Involvement of Neuronal Anion Exchange Proteins in Cell Death in Alzheimer’s Disease

Abstract
Anion exchange (AE) proteins are present in human neurons in the brain. Immunohistochemical data indicate that their apparent expression level increases with age, and especially with degeneration in Alzheimer’s disease-affected brain areas. The increase in immunoreactivity is probably caused by changes in AE structure that lead to an increased accessibility of hitherto hidden epitopes. These epitopes correspond to regions in the membrane domain that are involved in generation of senescent cell-specific antigen from AE1 in aging erythrocytes. Elucidation of the molecular nature of these changes and the underlying mechanisms, will lead to insight in the processes that govern aging- and degeneration-associated perturbation of membrane integrity. AE-mediated chloride/bicarbonate exchange is a major component in the regulation of intracellular pH. The functional consequences of changes in AE structure may range from acidosis, disturbance of cytoskeleton integrity, and untimely or impaired recognition of cells by components of the immune system, such as microglia. A molecular and physiological description of these changes will establish AE proteins as valuable tools in elucidating the processes of normal aging, and the disturbances in aging-related diseases such as Alzheimer’s disease.
Introduction

Proteins of the anion exchanger (AE) gene family have been found in all cells examined so far [1–3]. The AE proteins share a C-terminal, highly homologous membrane domain (~ 50 kD) that is mainly responsible for anion exchange, and a more variable N-terminal, cytoplasmic domain (50–80 kD) that binds cytoskeletal and other peripheral proteins. The functions of erythrocyte AE1 (or band 3), the most extensively studied member of this family, are the exchange of chloride and bicarbonate across the plasma membrane, thereby facilitating transport of carbon dioxide through the body, and anchoring the spectrin/actin cytoskeleton to the plasma membrane, by providing a binding site for ankyrin [2]. In addition, the cytoplasmic domain may play a role in regulation of anion transport activity [4], as well as in the regulation of cell metabolism by the binding of glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase [5, 6]. In other cells, the anion transport activity of AE1 and other AE proteins is involved in regulation of intracellular pH, chloride concentration, and cellular volume [7–9]. AE1 and AE3, but not AE2 or the truncated variants of AE1, have a high affinity for ankyrin [10]. The more extensive variation in the cytoplasmic domain may reflect the variation in cell type-specific cytoskeleton architecture.

AE1 has also been identified as the precursor of the senescent cell-specific antigen (SCA), responsible for recognition and subsequent removal of old and damaged erythrocytes by the immune system [11]. Changes in AE1 conformation, presumably triggered by oxidation and/or proteolytic degradation, occur during normal erythrocyte aging. These changes do not only affect anion transport and the interaction of AE1 with the cytoskeleton, but also result in the appearance of the neoantigen SCA. Binding of SCA by autologous IgG, possibly in a complex with C3b [12], leads to recognition and removal of old cells from the circulation by macrophages. The process that generates SCA from AE1, which may be accelerated during organismal aging [13], is active in other cell types as well [11].

Aging is the most important risk factor for Alzheimer's disease (AD): the neuropsychological and the neuropathological characteristics of AD show a broad overlap with the characteristics of normal, physiological aging [14–18]. Normal brain aging is accompanied by a number of changes, such as apparent neuron loss in hippocampus and cortex areas, accumulation of lipofuscin, an increase in the number of corpora amylacea, a gradual accumulation of amyloid plaques and neurofibrillary tangles, and an increase in number and/or reactivity of astrocytes [14–16, 18]. The neuronal membrane is probably the site at which aging-related changes most directly affect cellular homeostasis [19]. Aberrant processing and cleavage of amyloid precursor protein (BAPP), as well as abnormal phosphorylation of tau, are likely to be involved in the etiology of the AD-specific lesions amyloid plaques and neurofibrillary tangles [20, 21]. We propose that these events are secondary to and signposts of perturbation of neuronal membranes. This hypothesis is based on observations as: (1) the cleavage sites of the Aβ peptide, as well as the identified mutations in BAPP, are all located within or close to the putative membrane domain of BAPP [20]; (2) minute lesions in the neuronal membrane probably precede deposition of Aβ in diffuse plaques [22]; (3) structural and functional analyses have revealed AD-related alterations in neuronal membranes [19, 23–25]; (4) recently, mutations in two related genes that encode integral membrane proteins were identified as responsible for
over 80% of the cases of familial Alzheimer's disease [26, 27]. These mutations are located close to predicted membrane-spanning regions of these proteins. Together, these data suggest that aging-related phenomena are more pronounced in the AD-affected brain, and are concentrated at the neuronal membrane, but they provide no clues to the underlying mechanisms. It has been put forward that AD-related changes in cellular membranes could result in changes in signal transduction, similar to those observed during normal aging in neural and nonneural cells [28].

