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Spreading of Alzheimer's disease inflammatory signaling through soluble micro-RNA

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Abstract

Alzheimer's disease is a progressive, neurodegenerative disorder that develops within the limbic system, spreading radially into anatomically linked brain association areas as the disease progresses. Analysis of temporal-lobe association of neocortex-derived extracellular fluid and cerebrospinal fluid from Alzheimer's disease patients shows an abundant presence of micro-RNA (miRNA), including the proinflammatory miRNA-146a and miRNA-155. Using a novel and highly sensitive LED-Northern dot-blot focusing technique, we detected the secretion of potentially pathogenic amounts of miRNA-146a and miRNA-155 from stressed human primary neural cells. A conditioned medium containing miRNA-146a and miRNA-155 was found to induce Alzheimer-type gene expression changes in control brain cells. These included downregulation in the expression of an important repressor of the innate immune response, complement factor H (CFH). These effects were neutralized using anti-miRNA strategies. Anti-miRNA-based therapeutics may provide a novel and efficacious treatment to stem the miRNA-mediated spreading of inflammatory signaling involved in Alzheimer's disease.

Keywords

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; Alzheimer's disease; digoxigenin; inflammatory neurodegeneration; locked nucleic acids; micro-RNA; miRNA-146a; miRNA-155; spreading

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Conflicts of interest

There are no conflicts of interest.

Introduction

Alzheimer's disease shows a characteristic spreading of inflammatory neuropathology. The disease develops deep in the brain in the hippocampal region and then advances radially into more peripheral neocortical association areas; this pathogenic spreading is progressive and advances with age [1–5]

Materials and methods

Reagents, Alzheimer's disease tissues, and human neuronal-glia cocultures

The reagents used in these experiments were obtained from independent commercial suppliers and were used without further purification [15–19]. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; 161462 Sigma-Aldrich, St Louis, Missouri, USA) was used as described [20–22]. Human recombinant TNF- α (T6674) and A β 42 peptides (D2534; Sigma-Aldrich) were also used as described previously [15–18]. Alzheimer's disease and age-matched control human temporal lobe and association neocortical tissue and CSF were obtained from brain and tissue repositories including the Institute for Memory Impairments and Neurological Disorders and the University of California at Irvine; tissues were analyzed for total miRNA using miRNA arrays and LED-Northern blots and CFH abundance using western analysis [8,9,12–14]. All Alzheimer's disease cases were adult and all brain tissues were from the temporal lobe; the mean ($\pm$ 1 SD) age of the control brain group ($N=5$) was 72.1 $\pm$ 6.5 years and the mean post-mortem interval (PMI; death to brain freezing interval) was 2.2 h; the mean age of the Alzheimer's disease group ($N=5$) was 73.2 $\pm$ 5.5 years and the mean PMI was 2.15 h. There were no significant differences in the age, sex, or PMI between the Alzheimer and the control tissue groups. HNG cocultures were cultured as described previously and used at 2 weeks of culture [5,10–15].

Extraction of total RNA and protein and quality control

Total RNA and proteins were isolated simultaneously using TRIzol (Invitrogen, Carlsbad, California, USA) [9,12–14]. RNA quality was assessed using an Agilent Bioanalyzer 2100 (Lucent Technologies, Murray Hill, New Jersey, USA; Caliper Technologies, Mountain View California, USA) [15,16]. Typically, 1 μ l of total RNA sample was loaded on an RNA chip (6000 Nano Labchip; Caliper Technologies) and analyzed for quality control; RNA integrity numbers values were typically between 8.1 and 9.1 [14–18]. Protein concentrations were determined using the dotMETRIC microassay (sensitivity 0.3 ng protein/ml; Chemicon-Millipore, Billerica, Massachusetts, USA) [12,18].

miRNA array, LED-Northern dot-blot analysis, RT-PCR, and anti-miRNAs

miRNA labeling, hybridization, miRNA arrays, and reverse transcription polymerase chain reaction analysis were performed as described previously [8,9,12–19]. LED-Northern dot-blot analysis was performed using a modified Bio-Dot microfiltration blotting device (LED=LNA, EDC, and DIG; locked nucleic acids – EDC – digoxigenin; detection limit=0.05 fM of a single miRNA species; apparatus #170-6545, BioRad Life Science Research, Hercules, California, USA) [10–18,23]. LED-Northern dot blots are a significant advancement over

