Similarly, GFAP mRNA expression in the cerebral cortex, cerebellum, and hippocampus gradually increased during aging, although the GFAP mRNA content in the cerebral cortex was significantly lower than that in the cerebellum and hippocampus at all ages (Fig. 4B). GFAP expression in the cerebral cortex, cerebellum, and hippocampus at 30 months was about 4.2-fold, 3.1-fold, and 2.5-fold higher than in 3 month-old-mice, respectively. On the other hand, Nef3 mRNA expression was detected in the cerebral cortex, cerebellum, and hippocampus and was abundantly expressed in the hippocampus of mice at all ages tested. Moreover, Nef3 mRNA expression levels in all regions did not change during aging (Fig. 4C).

Immunohistochemical Localization of PAD2 in the Cerebral Cortex, Cerebellum, and Hippocampus

To establish firmly that PAD2-positive cells are present in the cerebral cortex, hippocampus, and cerebellum of 3-month-old mice, we performed immunohistochemical staining of serial sections by using PAD2-, GFAP-, and MAP2-specific antibody (Fig. 5). In the cerebral cortex and hippocampus, PAD2-positive signals were detected in neuronal cell bodies that costained with MAP2 (Fig. 5G,H,M,N) but not in dendrites. Not only was MAP2 staining positive in both the neuronal cell bodies and the dendrites, but the cerebral cortex, hippocampus, and cerebellum were also MAP2-positive (Fig 5M–O). However, GFAP-positive cells, which are considered to be reactive astrocytes, were PAD2 negative in the cerebral cortex, hippocampus, and cerebellum (Fig. 5G–L).

In the cerebellum, PAD2-positive staining was present on morphologically characteristic Purkinje-like cells along dense granule cell layers that were not positive for either MAP2 or GAFP (Fig. 5I,L,O). To confirm that these were actually Purkinje cells, we performed double immunostaining with PAD2 and calbindin, a known marker of Purkinje cells and limited to localization in those cells (Servais et al., 2005; Whitney et al., 2008; Fig. 6). Calbindin staining was evident as a light magenta coloration on the alkaline phosphate substrate (Fig. 6A). These Purkinje cells appeared as huge, round cell bodies located between the bottom of the molecule layer and surface of the granule cell layer of cerebellar tissue. PAD2 was stained brown by the DAB used as a chromogenic substrate (Fig. 6B). Double immunostaining allowed detection of both calbindin- and PAD2-positive cells in the same Purkinje cells from the cerebellum (Fig. 6C), thus ensuring the existence of PAD2 in clearly identified Purkinje cells of the cerebellum. Additionally, the characteristic localization of PAD2, GFAP, and MAP2 in the cerebral cortex, hippocampus, and cerebellum did not change during aging from 3 months to 30 months (data not shown).

DISCUSSION

We report here, for the first time, that PAD2 mRNA expression increases significantly in the cerebral cortex, hippocampus, and cerebellum during aging and that PAD2 localizes in neuronal cells of the cerebral cortex and Purkinje cells of cerebellum. Activation of the PAD2 enzyme is a known cause of protein citrullination (Watanabe et al., 1988; Vossenaar et al., 2003). The potential clinical importance of the findings presented here lies in the close association previously found between abnormal protein citrullination in the CNS and the neurodegenerative disorders AD (Ishigami et al., 2005), MS (Moscarello et al., 2007) and prion disease (Jang et al., 2008). However, until now, changes of PAD2 expression levels have not been linked with the aging process.

In this study, PAD2 mRNA expression was detected in the brains of mice after 15 days of embryonic development, and GFAP mRNA expression first became evident just 1 day later (Fig. 1B,C). Previous reports indicated that PAD2 appeared mainly in glial cells, especially astrocytes (Asaga and Ishigami, 2000, 2001), microglial cells (Asaga et al., 2002), and oligodendrocytes (Akiyama et al., 1999). However, we detected PAD2 earlier than GFAP, so PAD2 must be expressed in cells other than glial cells, possibly astrocytes. MAP2 and Nef3 were also expressed at an early embryonic stage in amounts that increased slightly until birth and remained almost constant until postnatal day 7 (Fig. 2A,D). Thus, PAD2 expression did not correlate with GFAP, MAP2, or Nef3 expression, indicating that PAD2 must appear at specific, but still unknown, stages and conditions of glial and neuronal cell differentiation.

In the cerebral cortex, cerebellum, and hippocampus, PAD2 mRNA expression increased significantly during the aging process (Fig. 4A). That is, PAD2 mRNA levels at the 30-month-old mark were 1.5-fold to 1.6-fold higher than in 3 month olds. Although GFAP mRNA expression also increased significantly during aging, the increase of GFAP did not correlate closely with that of PAD2, because GFAP in 30-month-old mice was 2.7-fold to 4.7-fold higher than that from 3 month olds, far exceeding the increase of PAD2. Moreover Nef3 mRNA expression did not change during aging. Because the change of PAD2 expression levels during aging did not correlate with those of GFAP and Nef3, PAD2 must be expressed only at certain times and under appropriate conditions by neuronal cells and glial cells, including astrocytes (Asaga and Ishigami, 2000, 2001), activated microglial cells (Asaga et al., 2002), and stage-specific immature oligodendrocytes (Akiyama et al., 1999).

Wood et al. (2008) reported that both PAD2 and PAD4 isoforms were present in myelin isolated from normal and MS white matter, and PAD4 was involved in histone citrullination in MS brain (Mastronardi et al., 2006). However, in this study, PAD4 protein was not detected at all by Western blot analysis with the PAD4-specific antibody we developed previously (Nakashima

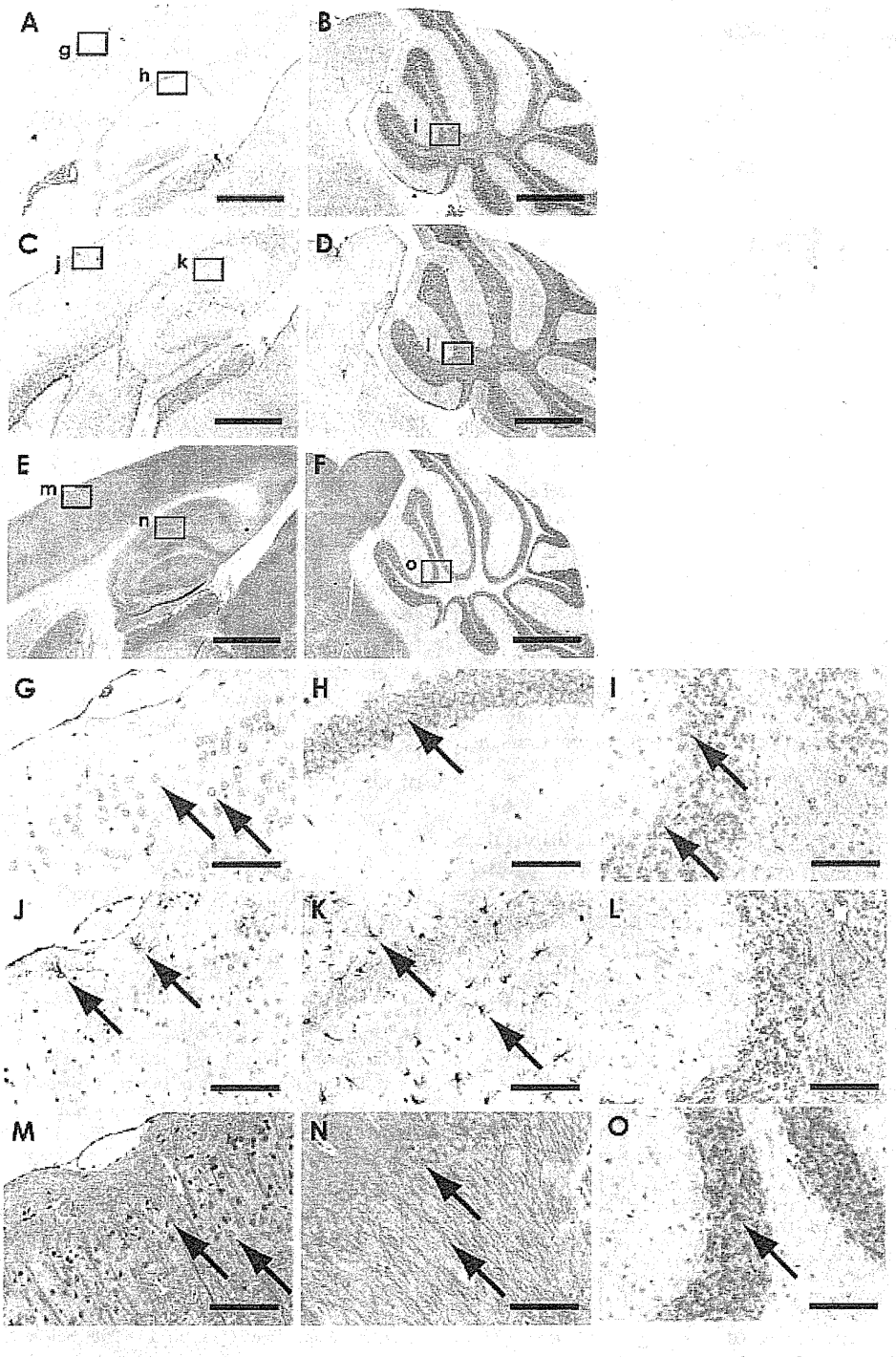

Fig. 5. Immunohistochemical staining of PAD2, GFAP, and MAP2 in the cerebral cortex, hippocampus, and cerebellum of 3-month-old mice. Each brain section was stained with PAD2 (**A,B,G–I**), GFAP (**C,D,J–L**), and MAP2 (**E,F,M–O**) antibody. The square area of g–i in A and B, j–l in C and D, and m–o in E and F were magnified for presentation in G–I, J–L, and M–O, respectively. Arrows indicate typical stained objects. Scale bars = 1 mm in A–F; 100 μm in G–O. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 1999). This discrepancy must be due to the different antibodies used. Moscarello et al. (1994) reported that approximately 20% of the total MBP was citrullinated in early developing human brain determined by protein sequencing; however, in this study, no citrullinated protein at all was found in the brain during normal development and aging by Western blot analysis with antimodified citrulline antibody (Senshu et al., 1992).

