Review

The dysregulation of the cell cycle and the diagnosis of Alzheimer’s disease

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Abstract

The ‘silent epidemic’ of Alzheimer’s disease is becoming a considerable social and economical problem in the developed countries. Especially so, because we still cannot diagnose the disease early enough, and there is no disease-modifying treatment. At present the only available therapeutic option is the use of cholinesterase inhibitors, which have mainly symptomatic short-term benefit for around one third of the patients [1,2]. The solution to the problem would be the evidence-based design of early therapies, which could reverse/halt the cellular mechanisms that precede the formation of the typical brain pathology. The development of new therapeutic strategies, however, is hindered by limited knowledge of the pathogenic mechanisms that lead to the development of the sporadic form of the disease. Additionally, by the time the disease can be diagnosed, using the currently available diagnostic protocols, the pathology has spread to large areas of the brain, causing irreversible damage and functional disability [3]. It is imperative therefore that we find early biomarkers for sporadic Alzheimer’s disease, which could identify patients before substantial pathology develops.

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1. Introduction

By definition biomarkers fall into two categories: biomarkers of ‘exposure’ and biomarkers of ‘disease’.

The Biomarkers of exposure (antecedent biomarkers) include measure of exposure to environmental risk factors and genetic risk factors. They are particularly useful for risk prediction. The biomarkers of ‘disease’ are indicators of biological factors which represent either a prodromal manifestation of the disease or a surrogate manifestation of the disease. These markers allow the identification of individuals who are in the pre-clinical stages of the disease and reduce heterogeneity in clinical trials. If the marker also reflects the progression of the disease it can be used as target for clinical trials [4].

1.1. The diagnosis of Alzheimer’s disease

At present the diagnosis of ‘definite’ Alzheimer’s disease can only be made by histopathological assessment after autopsy. While the patient is alive the clinical diagnosis carries varying amounts of uncertainty allowing the patients to fall into two categories: probable or possible Alzheimer’s disease (NINCDS-ARDRA). Either category requires the patient to show clinical signs of dementia. Additionally the clinical diagnosis relies mainly on the exclusion of other neurological disorders, which could cause dementia, rather than the identification of an Alzheimer-specific clinical profile. While the positive predictive value of clinical diagnosis of ‘probable AD’ is high (89–100%) the diagnostic categories described above have a very high false negative rate, excluding patients suffering from early/mid stages of the disease [5].

1.2. Existing biomarkers for Alzheimer’s disease

Because amyloid deposition in the brain is a defining feature of AD the measurement of markers related to APP and β-amyloid metabolism appeared attractive candidates as biomarkers for the disease. Despite much initial enthusiasm it transpired that measurements of β-amyloid from plasma (or serum) might relate to age or renal function but does not allow the discrimination between healthy elderly controls and AD patients (reviewed in [6]). Furthermore the plasma β-amyloid levels do not correlate to dementia severity and did not reflect...
the effectiveness of different types of therapeutic interventions targeted to reduce β-amyloid accumulation in the brain (reviewed in [6]). The possibility that the ratio of different APP isoforms from platelets could be used for diagnosis and monitoring disease progression and therapeutic effects was also raised. However, technical limitations make this marker difficult to standardise.

Cardiovascular risk factors, such as high cholesterol ApoE4 genotype hypertension and elevated plasma homocysteine levels were also identified as risk factors for sporadic AD. While they appear to be good antecedent biomarkers they do not fulfil the role of biomarkers of ‘disease’. The same is true for markers of oxidative stress (antioxidant levels and lipid peroxidation products) (reviewed in [6]).

As the CSF is regarded as a mirror of the biochemical changes occurring in the brain, considerable effort has been put into identifying biomarkers for AD in the CSF. In turn total tau and phospho-tau have been found to be elevated in AD patients relative to controls while β-amyloid is reduced in the CSF of the patient population. Although some studies claim good specificity and sensitivity for these markers in differentiating AD patients from controls, they do not differentiate AD from other types of dementia (reviewed in [7]). The main shortcoming of these markers is that they appear only once the disease has caused considerable accumulation of AD-type pathology, and as such they are relatively late markers.