classical Northern blotting techniques because they utilize LNA-stabilized miRNAs or anti-miRNAs (AMs) covalently linked to a nylon-based membrane matrix (using EDC) and are probed using DIG-labeled small RNAs with fluorescent reporters [19–22]. miRNA and/or AMs, as locked nucleic acid (LNA) oligonucleotides, included a control miRNA-183 (5'-TATGGCACTGGTAGAATTCAC-3'), anti-miRNA-155 (AM-155; 5'-AATTACGATTAGCACTATCCCCA-3'), and a scrambled control AM-155 (AM-155sc; 5'-TTAACATTAGACGATATCCCACC-3') were purchased from Applied Biosystems/Ambion, Austin, Texas, USA or Exiqon Inc., Woburn, Massachusetts, USA [11,16], and were used at a 5–20 nM ambient concentration that was replenished at every change of ABM (see above; Lonza, Basel, Switzerland) for a total treatment time of 12 and 36 h after the induction of TNF- α + A β 42 peptide. miRNA-146a and AM-146a sequences and their usage have been described previously [12–17].

Western analysis of CFH and β -actin in Alzheimer ECF and CSF

ECF samples were derived from high-speed supernatants of control and Alzheimer brain homogenates, and CSF was obtained from archived short PMI samples [12,17]. Western immunoblots were performed for the quantification of CFH and β -actin protein in control and Alzheimer's tissues using human-specific primary antibodies directed against the control protein marker β -actin (3598–100; Sigma-Aldrich) or human CFH (H-7; sc-166613 H-5; sc-166608; Santa Cruz Biotechnologies, Santa Cruz, California, USA) [15–19].

Statistical analysis and data interpretation

All miRNA array and LED-Northern dot blot data were analyzed as described previously [5–7,10–18]. Statistical procedures for protein (ELISA and western analysis) abundance were performed using a two-way factorial analysis of variance (P , ANOVA) using programs and procedures in the SAS language (Statistical Analysis Institute, Cary, North Carolina, USA) [16–19]. Only P -values less than 0.05 (ANOVA) were considered to be statistically significant. Figures were generated using Excel 2011 (Microsoft, Redmond, Washington, USA) and Photoshop CS2 version 9.0.2 (Adobe, San Jose, California, USA).

Results

Upregulation of miRNA-146a and miRNA-155 in Alzheimer's disease ECF and CSF

There were no significant differences in age, PMI, or RNA yield or quality between either the control or the Alzheimer brain tissue or CSF groups. Of the 12 different human micro-RNAs (hsa-miRNAs) measured to be significantly upregulated (Fig. 1) in ECF or CSF, miRNA-146a, and miRNA-155 showed the greatest significance in upregulation compared with age-matched controls ($P < 0.01$, ANOVA). At present, we cannot exclude the participation of other human brain-enriched miRNAs or other small-non-coding RNAs that may also contribute toward the proinflammatory neuropathological spreading mechanisms that help define the Alzheimer's disease process. Interestingly, miRNA-9 and miRNA-125b were also found to be upregulated ($P < 0.05$, ANOVA), and these two miRNAs also have binding sites in the CFH mRNA 3'-UTR (W.J. Lukiw, S. Bhattacharjee; unpublished observations) [24]. In summary, miRNA analysis showed consistent increases in miRNA-146a and miRNA-155 in five short PMI Alzheimer's disease ECF and CSF samples

when compared with five age-matched controls, and the proinflammatory miRNA-146a and miRNA-155 showed the greatest significance (Fig. 1).

Upregulation of miRNA-146a and miRNA-155 in stressed human neuronal-glial primary cocultures

We next studied the cytokine TNF- α -mediated and A β 42 peptide-mediated induction of proinflammatory miRNAs in a 2-week-old primary culture of HNG cells (Fig. 2a) using a LED-Northern technique (Fig. 2b). Using 5S RNA and miRNA-183 in the same sample as an internal control, the level of miRNA-146a was found to increase 2.4- and 4.2-fold over zero time controls 12 and 36 h after stress treatment, and miRNA-155 was found to increase 3.8- and 5.1-fold over zero time controls 12 and 36 h after stress treatment, and these results were highly significant (Fig. 2c). Bioinformatics analysis indicated that miRNA-146a and miRNA-155 have overlapping target recognition sites within the CFH mRNA 3'-UTR; both miRNA-146a and miRNA-155 are known to downregulate CFH expression through an miRNA-CFH mRNA 3'-UTR mechanism (Fig. 2d) [10–15,24].

