which corroborated that apoptosis was significantly increased in SH-SY5Y cells incubated