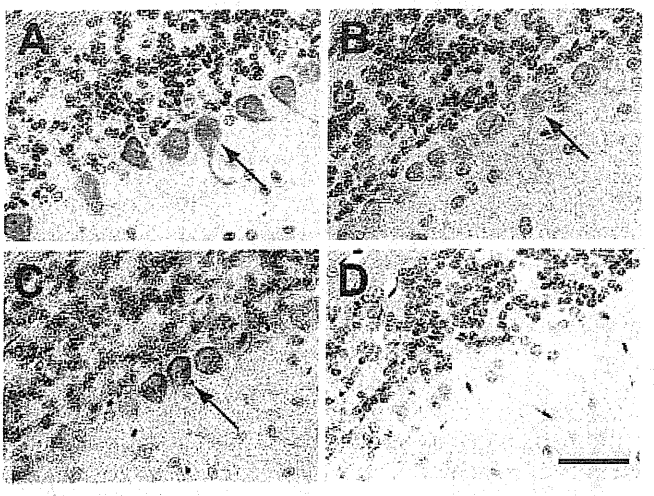

Fig. 6. Identification of PAD2-positive cells in the cerebellum by double immunostaining. Sections of cerebellum from 3-month-old mice were double immunostained with PAD2 and calbindin. Rabbit anticalbindin antibody was applied first, after which alkaline phosphatase-conjugated anti-rabbit IgG was applied for staining with the Red Alkaline Phosphate Substrate Kit I. Next, anti-PAD2 monoclonal antibody (hPAD2-2110) was applied; then, horseradish peroxidase-conjugated anti-mouse IgG was added to stain with DAB. The stained proteins are the following. **A:** Alkaline phosphate substrate as a chromogenic substrate (light magenta) for calbin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

This discrepancy must be due to the technical differences between protein sequencing and Western blot analysis.

As described in our previous reports, PAD2 was detected in activated astrocytes in brains from AD and scrapie-infected mice (Ishigami et al., 2005; Jang et al., 2008). Many reports have, in fact, indicated that PAD2 normally remains inactive but becomes active and citrullinates cellular proteins only when the intracellular calcium balance is upset during neurodegenerative changes such as AD, prion disease, and MS (Moscarello et al., 1994; Ishigami et al., 2005; Jang et al., 2008). Thus, citrullinated protein has become a useful marker for neurodegenerative disorders of humans.

For the first time, PAD2 has been detected in Purkinje cells of the cerebellum, as we found by applying double-immunohistochemical staining for PAD2 and calbindin (Fig. 6). The cerebellum functions as the center of learning and control over motion, sensory input, and cognition. Purkinje cells of the cerebellum are its sole output neurons and are important as the integrators and fine tuners of diverse input signals (Cheron et al., 2008). Accumulated evidence indicates that the dynamic movement of Ca^{2+} plays a key role in the function of Purkinje cells (Matsushita et al., 2002; Erickson et al., 2007). Intracellular Ca^{2+} concentrations become elevated via voltage-dependent calcium channels of plasma membranes or inositol-1,4,5-triphosphate-dependent Ca^{2+} release from intracellular Ca^{2+} storage sites such as the endoplasmic reticulum (Cheron et al., 2008). Elevations of intracellular Ca^{2+} activate an intracellular signal cascade leading to such functional events as neurotransmitter release (Cheron et al., 2008). The relevance of this background is that the PAD enzyme requires ~100-fold higher than the normal intracellular Ca^{2+} level for its activation (Inagaki et al., 1989). Insofar as Purkinje cells store large amounts of Ca^{2+} corresponding to physiological stimuli (Matsushita et al., 2002), conceivably those intracellular Ca^{2+} concentrations become elevated transiently in specific, limited areas, such as near the endoplasmic reticulum and plasma membrane. When such a condition prevails, PAD2 enzyme would become activated and citrullinate various proteins, leading to cell death (Asaga et al., 1998). In fact, quantities of PAD2 and citrullinated proteins have been shown to increase in the brain in vivo during such abnormal conditions as scrapie-infection of mice and AD of humans (Ishigami et al., 2005; Jang et al., 2008).

In conclusion, we detected PAD2 mRNA in the brains of mice beginning at embryonic day 15 and tracked its ever-increasing expression in the cerebral cortex, hippocampus, and cerebellum until the animals were 30 months old. Moreover, we found here, for the first time, that PAD2 localized in neuronal cells of the cerebral cortex and Purkinje cells of the cerebellum. PAD2 may play a role in the onset and progression of neurodegenerative disorders by abnormally disrupting Ca^{2+} homeostasis and thereby increasing the production of citrullinated proteins.

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ORIGINAL PAPER

Involvement of peptidylarginine deiminase-mediated post-translational citrullination in pathogenesis of sporadic Creutzfeldt-Jakob disease

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Abstract Peptidylarginine deiminases (PADs)-mediated post-translational citrullination processes play key roles in protein functions and structural stability through the conversion of arginine to citrulline in the presence of excessive calcium concentrations. In brain, PAD2 is abundantly expressed and can be involved in citrullination in disease. Recently, we have reported pathological characterization of PAD2 and citrullinated proteins in scrapie-infected mice, but the implication of protein citrullination in the pathophysiology in human prion disease is not clear. In the present study, we explored the molecular and biological involvement of PAD2 and the pathogenesis of citrullinated proteins in frontal cortex of patients with sporadic Creutzfeldt-Jakob disease (sCJD). We found increased expression of PAD2 in reactive astrocytes that also contained increased levels of citrullinated proteins. In addition, PAD activity was significantly elevated in patients with sCJD compared to controls. From two-dimensional gel electrophoresis and MALDI-TOF mass analysis, we found various citrullinated candidates, including cytoskeletal and energy metabolism-associated proteins such as vimentin, glial fibrillary acidic protein, enolase, and phosphoglycerate kinase. Based on these findings, our investigations suggest that PAD2 activation and aberrant citrullinated proteins could play a role in pathogenesis and have value as a marker for the postmortem classification of neurodegenerative diseases.

Keywords Citrullination · Peptidylarginine deiminase · Creutzfeldt-Jakob disease · Prion · Astrocytes

Abbreviations

PAD	Peptidylarginine deiminase
CJD	Creutzfeldt-Jakob disease
PrP	Prion protein
CNS	Central nervous system
2-DE	Two-dimensional gel electrophoresis
MALDI-TOF mass	Matrix-assisted laser desorption/ ionization-time of flight mass
BAEE	Benzoyl-L-arginine ethyl ester
SDS	Sodium dodecyl sulfate
anti-MC	Anti-modified citrulline
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

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GFAP	Glial fibrillary acidic protein
MBP	Myelin basic protein
AD	Alzheimer's disease

Introduction

Prion diseases are a group of progressive neurodegenerative diseases that affect the central nervous system (CNS) in humans and animals. These are rare, infectious, and fatal neurodegenerative diseases that are characterized by spongiform changes, neuronal degeneration, reactive gliosis, and accumulation of disease-associated misfolded prion proteins (termed PrP^{Sc}) in the CNS [51]. In prion diseases, PrP^{Sc} is a factor in causation and is thought of as an unconventional infectious agent. Sporadic Creutzfeldt-Jakob disease (sCJD) is the most common of the human diseases, accounting for approximately 85% of human prion cases; it occurs at a rate of approximately one per million [34]. The etiological factor of sCJD remains unknown; in contrast, variant CJD is transmitted from bovine spongiform encephalopathy, and familial CJD (fCJD) is caused by a point mutation at a codon of the prion protein [1, 51].

The post-translational modifications of various proteins are important events required in the regulation of many cellular processes. Aberrant and excessive modifications can provoke abnormal conditions; in particular, these modifications have emerged as key events of CJD development and pathogenesis. These modifications include glycosylation, nitration, phosphorylation, and lipoxidation [17, 20, 45, 46]. Among various post-translational modifications, citrullination (or deimination) is an irreversible process that converts protein-bound arginine residues to citrulline which results in loss of their positive charge, provokes a conformational change, and alters the isoelectric point (*p*I) value and electrophoretic-mobility [58]. Peptidylarginine deiminases (PADs) regulate this process by their activation along with up-regulation of intracellular calcium (Ca^{2+}) distribution [60]. PADs are found as five different isoforms (types 1–4, and 6) that are distinct in substrate and tissue specificity [60]. Among them PAD2 and PAD4 are localized in the CNS [24, 25, 36, 40, 60, 62] and PAD2 is also ubiquitously distributed in other mammalian tissues such as muscle, dermis, spleen, and hematopoietic cells [37, 60]. Especially PAD2 is abundantly expressed in brain which citrullinates various cytoplasmic proteins such as glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) [24, 38]. PAD2 has been reported to contribute to pathogenic events in abnormal conditions [5, 7, 24, 25, 32, 38], and is abundantly increased in reactive astrocytes during several neurodegenerative conditions [24, 25, 43]. Distinctively, PAD4 is the only type of PAD that has a nuclear localization signal sequence at N-terminal domain [2], resulting in localization in cell nuclei where the enzyme citrullinates histones [36, 41, 61]. Since the five Ca^{2+}-binding sites were found in PAD4 by structural analysis [2] and were conserved with several other isoforms [37], it is presumed that PAD2 also contains five Ca^{2+}-binding sites. Under abnormal conditions, PADs-mediated citrullinations have been shown to affect various biological functions, such as the change of proteolytic susceptibility, binding affinity to target molecules, inflammatory processes induced by autoantibodies, regulation of gene expression, and cellular structural changes [31, 35, 49, 50, 61].

Increased citrullination and/or upregulated PAD have been reported in a number of human diseases including multiple sclerosis [36, 38, 43], rheumatoid arthritis [23, 32], Alzheimer's disease (AD) [24], cancer [10, 11], dermatosis [37, 60], and an experimental mouse model of prion disease [25]. The occurrence of citrullinated proteins is associated with disease development or progression, and it could serve as a useful marker or therapeutic target for human diseases.

Recently, we reported pathological characterization of PAD2 and citrullinated proteins that were abnormally accumulated in various brain regions of ME7 scrapie-infected mice [25]. For human prion diseases, the role of citrullination remains to be assessed. In the present study, we explored the molecular and biological involvement of PAD2 and citrullinated proteins in frontal cortex of patients with sCJD.