Considering the central position of changes in structure and function of AE1 in erythrocyte aging, the emerging central role of AE proteins in cellular homeostasis, and especially the apparent changes in AE protein structure that occur during aging and degeneration in the human neuron, we see the AE proteins as the common denominators of physiological and pathological neuronal aging in the human brain. Here we will review the data that have led to this view, and present and discuss new data that support and extend it.

Materials and Methods

Neuropathology and Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue from control and AD patients (up to 10 h postmortem) was used for neuropathological examination and diagnosis as described before [29-34]. Plaques and tangles were encountered only sporadically in the brains of control patients. Immunohistochemistry was performed on formaldehyde or paraformaldehyde/sublimat-fixed tissue. Sections were counterstained with hematoxylin. Control incubations contained preimmune sera. Immunostaining was performed with the avidin-biotin-peroxidase technique [29-34].

Immunoblotting

Immunoblot analysis was performed on frozen tissue fractions from the same brains used for neuropathological and immunohistochemical analysis, using antiserum dilutions of 1:200–1:500, as described before [29-34].

Antibodies

The polyclonal antibody WP944 was raised against a soluble part of the cytoplasmic domain of erythrocyte AE1 [Bosman et al., unpubl. results]. The monoclonal antibody BIII.136 recognizes the amino acid residues 22–27 of human AE1 [35]. The antiserum against human erythrocyte ankyrin was a gift from Dr. J.C. Pinder (University of London, London, UK). The monoclonal antibodies 4.15 against amino acids 628–642 and 4A3 against amino acids 650–658 of human AE1 were gifts from Dr. I.W. Sherman (University of California, Riverside, Calif., USA).

pH Measurements

The contribution of the various ion transporters to the regulation of intracellular pH was determined with the fluorescent probe 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (Molecular Probes, Eugene, Oreg., USA), following the procedures described before [36, 37].

Cell Lines

The neuroblastoma cell line IMR-32 (American Type Culture Collection, Rockville, Md., USA) was cultured in RPMI medium (Dutch modification) containing 10% fetal calf serum (Life Technologies, Breda, The Netherlands).

Results and Discussion

Early in the recent explosion of research on Alzheimer's disease, it was postulated that accelerated cellular aging in general, and neuronal aging in particular, might lie at the origin of AD [14]. This hypothesis was difficult to falsify, as little was known about the normal neuronal aging process. In contrast, the aging-related changes in structure and function of erythrocyte AE1 constitute a useful paradigm for the final stages in the cellular aging process [11]. Measurement of aging-related parameters of erythrocyte AE1 suggested that the cellular aging process is disturbed in patients with Alzheimer's disease [38]. Comparison with other types of demen-
tia, including the dementia syndrome observed in elderly individuals with trisomy 21, indicated that perturbation of AE1 structure and function might occur early in life [39]. Recently obtained results, however, indicate a considerable variation in AE1 aging parameters between individuals, as well as variation in time within one individual. These variations largely obscure any AD-specific phenomena, especially as these variations may also occur in healthy, very old individuals [Bosman et al., in preparation]. This conclusion seems to apply to all findings in nonneuronal, peripheral cells [40].

