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NDM29, a RNA polymerase III-dependent non coding RNA, promotes amyloidogenic processing of APP and amyloid β secretion

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ABSTRACT

Neuroblastoma Differentiation Marker 29 (NDM29) is a RNA polymerase (pol) III-transcribed non-coding (nc) RNA whose synthesis drives neuroblastoma (NB) cell differentiation to a nonmalignant neuron-like phenotype. Since in this process a complex pattern of molecular changes is associated to plasma membrane protein repertoire we hypothesized that the expression of NDM29 might influence also key players of neurodegenerative pathways. In this work we show that the NDM29-dependent cell maturation induces amyloid precursor protein (APP) synthesis, leading to the increase of amyloid β peptide (Aβ) secretion and the concomitant increment of Aβ x-42/Aβ x-40 ratio. We also demonstrate that the expression of NDM29 RNA, and the consequent increase of Aβ formation, can be promoted by inflammatory stimuli (and repressed by anti-inflammatory drugs). Moreover, NDM29 expression was detected in normal human brains although an abnormal increased synthesis of this ncRNA is induced in patients affected by neurodegenerative diseases. Therefore, the complex of events triggered by NDM29 expression induces a condition that favors the formation of Aβ peptides in the extracellular space, as it may occur in Alzheimer's Disease (AD). In addition, these data unexpectedly show that a pol III-dependent small RNA can act as key regulator of brain physiology and/or pathology suggesting that a better knowledge of this portion of the human transcriptome might provide hints for neurodegeneration studies.

1. Introduction

Soluble and insoluble aggregates of Aβ or intracellular tangles of hyperphosphorylated Tau protein have been alternatively regarded as the most likely causes of neurodegeneration in AD (reviewed in [1,2]). In particular, the "amyloid hypothesis" originates by the observation that post mortem analysis of AD patients' cerebral cortices revealed amyloid plaques within the brain parenchyma [3]. This phenomenon is mainly due to an increased secretion of Aβ (the main component of amyloid plaques) in the extracellular space. In particular, an enhanced ratio between the two Aβ molecular variants (Aβ x-42 and Aβ x-40), favoring the formation of the less soluble Aβ x-42 variant, was observed. As a consequence, high amounts of a mixture of neurotoxic Aβ peptides accumulate in the brain. However, although increasing body of evidence supports a primary role of Aβ in the etiology of AD, little is known about the upstream events that trigger the amyloid cascade, so that the first disease-causing events still remain unknown. In this context several research approaches have been developed to investigate different possible pathogenetic events for AD. Among these, prolonged inflammatory stimuli are thought to be part of the complex pattern of events that ultimately lead to the onset of the disease-associated conditions, such as increased secretion and aggregation of Aβ peptides synaptic dysfunction, reactive gliosis and the generation of neurofibrillary tangles of hyperphosphorylated Tau [4–9].

Besides this working hypothesis, emerging evidence supports the possibility of incomplete, defective attempt to cell cycle re-entry as a possible cause of neuron death in AD [10,11]. In this view aberrant mitotic stimuli might be generated in neurons of AD patients and, as a consequence lead post-mitotic neurons to apoptosis [12]. This hypothesis is currently supported by experimental observations, showing an association between high aneuploidy rate, chromosomal missegregation and AD [13]. Interestingly, a link between the control of cell cycle and AD has been also recently proposed by epidemiological observations. Indeed, a diminished risk of AD in cancer patients and a highly reduced risk of cancer associated to AD have been documented [14].

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In a recent work we described Neuroblastoma Differentiation Marker 29 (NDM29), a RNA pol III-transcribed ncRNA whose synthesis is lead by an extragenic type 3 promoter composed by a TATA box, a Proximal Sequence Element (PSE) and a Distal Sequence Element (DSE) [15]. A preliminary expression analysis showed that NDM29 is actively transcribed in cells of the central nervous system, whereas cells characterized by epithelial-like phenotype synthesize a modest amount of this ncRNA. In order to investigate the functions of NDM29 we generated two genetically engineered SKNBE2 neuroblastoma (NB) cell lines that overexpress NDM29 [16,17]. Interestingly, we found a direct relationship between the expression levels of this ncRNA and cell differentiation toward a neuron-like phenotype. Indeed, the expression of NDM29 strongly restricts the malignant potential of NB cells leading to their maturation to a non-malignant neuron-like phenotype [16,17]. In particular, we demonstrated that NDM29 overexpression caused neuronal differentiation of S1.1 cells in terms of molecular markers (high increase in the transcription of GD2 synthase and NF68, while modest increase in glial markers) and electrophysiological behavior (we detected high levels of fast inactivating Na^+ current, able to generate mature action potentials and induction of the expression of functional GABA-A receptors on their membrane) [17]. These indicators show that NDM29 expression in S1.1 cells is accompanied by a well-coordinated differentiation process toward the neuron-like phenotype [17].

In this context, the aim of this study was to evaluate whether the expression of NDM29 might also influence the processing of APP and/or the amyloid cascade whose perturbation is involved in AD development.

We show that a sustained overexpression of NDM29 actively promotes the molecular processing and the secretion of Aβ peptides, thus influencing the main secretory pathway thought to be involved in AD development. High level of transcription of NDM29 increases APP synthesis and leading to the increase of Aβ secretion. Notably, the increase in Aβ production is mainly ascribed to the toxic insoluble form Aβ x-42, considered of primary relevance for AD etiology [18]. Finally, we demonstrate that the synthesis of NDM29 RNA can be promoted by inflammatory stimuli (and repressed by antiinflammatory drugs) and that this ncRNA, actually detected in human brains, is abnormally expressed in AD patient brains.