Materials and methods

Patients

Human brain tissues were obtained from the Biosafety Level-III Autopsy Center for CJD (Hallym University Sacred Heart Hospital, Republic of Korea). The sliced brain tissues were stored at −80°C until analysis. The study was approved by the Institutional Review Board at Hallym University. The pathologic features of the CJD patients are summarized in Table 1 and Fig. 1.

Western blot analysis

Brain tissues were homogenized in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM sodium vanadate, 1% Triton X-100, 1% Nonidet P-40, 0.25% sodium deoxycholic acid, and protease inhibitors (Roche diagnostics, Indianapolis, IN, USA). For detection of PrP^{Sc}, samples were digested with 20 μg/ml proteinase-K (PK)

Table 1 Clinical details of controls and CJD patients specimens

No.	Diagnosis	Sex	Age	Brain weight (g)	Postmortem interval (h)
1	Non-CJD	M	83	1,220	12.0
2	Non-CJD	F	67	1,400	4.0
3	Non-CJD	M	71	1,225	7.0
4	Non-CJD	M	55	1,280	12.0
CJD1	Sporadic	M	77	1,600	2.5
CJD2	Sporadic	M	49	1,150	120.0
CJD3	Sporadic	F	66	1,380	13.0
CJD4	Familial	F	66	1,450	13.5

All non-CJD cases are normal brains. Familial CJD has a point mutation of valine to isoleucine at codon 203 of the prion protein

for 40 min at 37°C and then were probed with mouse monoclonal anti-PrP (3F4, 1:500) [29]. For detection of citrullinated proteins, 50 μg of protein was subjected to 12% SDS–PAGE, transferred to PVDF membrane followed by incubation in modification reagent [1 v of a mixture of 1% diacetyl monoxime/0.5% antipyrine/1 M acetic acid, and 2 v of a mixture of 85% H_3PO_4/98% H_2SO_4/H_2O (20/25/55) containing 0.1% $FeCl_3 \cdot 6H_2O$] and probed with a rabbit polyclonal anti-modified citrulline antibody (anti-MC) at 1:1,000 (Upstate, Lake Placid, NY, USA) as described previously [54]. For the detection of other target proteins, the transferred PVDF membranes were directly probed with mouse monoclonal anti-PAD2 (1:5,000) [56], rabbit polyclonal anti-GFAP (1:7,000, Dr. Ishigami generation) or rabbit polyclonal anti-GAPDH (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then incubated with the appropriate secondary antibody-conjugated HRP. Bound antibodies were visualized by chemiluminescent substrate as described by the manufacturer (Amersham Biosciences, Piscataway, NJ, USA).

Immunohistochemistry

Neutral buffered formalin-fixed brains were cut into 6-μm thick slices and the sections were used for immunohistochemical staining. For staining of citrullinated proteins, the experiment was performed as described previously [25]. For negative control of staining of citrullinated proteins, the sections were incubated with dH_2O instead of 1 v of a mixture of 1% diacetyl monoxamine, 0.5% antipyrine, and 1 N acetic acid. After incubation with primary antibodies including mouse monoclonal anti-PrP (3F4, 1:200), rabbit polyclonal anti-GFAP (1:500, Dako, Copenhagen, Denmark), mouse monoclonal anti-PAD2 (2110, 1:100), and rabbit polyclonal anti-MC (1:400), the sections were washed and then treated sequentially with biotinylated anti-mouse IgG or anti-rabbit IgG, and then incubated with avidin–biotin peroxidase complex using the ABC kit (Vector, Burlingame, CA, USA), developed with 0.003% 3,3-diaminobenzidine and 0.03% H_2O_2 in 50 mM Tris buffer, and finally hematoxylin-counterstained sections were examined under light microscope (BX51; Olympus, UK). For immunofluorescence staining, primary antibodies-exposed sections were labeled with LRSC-conjugated donkey anti-rabbit IgG (1:200) or FITC-conjugated goat anti-mouse IgG (1:200) (Jackson ImmunoResearch, West Grove, PA, USA),

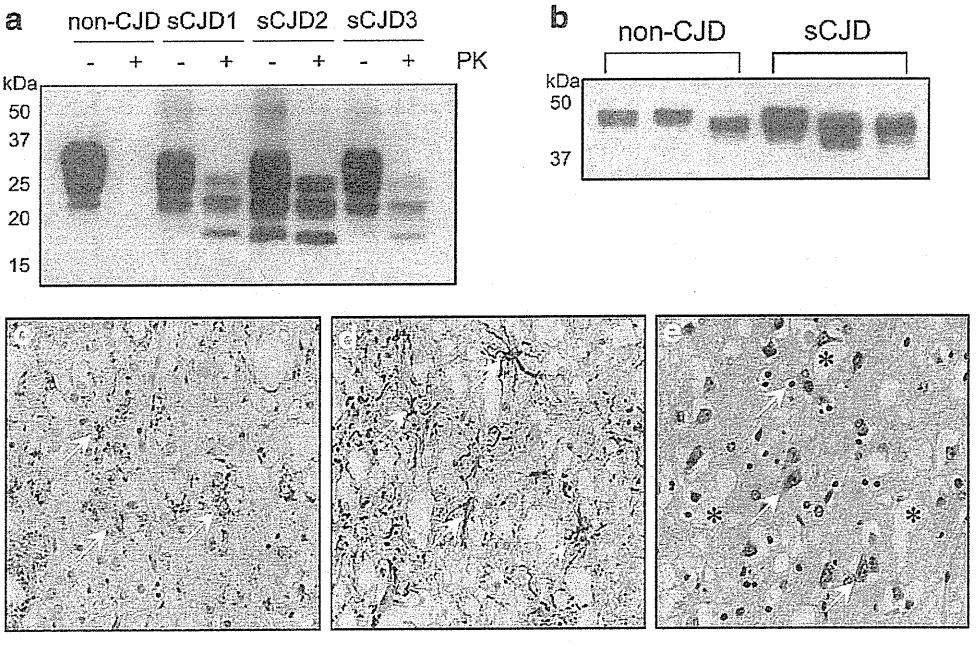

Fig. 1 Pathological characterization of brain samples from sCJD patients. **a** Proteinase K-(PK)-resistant PrP^{Sc} analysis by Western blotting using anti-PrP antibody. **b** Western blot analysis of GFAP expression using anti-GFAP antibody. **c–e** Histological characterizations including PrP^{Sc} deposition in the PK-treated brain slice (**c**), cell bodies and processes of reactive astrocytes (**d**), and vacuolation by hematoxylin–eosin staining (**e**) in frontal cortex of sCJD patients. *Arrows* indicate PrP^{Sc} (**c**), GFAP-positive astrocytes (**d**), and cell body and nucleus of neuronal cell or glial cell in the section. *Asterisks* indicate vacuoles as distinct holes. Original magnification ×20

and then observed with confocal laser scanning microscopy (LSM510; Carl Zeiss, Oberkochen, Germany).

Measurement of PAD activity

In order to determine PAD activity, 400 μg of brain proteins from non-CJD and CJD patients were incubated with the reaction mixture containing 100 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, 5 mM dithiothreitol (DTT) with or without 10 mM benzoyl-L-arginine ethyl ester (BAEE) (Sigma–Aldrich, St. Louis, MO, USA) at 50°C for 1 h. The reaction was then stopped by adding final 1 mol/L perchloric acid. Samples were cooled down on ice for 20 min and then centrifuged at 18,000×*g* for 5 min at room temperature. 80% (v/v) supernatants were mixed with color developing reagents [1 v of a mixture of 80 mM diacetyl monoxime and 2 mM thiosemicarbazide (Sigma) in dH_2O, and 3 v of a mixture of 85% H_3PO_4/98% H_2SO_4/H_2O (33/20/47) containing 0.1% $FeCl_3 \cdot 6H_2O$] and incubated at 95°C for 15 min. To determine the value of PAD activity, samples were cooled to room temperature and then the absorbance was monitored at 534 nm by ELISA reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). One unit of the enzyme is defined as the amount of enzyme that deiminates 1 μM of BAEE (Sigma, St. Louis, MO, USA) by 1 mg of brain homogenates in 1 min at 50°C. For detection of deiminated bovine serum albumin (BSA) by brain-derived PAD, 10 μg of each brain homogenate was incubated with 500 μg of BSA as a substrate in 100 mM Tris–HCl, pH 7.6 buffer containing 10 mM Ca^{2+} and 5 mM DTT at 37°C for 30 min to 2 h. Deiminated BSA (40 μg) was detected by Western blotting using an anti-MC antibody.

Two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass analysis

To perform 2-DE, 150 μg of protein was rehydrated with 7 cm immobilized pH gradient (IPG) strips (pH 3–10 or pH 4–7) in rehydration sample buffer (Bio-Rad, Hercules, CA, USA) for 12 h at 20°C. Isoelectric focusing was conducted at 50 V for 4 h rapidly, 250 V for 20 min rapidly, 2,000 V for 40 min linearly, and increased to a maximum of 4,000 V for 2 h linearly, and then run to accumulate a total of 18,000 Vhours rapidly using 2-D system (PROTEAN IEF CELL; Bio-Rad). Focused IPG strip was equilibrated and was then processed for 2-DE in 12% SDS–PAGE as per manufacturer's protocols. The 2-DE gels were stained by Coomassie brilliant blue G-250 (Bio-Rad) or by Western blot using anti-citrullinated antibody. To identify the citrullinated proteins, the immunoblotting-matched protein spots were excised, and then trypsin digested and MALDI-TOF mass spectrometry analysis was performed as previously described [25].

Data presentation and statistical analysis

Statistical graphs and data were displayed as mean ± standard error of the mean. The probability of statistical differences between non-CJD and sCJD groups was determined by a two-sample *t* test (two-sided) for means. Statistical differences were considered significant at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Results

Clinical features of CJD patients

To evaluate aberrant citrullination and PAD2 in CJD brains, we have tested PrP^{Sc} accumulation, astrogliosis and spongiform degeneration using brain tissues from normal conditions (non-CJD) and patients with sCJD, and fCJD (Fig. 1 and Table 1). The fCJD case possesses a point mutation of valine to isoleucine at codon 203 of the prion protein as demonstrated previously [48]. PK-resistant PrP^{Sc} was detected in all cases of sCJD (Fig. 1a), and PrP^{Sc} was widely accumulated in PK-treated brain slice of the frontal cortex of sCJD (Fig. 1c). We also confirmed the increased expression of GFAP (Fig. 1b, d), which showed a reactive state of astrocytes in sCJD brains. Next, spongiform degeneration in the sections of sCJD brain was observed by hematoxylin–eosin staining (Fig. 1e). Similar observations of neuropathological features have been found in other cases, including fCJD that were used in this study (data not shown).