Since the first description of the presence of band 3-related proteins in nucleated cells [41], proteins of the AE family have been found in all cells examined so far [3]. In neuronal cells, AE proteins seem to have the same functions as AE1 in erythrocytes, such as anion transport, ankyrin binding, and SCA generation [42]. Differentiation in vitro results in increased AE1 and SCA expression at the cell surface [43]. Various AE proteins (AE1, AE2 and AE0, an AE protein with structural elements of AE1 and AE2 [33]) are expressed in human neurons, and the neuronal expression of at least one of these proteins increases with age [33, 44]. Immunoabsorption studies indicate that SCA is generated on brain AE1, and SCA-antibodies label fibrillary structures in sections from old, but not from young brains [11].

In Alzheimer’s disease-affected brain areas, some anti-AE1 antisera show an increased immunoreactivity especially in large pyramidal cells with a degenerative morphology that do not (yet) contain mature neurofibrillary tangles [30, 45]. An increased immunoreactivity, both in the number of reactive cells and in the reactivity per cell, is observed especially with antibodies against epitopes of the membrane domain of AE1 (fig. 1D, E). Antibodies against the cytoplasmic domain of AE1 or AE2 do not show this increase (fig. 1A–C). These findings confirm and extend previous data [33, 45, 46]. Especially antisera against putative components of SCA in the AE1 membrane domain react with corpora amylacea in Alzheimer’s disease-affected brain [34]. Corpora amylacea are laminated hyaline bodies, with a protein part that contains proteins associated with the neuronal and glial cytoskeleton and membrane. Their number increases with age and AD [34]. We have not found significant quantitative or qualitative differences in AE expression at the protein level when comparing immunoblots of Alzheimer and control brain tissues (fig. 2, see also [30]). Preliminary results of semi-quantitative measurement of AE1 expression at the RNA level do not indicate increased expression of AE1 (or AE0 and AE2) in AD-affected temporal cortex tissue [Bosman et al., unpubl. observations]. However, qualitative differences in immunoblot protein patterns

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Fig. 1. Immunohistochemical analysis of Alzheimer and control hippocampus tissue with antibodies specific for various domains of anion exchange proteins and ankyrin. A Control hippocampus, monoclonal antibody BIII.136 against amino acids 22-27 of AE1. x 400. B Alzheimer hippocampus, BIII.136. x 400. C Alzheimer hippocampus, polyclonal antiserum WP944 against a soluble, cytoplasmic fragment of erythrocyte AE1. x 400. D Alzheimer hippocampus, monoclonal antibody 4.15 against amino acids 628-642 in the fourth extracellular loop of the membrane domain of AE1. x 400. E Alzheimer hippocampus, monoclonal antibody 4A3 against amino acids 650-658 in the fourth extracellular loop of AE1. x 400. F Alzheimer hippocampus, a polyclonal antiserum against erythrocyte ankyrin. x 400. Semiquantitative analysis shows no differences between Alzheimer and control tissues for antibodies BIII.136 (A, B) and WP944 (C), but a significantly increased immunoreaction in Alzheimer tissue for antibodies 4.14 (D) and 4A3 (E). The highly variable immunoreaction of the anti-ankyrin antiserum obscures any possible differences between Alzheimer and control tissue.
Fig. 2. Immunoblot analysis of control (C) and Alzheimer (A) hippocampus fractions with BIII.136 against the cytoplasmic domain of AE1. P = 40,000 g particulate fraction containing intracellular and plasma membranes; S = supernatant fraction containing cytosolic proteins and smaller vesicles. A main immunoreactive protein of the expected molecular weight of AE1 (95 kD) is found especially in the membrane fractions (P), without any significant differences between control (C_1 and C_2) and Alzheimer (A_1 and A_2) fractions.

have been reported with an antiserum against a peptide consisting of amino acids 812-827 of AE1 [45]. This antiserum also stained amyloid plaques, as did the antibody against a peptide in the fourth extracellular loop of AE1 (fig. 1D). In general, those antibodies against membrane-bound epitopes that react especially with polypeptides smaller than intact AE proteins on brain immunoblots, or with erythrocyte AE1 fragments generated in vitro, show increased immunoreactivity in sections of AD-affected brain areas when compared with control sections [34, 47]. The changes in immunoreactivity are accompanied by changes in anion transport [48].