A substantial number of imaging markers of the anatomy, chemistry, physiology and pathology of AD became available in recent years. Many of these have also been validated against clinical and pathologic measured of the disease. Most of these markers indicate a more rapid change over time in AD patients than in age-matched controls. It also appears that some changes in biomarkers indicate a more rapid change over time in AD patients from controls, they do not differentiate AD from other types of dementia (reviewed in [6]). The main shortcoming of these markers is that they appear only once the disease has caused considerable accumulation of AD-type pathology, and as such they are relatively late markers.

2. Cell cycle and AD

The cell cycle hypothesis for the pathogenesis of Alzheimer’s disease was originally based on the observation that neurones in the brain of elderly demented patients express cell division-related proteins [9–11]. The differential expression of these cell cycle markers suggested that the neurones in the adult nervous system are able to re-enter the cell division cycle [12–14]. In normal circumstances the cell cycle re-entry (from G0 to G1) is followed by cell cycle arrest in an early stage (before the G1/S transition point), when re-differentiation is still possible. It has also been suggested that in the healthy brain the transient re-entry into the cell cycle might be part of the synaptic remodelling process [15].

In Alzheimer’s disease the G1/S control mechanisms fail. Neurones are allowed to replicate their DNA [16] and progress into the late, G2 phase of the cycle [9]. Since the neurones are not able to divide, they remain in this very late stage of the cell cycle. In turn the cellular mechanisms activated in the G2 phase of the cell cycle drive the formation of neurofibrillary tangles and amyloid plaques [17, 18] in apoptosis incompetent neurones. Based on this hypothesis the development of Alzheimer’s disease is a ‘two stage’ process: cell cycle re-entry followed by regulatory failure at the G1/S transition point.

The hypothesis poses several questions. What are the mitogenetic stimuli that are able to drive mature differentiated neurones into the cell division cycle? What are the consequences of DNA replication in neurones that are 50–60 years old? What are the causes of the cell cycle regulatory failure at the G1/S transition point in some people but not others?

2.1. Mitogenic stimuli in the brain

The mitogenic stimulation of neurones is necessary to trigger cell cycle. Many of the identified risk factors for Alzheimer’s disease, such as elevated plasma homocysteine levels [19], ageing [15], menopause [20, 21], low thyroid levels [22, 23], low level prolonged oxidative stress [24, 25], head injury or low education, can either represent mitogenetic signalling for neurones or facilitate cell cycle re-entry in vulnerable neuronal populations (reviewed by Arendt [15]). The link between synaptic plasticity and cell cycle reactivation also explains how ApoE4 genotype, which is associated with reduced plastic capacity of neurones [26] is a risk factor for Alzheimer’s disease.

2.2. The G1/S regulatory failure

While cell cycle re-entry of neurones appears to be a prerequisite of Alzheimer’s disease, on its own is not sufficient to cause the disease [27, 28]. As discussed above, the cell cycle re-entry may just represent a short transient part of synaptic remodelling in the healthy elderly [15]. The difference between the healthy aging and AD seems to be the extent of cell cycle progression rather than cell cycle re-entry. This suggests that the transition from healthy ageing to AD is the point when neurones bypass the G1/S restriction point and replicate their DNA.

The cell cycle is driven by the sequential expression and activation of cyclin/cyclin dependent kinase (CDK) complexes and inhibited by the cyclin dependent kinase inhibitors (CDKIs). At each checkpoint different groups of cyclins kinases and CDKIs ensure that the cell responds adequately to the extra- and intracellular factors, which support either cell division or differentiation.
When the intracellular environment is not capable of supporting the events initiated by extracellular signals cell death is initiated.

At the G1/S transition point the cell cycle is driven by the activity of the cyclin E/CDK2 complex and inhibited by the members of the kip-cip protein family. There are three identified proteins in this family of CDKIs.

The gene for the p21cip1 (p21waf1, CDKN1A; 116899) has been mapped to 6p21.2. It is a short 21 kd protein, which inhibits the activity of CDK2 activated by cyclins D1, E and A, therefore inhibiting several phases of the cell cycle. Its expression is regulated at transcriptional level by p53, thus making this CDKI one of the effectors of DNA-damage induced cell cycle arrest at the G1/S and G2/M checkpoints. The successful cell cycle arrest, mediated by p21cip1 will protect cells against DNA-damage-induced apoptosis and conversely the inactivation of the protein will favour the induction of the p53-dependent apoptosis. Additionally the truncation of the C terminus of the protein by caspases 3 (and possibly other caspases) in apoptotic cells leads to a loss of the nuclear localisation signal, exit from the nucleus and loss of cell cycle inhibitor activity. As an inhibitor of the G1/S transition point it plays a part in the cell cycle exit and the activation of the differentiation programme in many cell types. The allelic variants of the gene are associated with elevated risk of tumours (http://www.ncbi.nlm.nih.gov/entrez/dispmim.cgi?id=116899).