Upregulation of PAD2 in reactive astrocytes in patients with sCJD

In an experimental mouse model of prion disease, the expression level of PAD2 was especially high at the end stage of scrapie incubation period and was correlated with disease progression [25]. To extend this finding to human diseased brains, we investigated the expression level of PAD2 by Western blot analysis using tissue from the frontal cortex. As shown in Fig. 2a, b, the expression of PAD2 was significantly increased in the brains of sCJD patients compared to non-CJD cases. Next, to investigate the cellular localization of PAD2, we carried out immunohistochemical and immunofluorescent staining. Increased immunoreactivity of PAD2 was detected in the brains of sCJD compared to non-CJD cases (Fig. 2c) and was predominantly found in reactive astrocytes (Fig. 2d). These results confirmed our previous finding that PAD2 was significantly increased in brain and mainly localized in reactive astrocytes of scrapie-

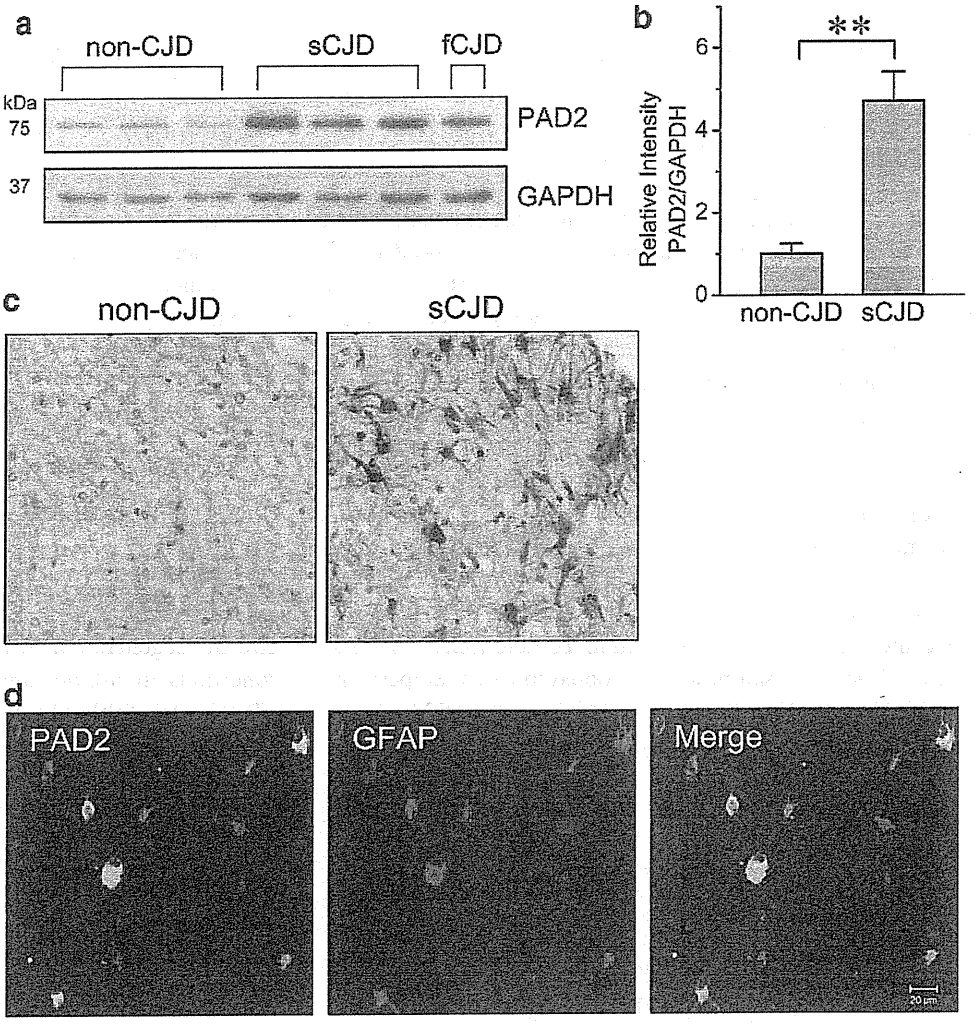

Fig. 2 Expression level of PAD2 and its cellular localization. **a** PAD2 protein was detected in frontal cortex of non-CJD and sCJD groups by Western blot analysis. GAPDH was used as a loading control. **b** PAD2 expression was normalized with GAPDH by Image J software (http://rsb.info.nih.gov/ij/). $^{**}P < 0.01$. **c** Immunohistochemical staining of PAD2 in frontal cortex of sCJD and non-CJD. Original magnification ×20. **d** Co-localization of PAD2 (*green*) and GFAP (*red*) in frontal cortex of sCJD. *Scale bars* 20 μm

infected mice [25]. Recently, it has been reported that both PAD2 and PAD4 are expressed in brains of multiple sclerosis patients [36, 62]. PAD4 was found in nuclei and at increased levels in myelin where there was an increase in citrullination of proteins. To confirm whether PAD4 is expressed and the expression level is changed in frontal cortex of patients with sCJD, we carried out subcellular fractionation to obtain cytosolic and nuclear fractions and then analyzed the expression level of PAD4 by Western blot analysis with two different PAD4 antibodies, which are specific for center or C-terminal region of PAD4. In this study, PAD4 was neither detected in whole brain homogenates nor in the nuclear fractions of brains of non-CJD or sCJD (data not shown). This result suggests that PAD2 is the main form of PADs expressed in frontal cortex of patients with sCJD.

Elevated PAD enzyme activity in frontal cortex of patients with sCJD

To establish if enzymatic activity is correlated with the increase in PAD2 expression, the enzyme activity of PAD was analyzed using brain homogenates from non-CJD and sCJD by in vitro citrullination assay with arginine analog, BAEE. As shown in Fig. 3a, PAD activity was significantly elevated by approximately 3.1-fold in sCJD brains (4.50 ± 1.09 units) compared with non-CJD brains (1.45 ± 0.13 units). In the case of fCJD, PAD activity was also slightly increased (2.52 unit) compared to non-CJD brains (data not shown). Next, to test and confirm the increased PAD activity in sCJD brains, we examined in vitro citrullination of BSA, a natural protein, using each brain homogenates under efficient Ca^{2+} concentration. To diminish intrinsic citrullinated proteins in human brain, the reaction was performed on the basis of the ratio of the protein amount in 1 μg of brain homogenates to 50 μg of BSA. The results show that the level of citrullinated BSA increased in a time-dependent manner when BSA was incubated with the homogenates of sCJD brains (Fig. 3b, c). In contrast, the level of citrullinated BSA exposed to non-CJD brain homogenate remained low throughout the incubation (Fig. 3b, c). Taken together, these results showed that both expression of PAD2

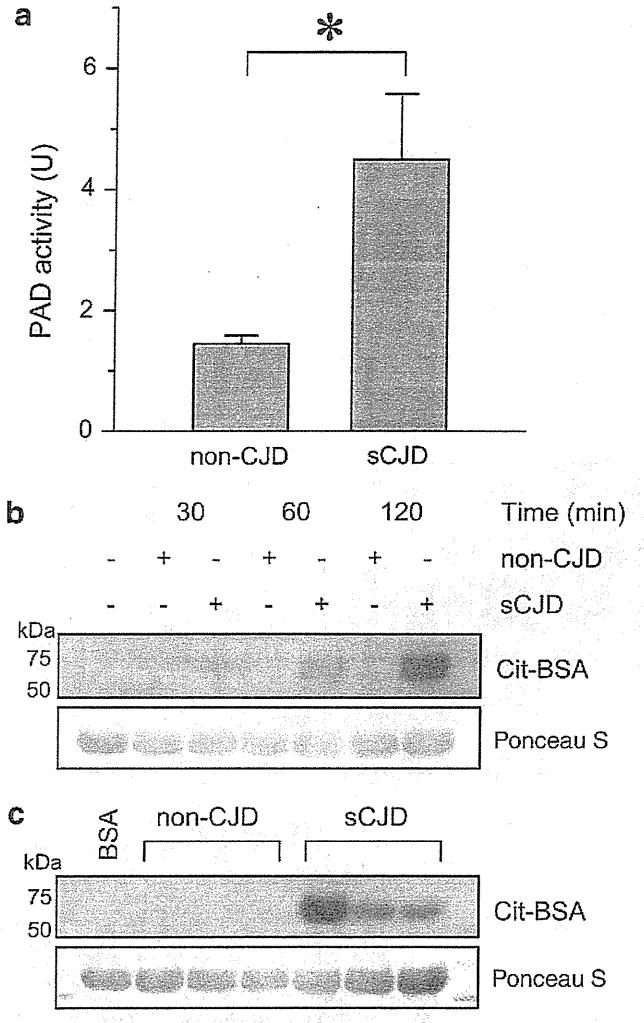

Fig. 3 Enzymatic activity of brain-derived PAD. **a** Comparison of PAD activity using BAEE as an arginine derivative in non-CJD and sCJD groups ($n = 3$/each group, $^*P < 0.05$); **b**, **c** Brain PAD-mediated deimination of BSA, which was used as a natural protein substrate. Time-dependent increase of BSA deimination (**b**) and its extensive deimination at 2 h (**c**) by incubation with sCJD brain homogenates. *Lane 1* BSA, *Lanes 2–7* BSA incubated with brains of non-CJD or sCJD. Deiminated BSA was confirmed by Western blotting with anti-MC antibody. Ponceau S staining shows equal loading volume

protein and its enzyme activity were increased in sCJD brains.