From such data we conclude that neuronal AE proteins undergo specific structural changes in the membrane of aging and especially degenerating neurons. Such changes result in a conformation that is recognized by antisera directed against epitopes of the membrane domain, which are not accessible in the healthy neuron. The strongest reactions are observed with antisera against components of the senescent cell antigen SCA that originates from AE1 in aging erythrocytes [11]. This conclusion indicates that the processes that lead to SCA generation from AE1 in aging erythrocytes may also occur in aging and degenerating neurons in the human brain. At present, there is only circumstantial evidence for the molecular nature of these processes, such as aging-associated oxidation, either in AE1 itself or in other membrane components, and/or activation of specific proteases [11]. A number of considerations, however, cloud the view sketched above. First, the known members of the AE family share a high degree of homology in their membrane domain [3], including the epitopes that display increased expression in aging and AD-affected neurons. As at least three AE proteins are present in human neurons [33], it is not clear which AE proteins in which type of neurons are responsible for the phenomena observed so far. Second, not all AE isoforms and variants may be equally detectable. We have observed in AD brains an immunoreaction in neurons, e.g. granule cells in the dentate gyrus, that were negative in nondemented brains [47]. In addition, we have seen AE immunoreaction only in neuronal cells, whereas others have found indications for the presence of AE proteins in astroglia as well [11, 49]. These results may be caused by the ability of antisera against membrane epitopes to reach these epi-
topes only in degraded and/or incomplete AE1 molecules [34]. Third, the increasing number of splice variants within the AE family constitutes another complicating factor. For example, not only are there a number of AE1 variants with different cytoplasmic domains [3, 50], some AE proteins may have a complete cytoplasmic domain, but a considerably shortened membrane domain [51]. The, mostly undocumented, reports of unexpected mRNAs that may lack variable parts of the membrane domain [1, 13] add to the suspicion that there may be a plethora of AE isoforms 'out there'. A careful combination of immunocytochemical and in situ hybridization methods will be necessary to distinguish between the numerous possibilities. Finally, various isoforms may end up in various cell compartments, depending on interaction of the cytoplasmic domain with cytoskeletal proteins such as ankyrin early in the synthesis pathway [50, 52], and/or the presence of specific sorting signals. It is noteworthy that an antiserum raised against a cytoplasmic fragment of AE1 is the only antiserum so far, that shows a reactivity concentrated at the plasma membrane (fig. 1C). Most other antisera display a mostly intracellular, dotted pattern [30, 53].

The anti-ankyrin antiserum stains both neurons and astrocytes (fig. 1F). This is, to our knowledge, the first immunohistochemical evidence for the presence of ankyrin-like proteins in the human brain. We could not demonstrate an Alzheimer-related degradation of ankyrin, as shown for fodrin, another neuronal cytoskeleton protein [45]. This is probably due to the sensitivity of ankyrin to degradation [Pinder, pers. commun.] that may occur even during very short (3 h) post-mortem delay and subsequent tissue fragmentation [Bosman et al., unpubl. results]. This conclusion is supported by the considerable interindividual variation that is shown by immunohistochemical analysis, as observed with various antisera against human erythrocyte ankyrin [Van Workum et al., unpubl. observations]. The ankyrin that is present in astrocytes (fig. 1F) may interact with AE proteins that are different from the neuronal isoforms. Aging and Alzheimer-related perturbation of AE structure may affect the interaction between AE proteins and cytoskeletal elements in brain cells, as in aging erythrocytes [11]. Proteins related to erythrocyte protein 4.1, one of the ligands of AE1, are present in neurofibrillary tangles [54], and preliminary data indicate that AE1 interacts with proteins of the neuronal cytoskeleton, that have been implied in the etiology of AD [Bosman et al., unpubl. results].