Another member of the family, p27kip1 (CDKN1B, 600778), was mapped to 12p13. In quiescent cells relatively high levels of the protein are expressed, but protein expression rapidly declines upon cell cycle induction. The CDK inhibitory activity of the protein is linked to its nuclear localisation, while the cytoplasmic accumulation of the protein is associated with high proliferative activity of certain cancers. The phosphorylation and ubiquitination of the protein leads to the segregation of p27kip1 in the cytoplasm followed by its degradation. The truncation of p27kip1 by caspase 3 in apoptotic cells leads to the deactivation of this CDKI similar to p21cip1. Germline variants of the gene have been found associated with increased risk of certain cancers (http://www.ncbi.nlm.nih.gov/entrez/dispmim.cgi?id=600778).

The third member of this CDKI protein family is p57kip2 mapped to 11p15.5 (CDKN1C; 600856). The CDK inhibitor region of the protein is highly homologous with the p21cip1 protein. However, unlike the previous two members of the family the p57kip2 is partially imprinted, the maternal allele being preferentially expressed. Loss of function (mutations on the gene, loss of maternal allele) was found to be associated with sporadic cancers as well as hereditary syndromes such as Simpson–Golabi–Behmel syndrome and Beckwith–Wiedemann syndrome (http://www.ncbi.nlm.nih.gov/entrez/dispmim.cgi?id=600856).

All three proteins have been found to be expressed in the cytoplasm of AD patients [29–32], often in tangle bearing neurones and distorted neuritic processes of plaques. The association with tangles and sequestration in the cytoplasm imply inactive CDKIs, which would explain the cell cycle regulatory failure at the G1/S transition point and uninhibited progression of neurones through DNA replication. However, the inconsistent expression pattern of the three proteins and large variations from one patient to another imply that AD is a heterogeneous disorder associated with the deactivation of different CDKIs in different patients. The question is, though what inactivates the CDKIs in neurones? Truncation by caspases in apoptotic cells is a tempting possibility. However, neurones appear to avoid the classical apoptotic process exactly due to the fact that they do not amplify their downstream cascade of caspases, including caspases 3 [33].

In summary, it appears likely that the inactivation of CDKIs plays a major role in the G1/S regulatory failure in neurones responsible for the development of AD-type pathology. However, the causes of this inactivation remain elusive.

3. The causes of the G1/S regulatory failure in AD

There is evidence in the literature suggesting that the cell cycle regulatory failure in AD is not restricted to neurones. It has been shown that there is an association between solid tumours and the incidence of AD [34]. Additionally peripheral cell, such as lymphocytes from AD patients showed altered response to mitogenic stimulation relative to control subjects [35,36]. Fibroblasts collected from AD patients also shown an aberrant cell cycle-dependent Ca response [37]. A more recent study has found that the differential phosphorylation of Erk1 and Erk2 in response to bradykinin in fibroblasts can distinguish AD patients from healthy elderly and patients suffering from non-AD dementias [38].

We have also found that this specific failure of the G1/S transition checkpoint is not restricted to neurons alone. Lymphocytes of AD sufferers also show signs of G1 regulatory failure [39]. Additionally, in subjects showing neuropsychological signs of pre-clinical AD, the lymphocyte response was similar to that seen in AD patients [39].

These findings also imply that the AD-related G1/S regulatory failure is probably due to genomic variations of the cyclin dependent kinase inhibitors (CDKIs) involved in the regulation of the G1/S transition.

In order to investigate this possibility we genotyped a relatively small but unselected group of AD patients and control subject (which included both healthy elderly people and patients suffering from other non-AD dementias). We have concentrated on known, mainly cancer-related SNPs on the p21cip1 p27kip1 and p57kip2 genes.