2. Materials and methods

2.1. Cell culture and transfection

Different cell lines were used: S1.1, S6, S7, M and M2 cells are SKNBE2 neuroblastoma cells (provided by the cell bank of the National Institute of Cancer Research (IST) Genoa, Italy and obtained from ECACC, [19]) stably transfected with pNDM29-EGFPN1 or pEGFPN1 empty vector respectively as described in [20] (both grown in RPMI supplemented with 10% FBS, 1% glutamine and 200 μg/mL G418) and transiently transfected with pNDM29-EGFPN1, pAntiNDM29-EGFPN1 (pS1.1/A) and pEGFPN1 (grown in DMEM supplemented with 10% FBS, 1% glutamine). HEK293-APP cells were a kind gift of Prof. Luciano D'Adamio (Albert Einstein College of Medicine, NY, USA). Cells were transfected using PEI (polyethylenimine) (SIGMA P3143).

2.2. Real time quantitative RT-PCR analysis

Total RNAs from samples were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, DNAseI-digested and subjected to reverse transcription by Transcriptor High Fidelity cDNA Synthesis Kit (Roche 05081955001) as described in [21]. The total RNA from the samples was measured by real-time quantitative RT-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) following manufacturer's instructions. The sequences of forward and reverse primers were: c-kit: 5′-GCAAGTCAGTGCTGTCGGAA and 5′-AAGATAGCTTGCTTTGGACACA-GA-3′; mmp-9: 5′-CACTCGCGTGTACAGCCG-3′ and 5′-TCGAAG-GGATACCCGTCTCC-3′; NF-68: 5′-CAAGGACGAGGTGTCCGAG-3′ and 5′-CCCGGCATGCTTCGA-3′; APP: 5′-TGGCCCTGGAGAACTACATCA3′ and 5′-CGCGGACATACTTCTTTAGCATATT-3′; NDM29: 5′-GGCAGG-CGGGTTCGTT-3′ and 5′-CCACGCCTGGCTAAGTTTTG-3′. For endogenous control the expression of Glyceraldehyde 3 phosphate dehydrogenase (G3PDH) gene was examined. The sequences for human G3PDH primers were 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′- GAAGATGG-TGATGGGATTTC-3′. Relative transcript levels were determined from the relative standard curve constructed from stock cDNA dilutions, and divided by the target quantity of the calibrator following manufacturer's instructions.

2.3. Immunofluorescence detection

S1.1 and Mock SKNBE2 cells grown on slides, were fixed with methanol, washed 3 times with PBS and incubated with the different primary antibodies in PBS plus 1% NGS for 1 h, and finally incubated with antigen-specific secondary antibodies (Alexa 488 or 568-conjugated, and AMCA from Invitrogen Corporation, Carlsbad, CA, USA). Cells were then mounted by Mowiol and analyzed on BioRad-MRC 1024 ES confocal microscope, equipped with a Nikon Eclipse TE 300 inverted microscope with a 60× objective lens as reported in [22]. Antibody for mouse GM130 (BD USA) was used at the dilution 1:100, mouse α-tubulin (Sigma) 1:1000 and Rabbit N-terminal APP (Sigma) 1:100; Alexa Fluor® 568 and Alexa Fluor® 488 (Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA) were used 1:200.

2.4. ELISA Aβ detection

The amount of secreted Aβ x-40 and Aβ x-42 were evaluated by Human Amyloid (1–40) Assay Kit (IBL 27713) and Human Amyloid (1–42) Assay Kit (IBL 27711) according to the manufacturer's protocol.

2.5. Western blots

Proteins were quantified using a commercial protein quantification kit (Protein Assay, Bio-Rad 500–0006) as described in [23]. The samples were subsequently analyzed by 10% SDS PolyAcrylamide Gel Electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane (Whatman, Inc.). The membranes were initially blocked by an incubation of 2 h in Tris-buffered saline Tween 20 (TBST; 50 mM Tris–HCl, 150 mM NaCl, pH 7.5, 0.05% Tween 20) containing 5% non-fat dried milk. The blots were incubated for 1 h with the appropriate primary antibodies: rabbit polyclonal anti-APP c-terminal (A 8717 Sigma) (1:1000); rabbit polyclonal anti-Presenilin1 (S182 Sigma) (1:2000); rabbit polyclonal anti-Presenilin2 (sc-7861 Santa Cruz Biotechnology) (1:200); rabbit polyclonal antibody anti-BACE1 (pc529, Calbiochem) (1:300). After washing with TBST membranes were incubated with peroxidase-conjugated secondary antibodies [anti-rabbit IgGs (A 0545, Sigma)(1:16000)] for 1 h at room temperature. After washing the reactive bands were revealed with ECL Plus Western Blotting Detection Reagents (Amersham RPN2132). In order to normalize the protein levels, western blot membranes were stripped with “Restore” (Pierce 21059), a western blot stripping reagent, then probed with a monoclonal antibody against-α-tubulin (T 5168 Sigma) (1:2000). Amyloid C-Terminal Fragment western blot analysis was performed by running protein samples on 14% Tris-Tricine gel. The membranes were blocked by an incubation of 1 h in Phosphate-buffered saline Tween 20 containing 5% non-fat dried milk. The blot was incubated overnight with the primary antibody, rabbit polyclonal anti-APP, c-terminal (A 8717 Sigma) (1:1000), diluted in 0.1% NaN_3 in PBS. The