Downregulation of CFH protein expression in neuronal-glial primary cocultures after treatment with a conditioned medium

We next examined the effects of this miRNA-146a and miRNA-155-containing CM from 36-h stressed primary cultures of HNG cells (Fig. 2c) on CFH expression in control HNG cells. Importantly, the CM was filtered to remove TNF- α and A β 42 peptide, and major constituents of the CM included miRNA-146a and miRNA-155 (data not shown). Incubation of control HNG cells with either A β 42 + TNF- α or this 'pathogenic' CM extract downregulated a known miRNA-146a and miRNA-155 mRNA target (Fig. 2d), CFH, to 0.15- and 0.18-fold the control values after 36 h (Fig. 3; three leftmost panels). This effect was suppressed by adding AM-146a or miRNA-155 (AM-155) to the neuronal-glial cells after the addition of the CM, restoring CFH expression to between 72 and 76% of the control (Fig. 3). AM-146a and AM-155 together restored CFH protein levels to 94% of the control. These observations indicated that miRNAs within the CM were able to induce CFH deficits known to lead to proinflammatory gene expression changes in human brain cells, an effect that was blocked using AM strategies (Fig. 3). ECF and CSF from Alzheimer's disease patients have also been shown to induce proinflammatory gene expression programs in neuronal-glial cells, including the specific upregulation of miRNA-146a and downregulation of CFH [10–18] (W.J. Lukiw, S. Bhattacharjee; unpublished observations). In sum, the results suggest that miRNAs carried by the CSF or ECF *in vivo* or CM *in vitro* promote the propagation of innate immune and pathogenic proinflammatory signaling known to drive the Alzheimer's disease process.

Discussion

The spreading mechanism for Alzheimer's disease inflammatory signaling throughout the higher function regions of the human brain is not well understood. Our results contribute four new observations to our understanding of the progressive and propagating nature of Alzheimer's disease inflammatory neuropathology: (a) that potentially pathogenic miRNAs are abundant in Alzheimer's disease ECF and CSF; (b) that the growth medium of stressed

primary HNG cells contains elevated miRNA levels; (c) that the miRNA-containing CM from stressed primary HNG cells can downregulate known proinflammatory regulatory glycoproteins such as CFH; and (d) that AM strategies can suppress the phenomenon of miRNA-mediated inflammatory spreading.

The initial work in this report indicates that aged, Alzheimer's disease-affected human brain cells appear to be exporting substantial quantities of miRNAs into the ECF and CSF, and that the complexities of these miRNAs – including the inflammation-relevant miRNA-146a and miRNA-155 – are known contributors to altered innate immune responses and inflammatory neuropathology [10–19]. The presence of stable miRNAs in Alzheimer's disease, ECF, and CSF at high enough concentrations to transmit inflammatory signaling suggests that miRNA bioactivities may extend well outside of the cell cytoplasm to further mediate homeostatic or pathogenic interneural and cell–cell communication [24–26]. Extracellular microvesicles and exosomes containing miRNAs suggest one plausible disease-proliferating mechanism [25]. Whether these externalized miRNAs are completely contained within these vesicles or exosomes or are secreted as free oligonucleotides is open to speculation [24–26]. Complex miRNA secondary and tertiary structures and/or miRNA-binding proteins may prolong the half-life of miRNA [18,25]. Using advanced LED-Northern type assays, it will be interesting to test whether other human neurological diseases with a progressive, age-related nature may use pathogenic miRNAs, in a paracrine manner, to spread pathological signaling throughout neighboring neural cells and throughout the CNS and other cell and tissue systems. Finally, it will be of interest to determine whether lower amounts of TNF- α and A β 42 peptide or ECF, CSF, or CM preparations over longer incubation periods can induce similar pathogenic miRNA generation, what individual brain cell types might be contributing to this multiple pathogenic miRNA output, and whether different brain cell types secrete a different repertoire of pathogenic miRNAs [13–17]. This knowledge may be profitably utilized with the aid of AM and related pharmacological approaches that have not yet been considered in the effective management of brain disease progression.

Conclusion

This study provides evidence (a) that the potentially pathogenic and proinflammatory miRNA-146a and miRNA-155 are abundant in Alzheimer's disease ECF and CSF; (b) that these miRNAs are secreted by human brain cells stressed with factors known to be elevated in Alzheimer's disease brain; (c) that a conditioned medium (CM) containing miRNA-146a and miRNA-155 will induce inflammatory gene expression in control brain cells, an effect that is mediated in part by the downregulation of the important immune system regulator CFH; and (d) that these effects are suppressed using AM. AM strategies may therefore be useful in the prevention of miRNA-mediated pathogenic spreading not only in Alzheimer's disease but perhaps also in other progressive, inflammatory degenerations of the human nervous system.

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Fig. 1.