Accumulation of citrullinated proteins and their cellular localization in the brains of sCJD patients

Based on the above results, we examined whether upregulated PAD2 in sCJD brains can be correlated with the generation of citrullinated proteins. As shown in Figs. 4a, b, accumulations of citrullinated proteins occurred more extensively in patients with sCJD compared to non-CJD. This finding is consistent with our previous result showing that the citrullinated proteins were abnormally accumulated at the end stage in brains of scrapie-infected mice [25]. In the next experiments, we performed immunohistochemical staining using serial sections of each brain to confirm the cellular localization of citrullinated proteins. The immunoreactive intensity of citrullinated proteins was higher in brains of sCJD patients than in brains of non-CJD patients; the staining was mainly localized in GFAP-positive astrocytes (Fig. 4c). Brain slices were not stained by anti-MC antibody when formalin-fixed sections were treated with dH_2O rather than a mixture of diacetyl monoxamine and antipyrine in acetic acid prior to exposure to antibody (Fig. 4d). This experiment demonstrates that citrullinated proteins were aberrantly accumulated in reactive astrocytes of sCJD brains.

To confirm whether the citrullinated proteins and PAD2 are co-localized in reactive astrocytes, we performed immunostaining using serial sections of sCJD brain. As shown in Fig. 5, immunoreactive signals for PAD2, citrullinated proteins, and GFAP were found colocalized in cells, i.e., astrocytes. This observation demonstrates that accumulation of citrullinated proteins by increased expression of PAD2 is a major event in reactive astrocytes in brains of patients with sCJD.

Identification of citrullinated proteins in brains of sCJD patients

To identify citrullinated proteins in brains of sCJD patients, we carried out 2-DE on pH 3–10 IPG strips using brain homogenates from non-CJD and sCJD followed by Coomassie staining (Figs. 6a, b, d, e) and Western blotting with an anti-MC antibody for detection of citrullinated proteins (Fig. 6c, f). As seen in Fig. 6c, citrullinated proteins were broadly distributed by *p*I value and molecular weights and were clustered in neutral and basic pH ranges. To optimize the resolution of the neutral pH spots, we performed isoelectric focusing using pH 4–7 IPG strips. In the pH 4–7 range (Fig. 6d–f), separation of citrullinated proteins increased, and they were more easily distinguished than in the pH 3–10 range. Using an antibody to modified citrulline, we detected at least 30 citrullinated spots in the brain of a sCJD patient that were not seen in non-CJD brains. By peptide mass fingerprint analysis using MALDI-TOF mass spectrometry, the citrullinated spots were identified as proteins that are listed in Table 2. Although we could not identify all citrullinated spots because of their low concentration and/or inability to match in a subsequent database search, we could identify various citrullinated candidates including vimentin, GFAP, enolase 1, aldolase A, MBP, cyclophilin A, and phosphoglycerate kinase. These candidates are also known to be citrullinated in various abnormal conditions such as AD, rheumatoid arthritis, glaucoma,

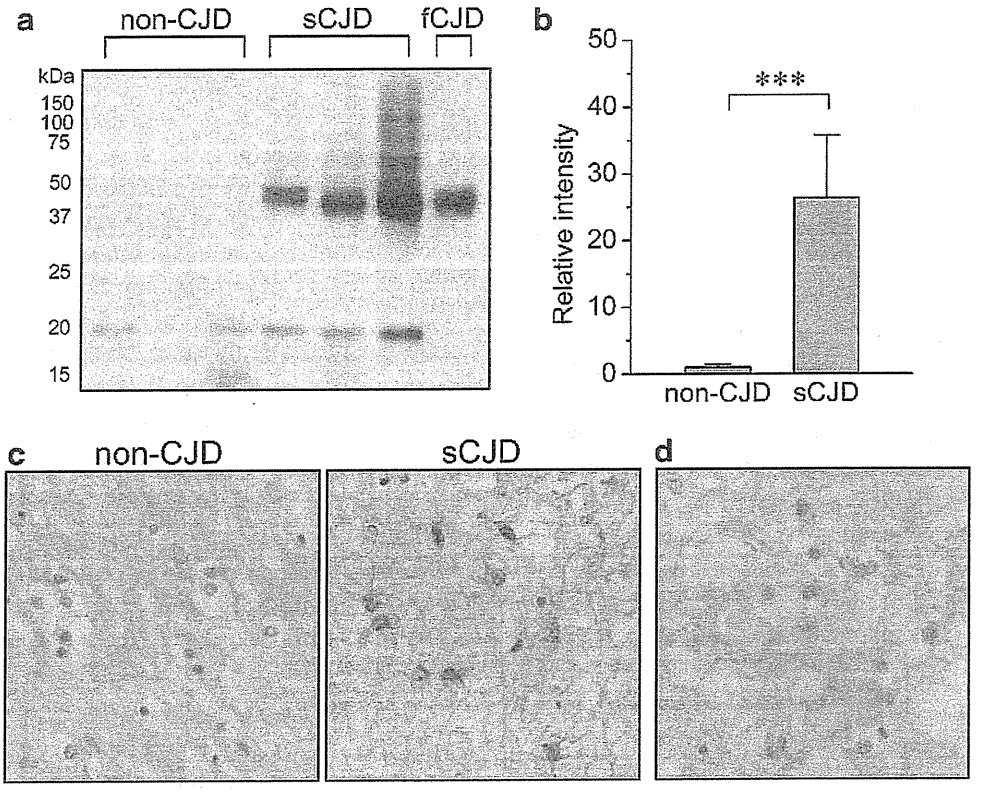

Fig. 4 Accumulation of citrullinated proteins and their cellular localization. **a** Detection of citrullinated proteins using chemically modified membrane labeled with anti-MC antibody. **b** Relative density in non-CJD and sCJD brain samples after normalization with GAPDH. Immunohistochemical staining of citrullinated proteins of non-CJD and sCJD brain samples (**c**) and negative control: section from a sCJD brain incubated with dH_2O rather than diacetyl monoxamine and antipyrine in acetic acid prior to staining with anti-MC antibody (**d**). Original magnification ×20

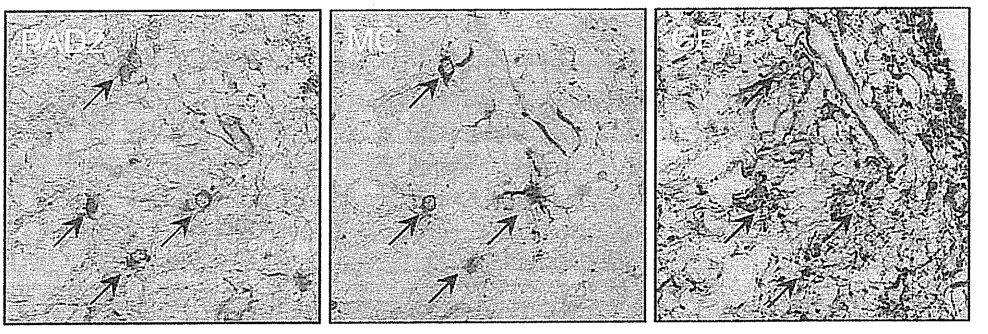

Fig. 5 Immunostaining of PAD2, citrullinated proteins, and GFAP using brain serial sections of sCJD patient. *Arrows* indicate co-localization of PAD2, citrullinated proteins (MC), and reactive astrocytes (GFAP). Original magnification ×20

scrapie infection, and multiple sclerosis [7, 24, 25, 32, 63]. Interestingly, spots including 2 and from 12 to 17, which were identified as GFAP, showed different migration pattern compared to theoretical value. Proteolytic cleavage of GFAP has been documented in various models of neurodegeneration [19, 39], which show marked astrocytic gliosis yielding GFAP fragments of molecular masses ranging from ~20 to 48 kDa. These results can explain this difference of migration pattern between theoretical information and the properties of proteins in nature.

Discussion

Although altered biochemical properties of several proteins following citrullination have been described [31, 35, 49, 50, 61], it is not clear how PAD-dependent citrullination leads to pathophysiological changes in cells. A number of researchers have evaluated the possible role of citrullination in the pathogenesis and diagnosis of diseases [23–25, 32, 37, 38]. Our recent study has revealed that prion infection induced the abnormal accumulation of citrullinated proteins by activated Ca^{2+}-dependent PAD2 in an experimental mouse model of prion disease [25], and it has led us to examine postmortem brains of a human prion disease, CJD. In the current study, we demonstrated that in brains of sCJD patients, citrullinated proteins increased, and this was associated with higher levels of PAD2 expression and increased enzymatic activity. It has been shown that prion infection induced Ca^{2+} dyshomeostasis and Ca^{2+}-mediated neurophysiological dysfunction by altering Ca^{2+} signaling molecules such as CaMK II and Ca^{2+} channels [27, 28, 53, 57]. It is clear that the activation of the deiminating activity of PAD requires Ca^{2+} as shown

