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Diagnostic pathology of Alzheimer's disease from routine microscopy to immunohistochemistry and experimental correlations☆

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ABSTRACT

The absence of any histologic correlate for Alzheimer's disease despite its commonness and severe clinical sequelae may offers clues to its etiology. Recent evidence strongly suggests that the central event of this disease is the hyperphosphorylation of neuronal tau protein and not the beta amyloid precipitates. In each case, essential and soluble neuronal proteins derivatives form insoluble aggregates that can readily be detected by immunohistochemistry using antibodies specific for the misfolded proteins. Immunohistochemistry also demonstrates that neurons with hyperphosphorylated tau protein are viable. Experimental evidence using neuronal cell cultures suggests that the affected neurons in Alzheimer's disease may have undergone molecular changes that include accumulation of anti-apopotic proteins MCL1 and cFLIP that do not allow the cell to undergo programmed cell death but, rather, to "immortalize" and thus accumulate hyperphosphorylated tau protein in the neuronal cell body and beta amyloid in downstream dendrites. We describe a simplified protocol to demonstrate such changes based on tagged LNA modified microRNA/antimicroRNA oligomers and cell cultures. Co-expression showed that the tagged antimiR-512 strongly localized with the markedly up-regulated proteins MCL1 and cFLIP with concomitant accumulation of hyperphosphorylated tau protein. The data underscore to the anatomic pathologist that the diagnosis of Alzheimer's disease is best accomplished by simple immunohistochemistry tests correlated to the clinical history and the key role pathologists can play in understanding the cause of the disease.

1. Introduction

Alzheimer's disease is the most common form of dementia with about 50 million cases worldwide [1-5]. The commonness of the disease, dramatic and horrific clinical sequelae, and intense research to understand its biology and pathology has resulted in several thousand publications on the pathology alone based on a recent MEDLINE search. The gross pathology is straightforward. There is brain atrophy affecting specific regions including the temporal and parietal lobes. This led to the logical conclusion that there must be concomitant neuronal degeneration and apoptosis. Here the literature is both controversial and changing in that the evidence of neuronal apoptosis that includes the TUNEL assay and demonstration of caspase activation in many of the earlier pathology papers has been replaced by a consensus that neuronal death is not a feature of Alzheimer's disease [1-5]. If not, then what is the pathologic basis for the gross atrophic changes?

Neural plaques (precipitates of beta amyloid) and neurofibrillary tangles (precipitates of hyperphosphorylated tau protein) have long been recognized as essential features of Alzheimer's disease [1-5]. They each dominate in the specific areas of the brain that show the gross atrophic changes and where clinical symptomology correlates. Another controversy in the pathology of Alzheimer's disease is whether neural plaques/tangles occur in the brain from people with no evidence of dementia? Yet another controversy based in the pathology is whether the neural plaques or tangles represent the primary, etiologic epicenter of the disease. It was long assumed that neural plaques were the primary, causal concomitant of Alzheimer's disease with the tangles representing a secondary change. This led to many clinical trials using agents that either block beta amyloid formation (semagacestat), or assist in its removal by acting as beta amyloid "sinks" (aducanumab, solanezumab) [1-5]. Each study based on beta amyloid failed to show clinical efficacy [5-9]. Similarly, a vaccine developed against a synthetic beta amyloid had to be terminated because of an unacceptable incidence of autoimmune encephalitis [5-9].

The purpose of this paper will be to discuss recent advances in the understanding of the pathology of Alzheimer's disease. It can be argued that the pathologist only needs an immunohistochemistry test against hyperphosphorylated tau protein and beta amyloid 42 for diagnostic

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purposes. Importantly, we will show that simple pathological analysis can shed much light on the pathophysiology of the disease by focusing on microRNA changes and concomitant mRNA dysregulation that strongly co-express with the dysfunctional neurons defined by hyperphosphorylated tau protein [10].

2. Materials and methods

2.1. Tissue procurement

We obtained both frozen (n = 20) and formalin fixed, paraffin embedded tissues (n = 20) brain tissues from people with early stage (BRAAK III or IV) Alzheimer's disease with the same number of age matched controls. The tissues were equally divided between the hippocampus and frontal or parietal cortex. All samples were de-identified from the patient's name, hospital where autopsy was performed, and date of birth (though birth year was maintained).

2.2. Immunohistochemistry

Immunohistochemistry was done using the Leica Bond Max automated platform (Leica, Buffalo Grove, IL) with the modification of substituting the Polyview polymer-peroxidase conjugate from Enzo Life Sciences (Farmingdale, NY) [11,12]. Immunohistochemistry was performed for MCL1, cFLIP as well as the downstream targets BAK, BAX, caspase 3 and caspase 8 (cFLIP). The tissues were also tested for beta amyloid 42, hyperphosphorylated tau protein, pyruvate dehydrogenase and Sur 1. The sources of the primary antibodies were Enzo Life Sciences, Farmingdale NY (MCL1, BAK, BAX), and ABCAM, Cambridge MA (cFLIP, caspase 3, hyperphosphorylated tau protein, beta amyloid 42, pyruvate dehydrogenase, Sur 1, and caspase 8). The optimal conditions for cFLIP, beta amyloid 42, and the hyperphosphorylated tau protein was protease digestion (proteinase K solution, Enzo Life Sciences) for 4 min at room temperature. All other antibodies gave optimal signals using antigen retrieval for 30 min with AR solution #2 (Leica Biosystems).