There are two variants on the p21cip1 gene associated with increased tumour frequency [40–42]. One of the variants is a Ser-to-Arg substitution in codon 31 while the other was a C-to-T change in the 3-prime untranslated region of the p21cip1 gene 20 bp following the stop codon. The amino acid substitution is within the CDKI region of the protein, and probably affects the binding of the inhibitor to the cyclin CDK complex. The role of the intronic SNP is less clear, but it has been suggested that it might affect the stability of the mRNA and alter protein levels in the cytoplasm.

We have genotyped the patients using the methods described previously [40]. No homozygous patient occurred in our cohort. The simultaneous presence of both variants increased the risk of developing AD slightly, albeit significantly (Table 1). Inte-
restingly however, the effect of the genotype was gender dependent (Table 1), similar to IGF-1R[43] ABCA1 [44] and ESR2 [45].

We have also found that the presence of the two SNPs on p21cip1 was associated with a reduced age of onset (\(p=0.002\)) (Fig. 1) in AD patients (both males and females).

In late (neocortical) stage AD patients (\(n=59\), of which 9 had variant p21cip1) the number of Cyclin B positive (G2 phase) neurones was significantly higher in patients with the p21cip1 variant (Fig. 2), indicating that the variant is associated with the progression of the cell cycle in neurones. In early stage (entorhinal) patients (\(n=40\), of which 5 had variant p21cip1) however, the p21cip1 variant was associated with significantly lower levels of synaptophysin (Fig. 3).

The p21cip1 protein is also responsible for the cell cycle arrest in response to oxidative stress induced DNA damage downstream of p38. Thus the functional loss of p21cip1 may lead to deficiencies in the neuronal stress-response. In this context it is interesting that earlier studies found that both mitogenic stimulation and oxidative stress are required for the progression of AD [27].

For p27kip1 we analysed the effect of the Gly-to-Val polymorphism on codon 109. The VV genotype has been found to be associated with increased risk of cancer [46]. The polymorphism lies in the p38jab1 binding domain of the protein and probably reduces the CDKI activity of the protein by altering its degradation. We found that the VV genotype also increases the risk of AD (Odds Ratio 1.72, \(p=0.049\)) (Table 2). However, this SNP had no effect on the age of onset of dementia in our AD patients (data not shown).

There are no SNPs on the p57kip2 gene known to be associated with cancer and the known SNPs are not characterised in terms of population diversity. We have found that the SNP on codon 159, which leads to an Ala-to-Val substitution, is associated with a significantly reduced age of onset in our AD patients (Fig. 4). The polymorphism does not lie within the CDKI domain of p57kip2. The function of the region affected is not known. The interpretation of these, in terms of their functional significance, is made more complex by the fact that p57kip2 is imprinted.

### 4. New early diagnostic possibilities for Alzheimer’s disease

The discovery of the pathogenic pathway responsible for the development of Alzheimer’s disease holds the promise of early diagnostic protocols, novel biomarkers and disease-modifying therapies for Alzheimer’s disease.

As mentioned earlier, there are two types of biomarkers. The antecedent biomarkers measure the exposure to environmental risk and genetic risk factors, allowing a more accurate risk prediction for an individual patient.

In this sense all the factors which contribute to the mitogenic stimulation of mature neurones can be regarded as antecedent biomarkers. Elevated plasma homocysteine levels, low thyroid and oestrogen levels can all be included in this category.

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**Table 1**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>p21cip1</th>
<th>Relative frequency</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>21</td>
<td>184</td>
<td>1.64</td>
</tr>
<tr>
<td>Men</td>
<td>10</td>
<td>84</td>
<td>1.97</td>
</tr>
<tr>
<td>Women</td>
<td>11</td>
<td>100</td>
<td>1.42</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>288</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>8</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>12</td>
<td>155</td>
<td></td>
</tr>
</tbody>
</table>

Odds ratio calculations indicate an association between the variant and Alzheimer’s disease. This association is stronger in men.

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**Fig. 1.** The number of cyclin B positive neurones has been assessed using a semiquantitative scale (0, occasional=0.1, some=0.5, many=1). The average number of cyclin B positive neurones (y axis) is significantly larger in patients with the variant p21cip1. Error bars represent the standard error of the means.