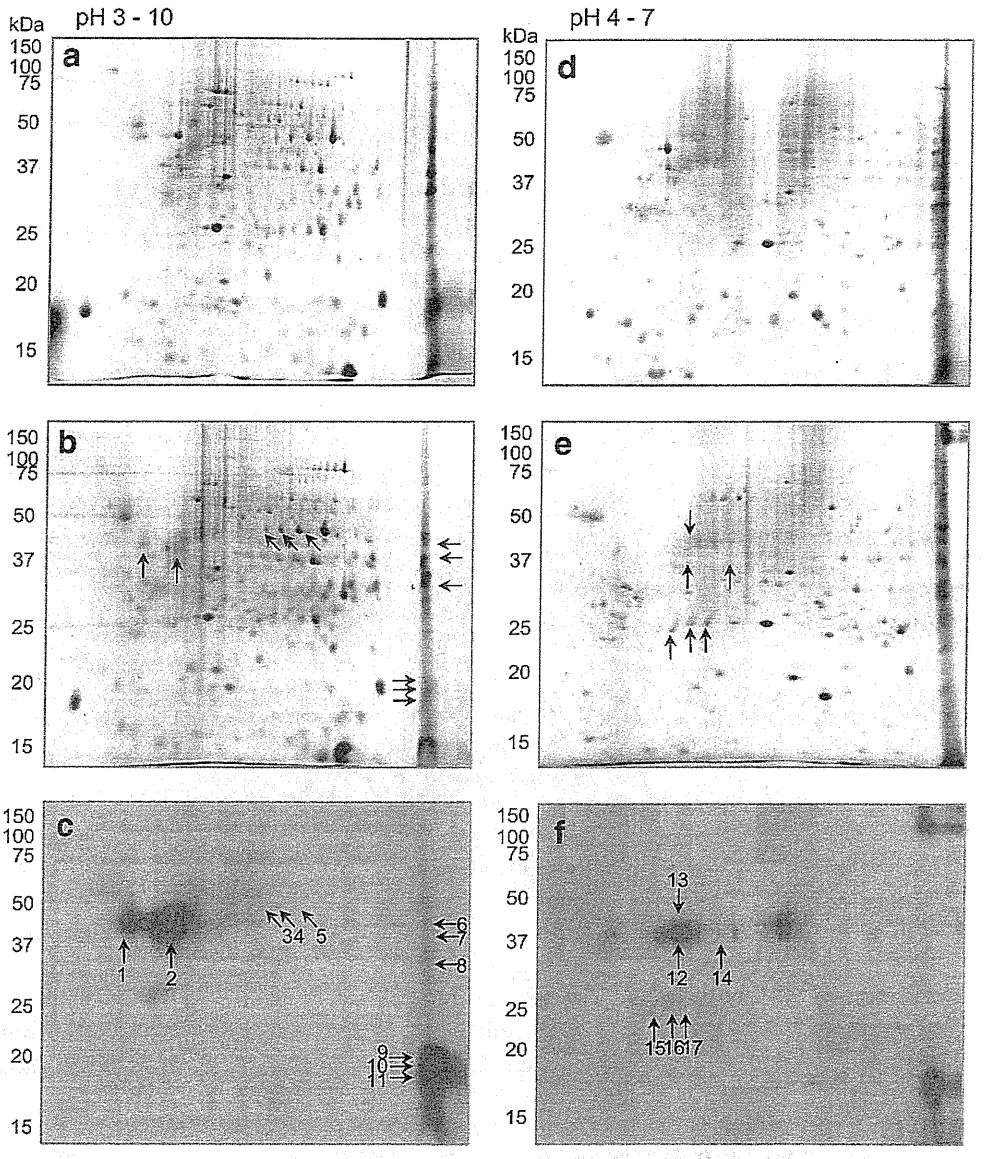

Fig. 6 2-DE analysis of brain proteins from non-CJD and sCJD. Proteins were separated on pH 3–10 (**a–c**) and pH 4–7 (**d–f**) IPG strips. **a**, **b**, **d**, **e** Coomassie staining of 2-DE gels. **c**, **f** Anti-MC antibody-labeled brain samples from patients with sCJD. **a**, **d** Control. **b**, **c**, **e**, **f** sJCD. *Arrows* indicate matched citrullinated spots and protein spots, and serial numbers were used to distinguish subsequently identified citrullinated proteins

by the fact that in vitro PAD activation is blocked by EDTA. In addition, Ca^{2+} seems to play a role in the regulation of PAD transcriptional levels [6]. Taken together, these findings along with our results indicate that Ca^{2+} imbalance in human prion diseases including sCJD may control PAD2 expression and its activity leading to citrullination of various proteins.

PAD2 is expressed at a high level in brain and has been found in glial cells [24, 25, 60]. In normal status, astrocytes play a decisive role as the linker between neurons and blood vessels [65] and act to supply the oxygen and energy sources via internal stores and vasodilatation to support neuronal activity [8, 65]. These mechanisms are involved in increases of intracellular Ca^{2+} in astrocytes, and these events trigger Ca^{2+} waves to neighboring astrocytes [12, 16, 65]. Ca^{2+} signaling in astrocytes may lead to activation of PAD2 and citrullination of its intracellular targets.

Glial cell activation is a prominent response to brain injury; astrocytes show altered shapes, enlargement of cell bodies and thickened cell processes [47]. These pathologic changes are characteristic of the astrocytosis seen in prion diseases. In our previous [25] and current immunohistochemical analyses, we demonstrated that PAD2 and citrullinated proteins were predominantly localized in reactive astrocytes. Reactive astrocytosis is accompanied by activation and upregulation of various proteins with potent biological effects: L-type Ca^{2+} channels, various ionotropic/metabotropic receptors, PAD2, and its well-known substrates including GFAP and vimentin [3, 14, 18, 21, 25]. In both human and mouse models of prion disease, increased PAD2 expression and high levels of accumulated citrullinated proteins are principally found in reactive astrocytes. The reactive status reflects abnormal brain changes, such as those in prion diseases. The current

Table 2 Summary of identified citrullinated proteins in frontal cortex of sCJD

Spot no.	Identification	Sequence coverage (%)	*p*I	kDa	NCBI accession no.	*Z* value
1	Vimentin	20	4.8	41.66	AAA61281.2	1.55
2	Glial fibrillary acidic protein	21	5.5	49.79	AAH62609.1	1.15
3	Enolase 1	15	7.0	47.49	NP_001419.1	1.57
4	Enolase 1	33	7.0	47.49	NP_001419.1	2.36
5	Enolase 1	32	7.0	47.49	NP_001419.1	2.35
6	Phosphoglycerate kinase 1	22	8.6	44.98	CAG32997.1	1.05
7	Aldolase A	22	8.7	39.71	CAA30979.1	1.34
8	Carbonyl reductase 1	24	8.9	30.64	NP_001748.1	1.37
9	Neuropolypeptide h3	57	9.0	16.06	AAD14234.1	1.86
10	17.3K Myelin basic protein	19	11.1	17.33	AAA59559.1	1.37
11	Cyclophilin A	33	8.1	18.21	CAG32988.1	1.65
12	Glial fibrillary acidic protein	22	5.4	49.79	AAH62609.1	1.73
13	Glial fibrillary acidic protein	24	5.4	49.79	AAH62609.1	1.20
14	Glial fibrillary acidic protein	19	5.4	49.79	AAH62609.1	1.48
15	Glial fibrillary acidic protein	24	5.4	49.79	AAH62609.1	1.15
16	Glial fibrillary acidic protein	23	5.4	49.79	AAH62609.1	1.26
17	Glial fibrillary acidic protein	28	5.4	49.79	AAH62609.1	2.32

Z value and its corresponding confidence are following: 1.037, 85%; 1.282, 90.0%; 1.645, 95.0%; 2.326, 99.0%; 3.090, 99.9%. *p*I, isoelectic point. The values of *p*I and molecular weight (kDa) follow theoretical value

findings support the concept that increased expression of PAD2 and the associated aberrant citrullination are involved in the induction of pathologic changes seen in patients with sCJD.

In the normal brain, there is citrullination of GFAP and MBP [42, 63], however, in various acute and progressive neurodegenerative diseases, hypercitrullination of various proteins including GFAP and MBP is seen [5, 24, 25, 38]. In our and other studies, brain-expressed PAD readily deiminates several structural and glycolytic proteins such as vimentin, GFAP, MBP, and enolase [24, 25, 38]. In CJD patients, glial cells including astrocytes and oligodendrocytes are the primary responders to neurological stress. Disturbed Ca^{2+} homeostasis in these cell types can lead to PAD activation which, in turn, can exacerbate abnormal accumulation of citrullinated proteins. Nevertheless, it is unknown whether these citrullinated proteins play a key role in pathophysiological status of reactive astrocytes and oligodendrocytes or are merely concomitant effects of activation of PAD. In addition, although citrullinated forms of astrocyte-specific GFAP have been reported in various neurodegenerative conditions [24, 25, 43], a functional role for citrullinated GFAP in CNS has not been elaborated. In Figs. 1- and 2-DE results, the GFAP protein actually runs on gels at ~25 and 37–50 kDa compared to molecular weight value of 50. GFAP has been known to yield bands at lower molecular weights [44], which are thought to be proteolytic fragments induced by Ca^{2+}-mediated protease [13, 19] and caspase 3 [39]. Thus, further characterization of the effect of citrullinated GFAP on its proteolytic processing should be addressed.

PAD2 has been considered the main type of PADs in brain, but PAD4, the isotype highly expressed in white blood cells [41, 59, 60], was recently found in fractions of nuclear and myelin from brains of multiple sclerosis patients and demyelinating animal models [36, 40, 62]. PAD4 contains a classical monopartite nuclear localization signal sequence at N-terminal [2] and is thus involved in citrullination of nuclear proteins such as histone H2A, H3, and H4 [36, 41, 61]. In addition, it has been reported that the increased PAD2 and PAD4 are important factors in increased citrullinated proteins as well as in the pathogenesis of MS [62]. However, in our expanded study, we could not detect either PAD4 or citrullinated histone H3 in frontal cortex of control or CJD brains using two different PAD4-specific antibodies (data not shown). Therefore, it is likely that the major citrullination-inducing PAD isotype in the brain of CJD in our study is PAD2, as shown by the finding that PAD2 knock-out mice did not show citrullination in brain [52], and brain-derived PAD primarily targets arginine residues of cytoplasmic proteins for citrullination [5, 25]. However, the involvement of PAD4 cannot be excluded.

Although abnormal accumulation of citrullinated proteins has been reported in various neurodegenerative conditions including prion diseases, AD, multiple sclerosis,

and kainic acid administration [4, 5, 24, 25, 36], it remains unknown whether the accumulation level of citrullination is different and whether specific citrullinated proteins are present in these neurodegenerative conditions. In our expanded study for this question, we compared the levels of citrullinated proteins in frontal cortex between patients with sporadic CJD and AD. But we could not find significant differences of accumulation levels of citrullinated proteins in this region using Western blot analysis with anti-modified citrulline antibody (data not shown). Although we could not test various other neurodegenerative diseases, this result suggests the possibility that citrullination may reflect glial cell activation and result in a common phenomenon in many neurodegenerative diseases. Nevertheless, more detailed study of PAD and citrullination between CJD and other neurodegenerative conditions as well as the development of specific antibody against each of the newly identified citrullinated proteins may contribute to our understanding of citrullination-related pathogenesis of neurodegenerative diseases.

It is not clear why PADs, especially PAD2, is activated and upregulated in neurodegenerative conditions. In MS, the inflammatory cytokine tumor necrosis factor alpha (TNF-α) induces PAD4 nuclear translocation, in which histone H3 is hypercitrullinated and apoptosis of oligodendrocytes is induced [36]. In addition, PAD2 transgenic mice showed astrocytes and macrophage activation, and increased production of TNF-α [40]. In studies including our previous work and others [30, 33], upregulation of inflammatory cytokines such as interleukin 1α (IL-1α), IL-1β and TNF-α in the brains of experimental scrapie and CJD mice correlated with the onset and progression of clinical disease. Thus, it is possible that the induction of the proinflammatory cytokines during the progression of prion disease may activate and upregulate PAD enzymes.