As discussed above, structural changes in the AE membrane domain are the most conspicuous phenomena in AE proteins in the degenerating human neuron. The membrane domains of the known AE proteins are all involved in chloride/bicarbonate exchange-related regulation of intracellular pH [1, 2]. In order to assess the relative importance of intact AE activity in maintenance of pH, we measured the capacity of IMR-32 neuroblastoma cells - a cell line that is also used as a model system to investigate AD-related processes [55] - to regulate pH, we measured the capacity of IMR-32 neuroblastoma cells - a cell line that is also used as a model system to investigate AD-related processes [55] - to regulate pH in various circumstances. In these cells, pH recovery after acidosis is incomplete in the absence of bicarbonate (fig. 3A), but cells do recover in the presence of bicarbonate (fig. 3B). This recovery is completely inhibited by DIDS, a high-affinity ligand of AE proteins (fig. 3C), and only partially dependent on extracellular sodium (fig. 3C). These data indicate that bicarbonate transport across the neuronal membrane is the major pH-regulatory system in neuronal cells, and that at least part of the bicarbonate transport can be contributed to AE-catalyzed chloride/bicarbonate exchange. Intracellular pH plays an important role in
Fig. 3. Contribution of AE-mediated chloride/bicarbonate exchange to recovery of intracellular pH in IMR-32 neuroblastoma cells after acidosis. A Cells do not recover from an acid load using the NH₄Cl prepulse method [36, 37] in the absence of external bicarbonate. B Cells do recover when bicarbonate is present. Recovery is completely inhibited by DIDS, an inhibitor of chloride/bicarbonate exchange, and a high-affinity ligand of AE proteins. This inhibition is, at least partly, reversible. C Recovery from an acid load is partially independent of the presence of extracellular sodium, indicating the activity of a sodium-independent chloride/bicarbonate exchange system.
cellular homeostasis in general [56]. In neurons, regulation of pH$_i$ is involved in regulation of voltage-activated calcium signals, and neurotransmitter release and uptake [57]. These results warrant the conclusion that a major consequence of aging- and degeneration-associated changes in AE proteins in neuronal cells will be a disturbance of the regulation of intracellular pH, with deleterious effects on neuronal homeostasis and function.

**Conclusions**

Neuronal aging and degeneration are accompanied by changes in AE structure. These changes are most conspicuous in the membrane domain, and resemble the alterations that occur in aging erythrocytes. In the latter, changes in AE1 structure lead to a decrease in anion transport capacity, to decreased binding of the cytoskeleton protein ankyrin, and to generation of a neoantigen. This senescent cell-specific antigen is recognized by the immune system, which results in phagocytosis of old and damaged cells. Against this background, establishment of the identity of the AE protein(s) involved in neuronal aging and degeneration, together with a molecular description of the accompanying changes in AE structure, will illuminate the processes that are involved in aging-related loss of membrane integrity. In our view of the central position of AE proteins in cellular homeostasis in general, the observed structural changes in human neurons may have severe functional consequences, similar to those observed in aging erythrocytes. A more extensive discussion of the putative mechanism(s) of cell death in Alzheimer's disease, i.e. apoptosis and/or necrosis, is beyond the scope of this article [58]. However, the major processes in both necrosis and apoptosis may well be associated with a compromised AE integrity. For example, AE-mediated anion transport is a major factor in regulation of intraneuronal pH, and disturbance of pH regulation has been suggested to be causally associated with various stages in the apoptosis process, e.g. through activation of a specific endonuclease [59, 60]. Also, perturbation of the membrane-cytoskeleton interaction has been postulated to play a major role in the initial stages of neuronal degeneration in AD [61]. The first events in this perturbation may very well be sought in the interaction of the cytoplasmic domain of an AE protein and one or more cytoskeletal proteins. The more so, as this interaction is regulated by intracellular calcium concentration and state of phosphorylation [62, 63], both of which are considered to be important players on the Alzheimer stage [64, 65]. Another important role, in physiological apoptosis and in the Alzheimer pathology, is played by microglia, the macrophages of the brain [66]. Normally, plasma membrane integrity is maintained until late in the course of the apoptotic process, until membrane-bound cellular remains are removed by macrophages, thereby minimizing inflammation. When the apoptosis load is high, as may be the case in AD [58], the microglia clearance system may fail, resulting in necrosis, induction of inflammatory responses, and further tissue damage. The putative role of AE-related antigens in the impaired or untimely recognition of dying and dead cells (or fragments) by microglia is an as yet unexplored, but fascinating avenue.
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