2.3. Co-expression analysis and quantification of results

The co-expression analysis protocol used has been previously published and is based in the Nuance and InForm systems from Perkin-Elmer, Waltham MA [10-12]. The Nuance system can separate each chromogenic signal, convert to a fluorescence based signal, then merge these signals to determine what percentage of cells were expressing the two targets of interest via the companion InForm system.

2.4. Cell lines/transfection

The following cell lines each obtained directly from the ATCC (Manassas, VA) were studied: N1E-115 (ATCC CRL-2263; neuroblasts), Jurkat (ATCC TIB-152, leukemia), and HSB-2 (ATTC CCL-120.1, leukemia). Cell culture conditions were as specified by the ATTC.

Transfection was accomplished by adding 0.5 ml of growth media to each well that contained either the scrambled tagged LNA modified oligonucleotide or the miRNA/antimiRNA (Exiqon, Woburn, MA). These oligonucleotides have two key features: 1) a 5′ digoxigenin tag and 2) locked nucleic acid (LNA) modified nucleotides. The sequence of the mature miR-512-5p (miR-512 mimetic) as determined from the web site miRBase (http://www.mirbase.org/index.shtml) is 5′ CACUCAGCCUUGAGGGCACUUUC. Thus, the sequence of the antimiR-512 is: 5′ GAAAGTGCCCTCAAGGCTGAGTG. The cell cultures were then monitored daily for morphologic changes. After 48 h the cells were fixed by the addition of 3 ml/each well of 10% neutral buffered formalin for 3–5 h, followed by collecting the cells, washing in sterile RNase free water, and diluting the cell pellet in 250 μl water. Ten microliter of the cell suspension was added to a positively charged slide, air dry, and store at room temperature.

2.5. Detection of digoxigenin

The digoxigenin tag was detected by incubation with an anti-digoxigenin-alkaline phosphatase conjugate (Roche Diagnostics, Indianapolis, IN) followed by incubation with the nitro-blue tetrazolium and 5-bromo-4-chloro-3″-indolyphosphate (NBT/BCIP) substrate (ThermoFisher Scientific, Waltham, MA) [10-12].

3. Results

3.1. Formalin fixed, paraffin embedded tissues

First, we compared the distribution of hyperphosphorylated tau protein and beta amyloid 42 in twenty tissues from people with Alzheimer's disease (10 hippocampus and 10 frontal or parietal cortex) and forty aged matched normal controls. Each of the hippocampal tissues from the Alzheimer's disease patients showed cells positive for both hyperphosphorylated tau protein and beta amyloid 42 although individual tissues varied from 1+ to 3+ in the number of positive cells. The Alzheimer's disease cortical sections showed from 0 to 2+ signal for each hyperphosphorylated tau protein and beta amyloid 42, which is as expected since the patients had BRAAK III or IV disease. Importantly, not one of the forty normal tissues (hippocampal and cortex from aged matched controls) showed any neural plaques or tangles.

Fig. 1 shows representative images of the immunohistochemistry for hyperphosphorylated tau protein and beta amyloid 42. Note that the plaques and tangles are in different gray matter regions of a given hippocampal section which likely reflects that the plaques concentrate in dendritic areas while the tangles are found in the neuron cell body. Also note that the number of plaques tends to be greater than the number of neurons with hyperphosphorylated tau protein.

We have previously shown that neither caspase 3 nor caspase 8 is activated in the areas of the brain where neural plaques and tangles dominate [10]. The next question that was addressed was whether the neurons with abundant hyperphosphorylated tau protein were viable. To this end, we did immunohistochemistry for two proteins that are highly expressed in normal neurons because they are involved in aerobic energy production. These are pyruvate dehydrogenase and Sur1. As seen in Fig. 2, the neurons with abundant hyperphosphorylated tau protein express high levels of both pyruvate dehydrogenase and Sur1 in amounts that are indistinguishable from neurons in the hippocampus of healthy controls.

We had previously shown that several microRNAs, including miR-512, are markedly reduced in the regions of Alzheimer's disease where hyperphosphorylated tau protein dominates [10] and that two targets of miR-512, MCL1 and cFLIP [13,14], were each increased [10]. Analysis of serial sections for MCL1 and cFLIP protein by immunohistochemistry confirmed that these proteins were most prevalent where hyperphosphorylated tau protein dominated. MCL1 and cFLIP protein are rarely evident in normal brain tissues. Thus, we have further evidence that the neurons with hyperphosphorylated tau protein that are at the epicenter of the neurofibrillary tangles are viable and synthesizing other proteins not normally found in the brain.

3.2. Cell lines

Next experimental evidence was sought to support the in vivo data that microRNA dysregulation could, in turn, lead to increased anti-apoptotic proteins (cFLIP and MCL1) that allow the cell to “immortalize” and thus accumulate neurotoxins.

We studied the human T cell leukemia cell lines Jurkat and HSB-2. It has been documented that each cell line expresses MCL1 and cFLIP; the