The presence of PrP^{Sc} is a marker of prion pathogenesis and can be used as a diagnostic marker, but it is only useful for biopsy- or autopsy-derived brain samples. Accordingly, many researchers have tried to find useful diagnostic factors in brain, cerebrospinal fluid, blood, and urine, and various molecules have been suggested, such as PrP^{Sc}, 14-3-3 family, tau, alpha1-antichymotrypsin, and neuron-specific enolase [9, 15, 22, 26, 55]. In a recent report, in vitro deimination of ovine PrP showed PrP^{Sc}-like characteristics, such as an increase of beta-sheet structure and PK-resistant form [64]. Further characterization of newly identified citrullinated proteins that were identified in this study and other citrullinated proteins found in body fluids might provide markers for the pre-clinical phase of prion diseases.

In summary, citrullinated proteins and increased PAD2 were observed in brains of sCJD patients; by immunohistochemistry, these proteins were found predominately in reactive astrocytes. The level of enzymatic activity of brain-derived PAD from sCJD patients was increased significantly compared to non-CJD controls. Finally, we suggest that the increased protein citrullination by activated PAD could be involved in the pathogenesis of prion diseases and may be an aid in the postmortem classification of human prion diseases.

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REVIEW ARTICLE

Importance of research on peptidylarginine deiminase and citrullinated proteins in age-related disease

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Peptidylarginine deiminases (PAD) are a group of post-translational modification enzymes that citrullinate (deiminate) protein arginine residues in a calcium ion-dependent manner. Enzymatic citrullination abolishes positive charges of native protein molecules, inevitably causing significant alterations in their structure and functions. Citrullinated protein has an important physiological purpose; the formation of a cornified layer of skin that covers the human body. Despite this beneficial function, citrullinated protein also has a negative side, because this protein's accumulation in the brain is a possible cause of Alzheimer's disease. In the present review, we introduce PAD and their protein citrullination function, now considered critical for advancing research on aging and disease. **Geriatr Gerontol Int 2010; 10 (Suppl. 1): S53–S58.**

Keywords: aging, Alzheimer's disease, citrullinated proteins, epidermal differentiation, peptidylarginine deiminase.

Introduction

Numerous post-translational modification enzymes participate in age-associated diseases. However, little attention has been paid to one group of post-translational modification enzymes, the peptidylarginine deiminases (PAD, EC 3.5.3.15).[1–4] These PAD function to citrullinate (deiminate) protein arginine residues in a calcium ion-dependent manner, yielding citrulline residues. Enzymatic citrullination abolishes positive charges of native protein molecules, inevitably causing significant alterations in their structure and functions.[5–7] Citrullinated protein carried out the important physiological act of cornification, which thickens the protective layer of skin that covers the human body.[8,9] However, this protein's detrimental aspect is its accumulation in the brain, constituting a possible cause of Alzheimer's disease (AD).[10] Research on PAD and citrullinated proteins is devoted to untying the threads of this pathway precisely and usefully in the expectation of contributing to humanity the capacity for healthy longevity. The present review describes the current state of studies on PAD expression and protein citrullination; the understanding of which is critical for advancing research on the diseases associated with aging.

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Peptidylarginine deiminases

The group of enzymes collectively called PAD convert protein arginine residues to citrulline residues in the presence of calcium ions (Fig. 1).[1–4] Early reports described three types of PAD termed "PAD I" or "epidermal type", "PAD II" or "muscle type", and "PAD III" or "hair follicle type", each of which differs in relative activities towards synthetic substrates, antigenic properties and distribution in mammalian tissues.[11,12] Subsequently, cDNA cloning analyses showed the existence of five isoforms of PAD (PAD1, PAD2, PAD3, PAD4/5 and PAD6) in rodents.[13] These isoforms showed nearly identical amino acid sequences,[13–17] but appeared to

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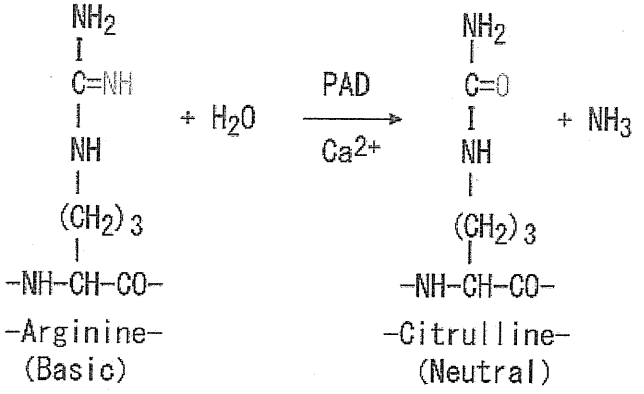

Figure 1 Conversion of arginine residues in proteins to citrulline residues catalyzed by peptidylarginine deiminase. Calcium ion is essential for enzyme activation.

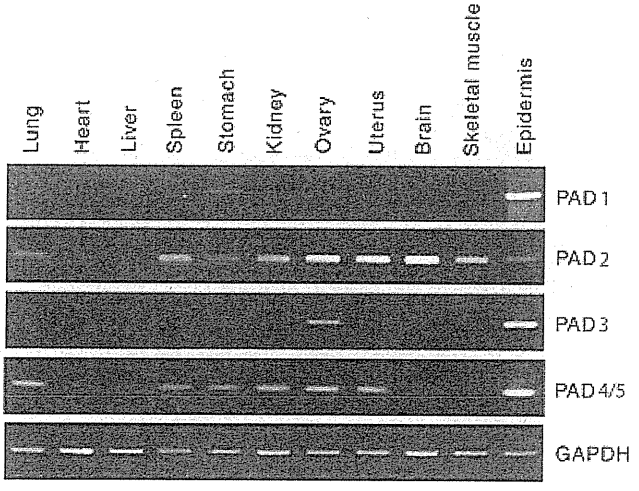

Figure 2 Expression of PAD1, PAD2, PAD3 and PAD4/5 transcripts in various rat tissues analyzed by RT–PCR.[18] Expected sizes were 631 bp for rat PAD1, 428 bp for rat PAD2, 648 bp for PAD3, 205 bp for PAD4/5 and 788 bp for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

have different tissue-specific expression, as evident by reverse transcriptase-polymerase chain reaction (RT–PCR) or Northern blot analysis.[18] Rat PAD1 mRNA was detected only in the epidermis and stomach; that of rat PAD3 appeared mainly in the epidermis, ovary and hair follicles, whereas rat PAD2 and PAD4/5 were more widely expressed, for example, in the epidermis, lung, spleen, stomach, kidney, ovary and uterus (Fig. 2). Only in the epidermis were four PAD mRNA identified, indicating that PAD play functionally important roles during terminal differentiation of epidermal keratinocytes.

Concerning human tissues, five types of PAD have been cloned to date, that is PAD1,[19] PAD2,[20] PAD3,[21] PAD4/5,[22] and PAD6.[23] However, the tissue-specificity of these human PAD is poorly delineated. PAD3 was found in both the inner and outer root sheaths of the hair follicles, where citrullination of trichohyalin occurs in the process of keratinization.[21] PAD4/5 was present in human myeloid leukemia HL-60 cells induced to differentiate into granulocytes by retinoic acid and later found in peripheral blood granulocytes.[22,24]

PAD expression and protein citrullination during normal cornification of keratinocytes

The process of normal epidermal differentiation is characterized by a series of morphological changes as keratinocytes progress from the germinative basal layer through the spinous and granular layers to the outer cornified layer. This process climaxes in a transition from the granular cells to the cornified cells, during which a number of proteins are subjected to various post-translational modifications. Citrulline-containing proteins were first described in the hardened inner root sheath of guinea-pig hair follicles by Rogers and Simmonds.[2]

We found that multiple citrullinated proteins, composed largely of keratins and filaggrin, which is a keratinocyte terminal differentiation marker synthesized in granular cell layers, were present and localized in the granular and cornified cell layers of the epidermis.[9] The presence of citrullinated proteins in such a restricted region of the epidermis strongly suggests that PAD enzymes are involved in the cornification of epidermal keratinocytes. Although PAD activity can be identified in the whole epidermis of newborn rats, its presence is difficult to determine in the individual basal, spinous, granular and cornified cell layers, which resist precise separation. Therefore, to investigate in detail the distribution of citrullinated proteins and the expression of PAD during the cornification of keratinocytes, we used a cell line of epidermal keratinocytes from a newborn rat.[25,26] Such cells, when inoculated into cultures at a density of 1.3×10^4 cells/cm^2, became confluent after 6 days, began to construct stratified colonies at 8 days and formed multiple cell layers at 15 days (Fig. 3). Citrullinated proteins were initially detected on the 11th day, then gradually increased as the cell layers multiplied (Fig. 4). However, PAD activity was detected 5 days earlier than the appearance of citrullinated proteins, when cell homogenates were incubated in the presence of 1 mmol Ca^{2+}. To elucidate the precise stage of growth and differentiation when keratinocytes express PAD, we tested for filaggrin, which is a terminal differentiation marker synthesized in granular layers of keratinocytes.[27] Filaggrin content was represented by its high-molecular-weight precursor protein, profilaggrin. Profilaggrin was initially detected on the 6th day of culture and reached maximum production at 8 days. Processed intermediate filaggrins and filaggrin