**Fig. 2.** Synaptophysin expression in the CA1 region of the hippocampus. Synaptophysin levels were measured using radioimmunohistochemistry and image analysis. The amount of synaptophysin is expressed as nCi/g tissue (y axis). Error bars represent the standard error of the means.
Table 2
The relative frequency of the variant p27\(^{kip1}\) gene in AD patients and age matched control subjects

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>AA109 G/G</th>
<th>AA109 V/G</th>
<th>AA109 V/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>3 (4.05%)</td>
<td>34 (45.94%)</td>
<td>37 (50%)</td>
</tr>
<tr>
<td>Control</td>
<td>9 (11.39%)</td>
<td>41 (51.89%)</td>
<td>29 (39.18%)</td>
</tr>
</tbody>
</table>

Patient numbers and the relative frequency values (expressed in %).

Since the G1/S regulatory failure is important for disease development the genetic polymorphisms on the CDK1 genes described above also constitute antecedent biomarkers. However, since neither the potential mitogenic stimuli nor the G1/S regulatory failure lead to AD-type changes in neurons on their own, the real risk of developing AD is only present in individuals where both types of risk factors are present. In this context risk assessment in any individual patient would require multiple tests, including genotyping for several genes, and the determination of complex risk-profiles.

The disease biomarkers indicate the presence of phenomena or factors which are either a pre-clinical or a surrogate manifestation of the disease. These markers allow early diagnosis of individual patients but they are also important for the stratification of clinical trials.

Recent studies suggest that the identification of disease biomarkers, based on detecting the AD-type cell cycle deregulation, is possible.

The proliferative capacity and survival of cultured lymphocytes is altered in AD patients relative to age-matched controls [35,47]. The apparent discrepancy between the two reports may have several reasons. Besides the possible differences in the selection/inclusion criteria for AD patients and controls, the experimental design and measures of lymphocyte proliferative capacity in the two studies were different. It is also known that the proliferative potential of peripheral lymphocytes is affected by age, therefore even slight differences in the age of AD patients and controls will affect the outcome of these studies. In addition, in an individual patient, the use of COX-2 inhibitor NSAIDS, or a simple cold might alter the response of the lymphocytes to mitogenic stimulation in vitro. Larger controlled studies would be required to clarify the cause of the apparent discrepancy.

The specific G1/S regulatory failure, typical to neurons in AD, is also detectable from lymphocytes [39]. The study was aimed specifically at the detection of any defects of the G1/S transition control. We found that in response to cell cycle inhibitors the relative lengthening of the G1 phase in exponentially proliferating peripheral lymphocytes was significantly reduced in AD patients even when the clinical picture was atypical due to other co-existing disease processes. The test also identified the early, pre-clinical stage AD patients. It is reassuring that the results of patients suffering from non-AD dementias were similar to the control group. The effect of the cell cycle inhibitor rapamicin used to induce G1/S arrest depends on the integrity and expression of the p27\(^{kip1}\) CDKI. The presence of the polymorphism on p27\(^{kip1}\) described earlier could explain the lack of response to rapamycin. The other finding of the study was that in lymphocytes from AD patients sub-lethal oxidative stress did not induce cell cycle arrest to allow for DNA-repair. This finding was later replicated in fibroblasts from AD patients [48]. At least in a subset of patients this phenomenon might be due to the p21\(^{cinc}\) polymorphism associated with increased risk of AD and an earlier onset. Since oxidative stress is one of the factors believed to be necessary for the development of AD [27], the lack of the repair mechanisms could certainly explain the earlier appearance of the pathology.

The recent finding of differential Erk1 and Erk2 phosphorylation in response to bradykinin [49,50] (inflammatory agonist and G1 inhibitor) in fibroblasts from AD patients [38] further strengthens the hypothesis that that p27\(^{kip1}\) mediated cell cycle responses are aberrant in AD patients and could be used as an early diagnostic marker for the disease.

The early peripheral markers of the G1/S regulatory failure appear to perform well as disease biomarkers and would be equally useful in clinical practice and drug trial design. However, more controlled studies are necessary to validate and standardise the protocols designed to identify patients with AD-type cell cycle regulatory deficits. The main caveat of course of these markers is that, although they identify AD patients accurately and early, they do not reflect the progression of the disease and cannot be used as outcome measures in clinical trials.

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