(a) miRNA contents of human extracellular fluid (ECF) and cerebrospinal fluid (CSF) show an abundant representation of miRNA-146a, miRNA-155, and other Alzheimer's disease-enriched small-non-coding RNAs (sncRNAs) compared with age-matched controls [10–19]. miRNA array data for the 12 most significantly upregulated miRNAs in control and AD ECF are shown in (a) and quantified in (b); similarly, significantly upregulated miRNAs in control and AD CSF are shown in (c). ECF and CSF miRNA profiles were derived from high-speed supernatants of previously characterized age-matched control and AD brains

[17–19]. PMIs for age-matched control or AD human brain tissues and CSF were all less than 2.2 h; all tissues were from the superior temporal lobe neocortex; the study group (controls $N=5$; AD $N=5$) of tissues showed no significant differences in age (72.1 ± 6.5 vs. 73.2 ± 5.5 years, $P<0.85$), PMI (mean 2.1 ± 2.1 vs. 2.2 ± 1.4 h, $P<0.95$), RNA $A_{260/280}$ indices (2.10 ± 0.5 vs. 2.05 ± 0.4 , $P<0.95$), or RNA 28S/18S (1.5 vs. 1.45 , $P<0.92$) in age-matched control and AD, respectively. RNA integrity numbers (RIN) ranged between 8.1 and 9. No significant differences were found in the total RNA yield between the control and the AD groups; the dashed horizontal line at 1.0 indicates the control miRNA-183 signal in the ECF or CSF for ease of comparison. miRNAs were analyzed by LC Sciences (Houston Texas, USA) using miRNA array panels containing 1898 individual human miRNA targets; interestingly, miRNA-9, miRNA-125b, miRNA-146a, and miRNA-155 all have binding sites within the CFH mRNA 3'-UTR. AD, Alzheimer's disease; ANOVA, analysis of variance; CFH, complement factor H; miRNA, micro-RNA; PMI, postmortem interval. $*P<0.05$; $**P<0.01$ (ANOVA) [10–18].

Fig. 2.

(a) HNG cells 2 weeks in culture; $\times 20$; cells are triple stained using antibodies to glial fibrillary acidic protein (GFAP), a glial-specific marker (green fluorescence; $\lambda_{\text{max}}=556$ nm), with β TUBIII, a neuron-specific marker (red; $\lambda_{\text{max}}=702$ nm), and Hoescht 33258 to highlight cellular nuclei (blue; $\lambda_{\text{max}}=461$ nm) [12–15]; (b) representation of a typical result, this panel depicts LED-Northern dot blots of miRNA-146a and miRNA-155 in control and stressed HNG cells using unchanging 5S RNA and miRNA-183 as internal control markers [10]; a ‘halo’ around each dot appears to be associated with increased sensitivity of the LED Northern protocol (unpublished observations). (c) graphed results of 5S RNA, miRNA-183, miRNA-146a, and miRNA-155 in stressed HNG cells indicating relative levels; the dashed horizontal line at 1.0 indicates 5S RNA control levels for ease of comparison (loaded at 1/20th the concentration of the miRNAs); $N=5$; * $P<0.05$; ** $P<0.01$ (ANOVA); (d) overlapping miRNA-155 and miRNA-146a high-affinity binding sites in the 232 nucleotide CFH mRNA 3'-UTR (Genbank BC142688); an energy of association for the miRNA–mRNA of less than -22 kcal/mol defines an exceptionally stable miRNA–mRNA interaction and a potential CFH mRNA 3'-UTR miRNA-regulatory control (MiRC) region 5'-TTTAGTATTAA-3' (white letters on a black background) [24]; ‘|’ indicates a full hydrogen bond between the miRNA and CFH mRNA 3'-UTR and ‘:’ indicates a partial hydrogen bond; vertical black arrows show an miRNA-146a–miRNA-155 interaction domain in the CFH mRNA 3'-UTR (highlighted in gray; see text) [16,17,24]. ANOVA, analysis of variance; CFH, complement factor H; HNG, human neuronal-glial; miRNA, micro-RNA.

Fig. 3.

Results of a Western analysis of complement factor H (CFH) protein levels in control and stressed HNG cells in primary culture, and rescue to homeostatic levels by AM-146a and AM-155; a dashed horizontal line at 1.0 indicates the control CFH protein levels for ease of comparison; stress increases both miRNA-146a and miRNA-155, thus reducing the expression of CFH [9–19]; conditioned medium (CM) contained only trace levels of TNF- α and the A β 42 peptide; the major small RNA constituents of the CM were miRNA-146a and miRNA-155 (data not shown) [14–16]; AM-146a and AM-155, either separately or together, reverse this effect and restore CFH back to homeostatic levels; $N=5$; ANOVA, analysis of variance; HNG, human neuronal-glial; miRNA, micro-RNA; TNF- α , tumor necrosis factor- α . * $P<0.05$; ** $P<0.01$ (ANOVA).