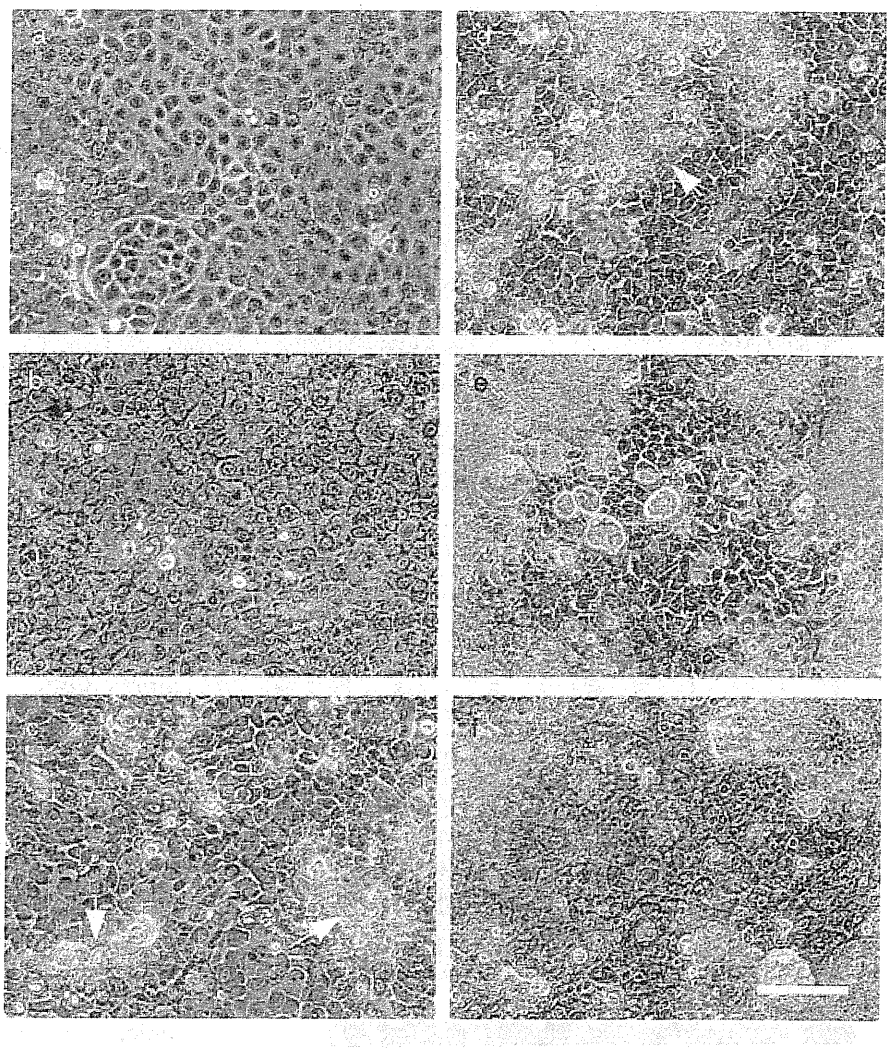

Figure 3 Morphological changes during cultivation of an epidermal keratinocyte line originating from a newborn rat.[26] The initiating inoculum contained 1.3×10^4 cells/cm^2 and cultivation followed for (a) 4 days; (b) 6 days; (c) 8 days; (d) 11 days; (e) 15 days; and (f) 20 days. The arrow indicates stratified colonies (bar, 100 μm).

monomers were also detected at 8 days. Because profilaggrin and PAD were expressed after the same interval of cultivation, these proteins must arise at the same point of keratinocytes' terminal differentiation. The foregoing results indicate that protein citrullination is involved in the cornification of epidermal keratinocytes and that PAD is expressed during their terminal differentiation process.

Abnormal accumulation of citrullinated proteins in brains from patients with Alzheimer's disease

Numerous proteases and post-translational modification enzymes participate in neurodegeneration, such as that in patients with AD and Parkinson's disease.[28,29] In mammalian tissues, only PAD2 is a proven occupant of the rat central nervous system.[3,11,12] Immunocytochemical studies have localized PAD2 in glial cells, especially astrocytes,[30–32] microglial cells[31,33] and oligodendrocytes.[34] Because citrullinated proteins were rarely located in the enzyme-positive glial cells examined with our sensitive detection method,[35] we assumed that PAD2 is normally inactive.[30,32,33] However, glial fibrillary acidic protein (GFAP) was highly susceptible to the attack of PAD2 in excised rat brains deliberately left at room temperature.[36] These findings provided a clue that PAD2 normally remains inactive, but becomes active and citrullinates cellular proteins when the intracellular calcium balance is upset during neurodegenerative changes.

The pathological presentation of AD involves the selective death of pyramidal neurons and an accumulation of two main abnormal protein aggregates, senile plaques (SP) and neurofibrillary tangles (NFT).[37,38] Although NFT and SP are found in many areas of the cerebrum, they are concentrated mainly in the hippocampus and cerebral cortex. The former site actually appears to be more important, because pathological indices are first localized in that region.[29] Our report indicates that levels of PAD2 are more than threefold higher in the hippocampus than the cortex of rat brains.[30] Furthermore, PAD2 activates and citrullinates various cerebral proteins under hypoxic conditions[30]

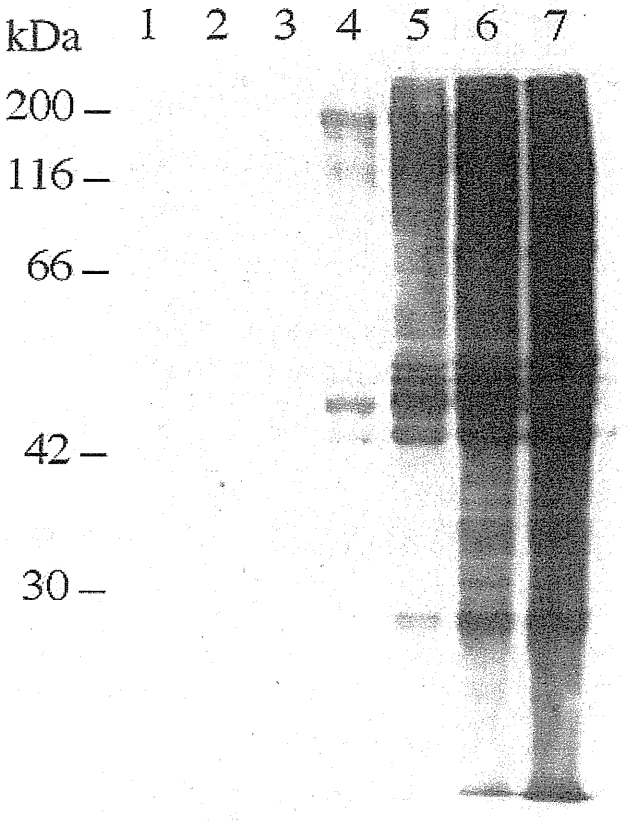

Figure 4 Western blot analysis of citrullinated proteins in keratinocytes during cultivation.[26,35] Lane 1. 4 days; lane 2. 6 days; lane 3, 8 days; lane 4. 11 days; lane 5, 15 days; lane 6, 20 days; and lane 7, 30 days.

and during kainic acid-evoked neurodegeneration,[32,33] suggesting the involvement of protein citrullination in neurodegenerative processes.

To elucidate the involvement of protein citrullination in the progress of AD, we examined whether citrullinated proteins are produced in the brains from patients with AD.[10] By Western blot analysis using anti-modified citrulline antibody, citrullinated proteins of varied molecular weights were detected in hippocampal tissues from patients with AD but not normal subjects (Fig. 5). Two of the citrullinated proteins were identified as vimentin and GFAP by using two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry. Interestingly, PAD2 was detected in hippocampal extracts from AD-positive and normal brains, but the amount of PAD2 in the AD tissue was markedly greater. Histochemical analysis showed citrullinated proteins throughout the hippocampus, especially in the dentate gyrus and stratum radiatum of CA1 and CA2 areas (Fig. 6). However, no citrullinated proteins were detected in the normal hippocampus. Nevertheless, PAD2 immunoreactivity was ubiquitous throughout both the AD-affected and normal hippocampal areas. Still, PAD2-enrichment coincided well with citrullinated protein-positivity. Double immunofluorescence staining showed that citrullinated protein- and PAD2-positive cells also coincided with GFAP-positive cells, but not all GFAP-positive cells were positive for PAD2. Like GFAP, which is an astrocyte-specific marker protein, PAD2 is distributed mainly in astrocytes. These collective results, the abnormal accumulation of citrullinated proteins and abnormal activation of PAD2 in hippocampi of patients with AD, strongly suggest that PAD has an important role in the onset and progression of AD, and that citrullinated proteins might become a useful marker for human neurodegenerative diseases.

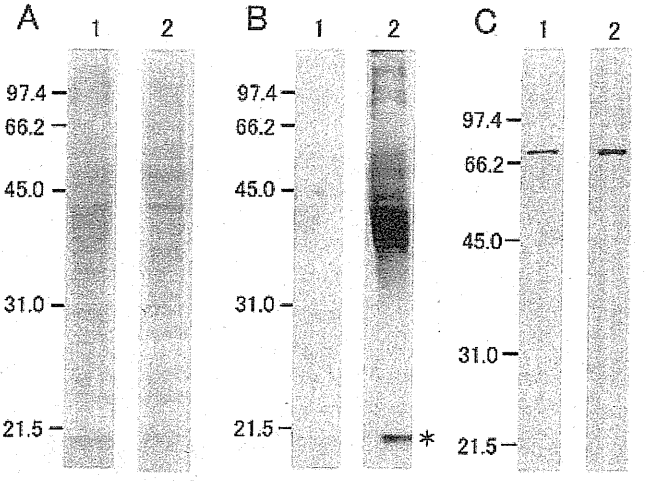

Figure 5 Western blot analysis of citrullinated proteins and PAD2 in hippocampi from the brains of Alzheimer's disease patients and normal controls. (a) Typical protein profiles detected by Amido black staining. (b) Citrullinated protein profiles. (c) Immunoreactive PAD2 profiles. Lane 1, age-matched control; lane 2, Alzheimer's disease. Asterisk indicates the citrullinated myelin basic protein.

Aspects of PAD2 expression and protein citrullination in neurodegenerative disorders

The mechanism(s) by which citrullinated proteins occur in the hippocampus during AD remains unclear. It is possible that PAD2 becomes activated, abundant and functional only in the presence of AD, because the amount of PAD2 increased notably in hippocampi of the patients with AD we assessed compared with that in normal subjects. Although PAD2 was also present in hippocampal extracts from normal subjects, that enzyme remained in a steady state during which no enzyme activation occurred. For enzyme activation, the intracellular calcium concentration must become elevated. To the best of our knowledge, no other factors can regulate PAD activity *in vivo* or *in vitro*. A loss of neuronal calcium homeostasis leading to increases in the intracellular calcium concentration has been