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-

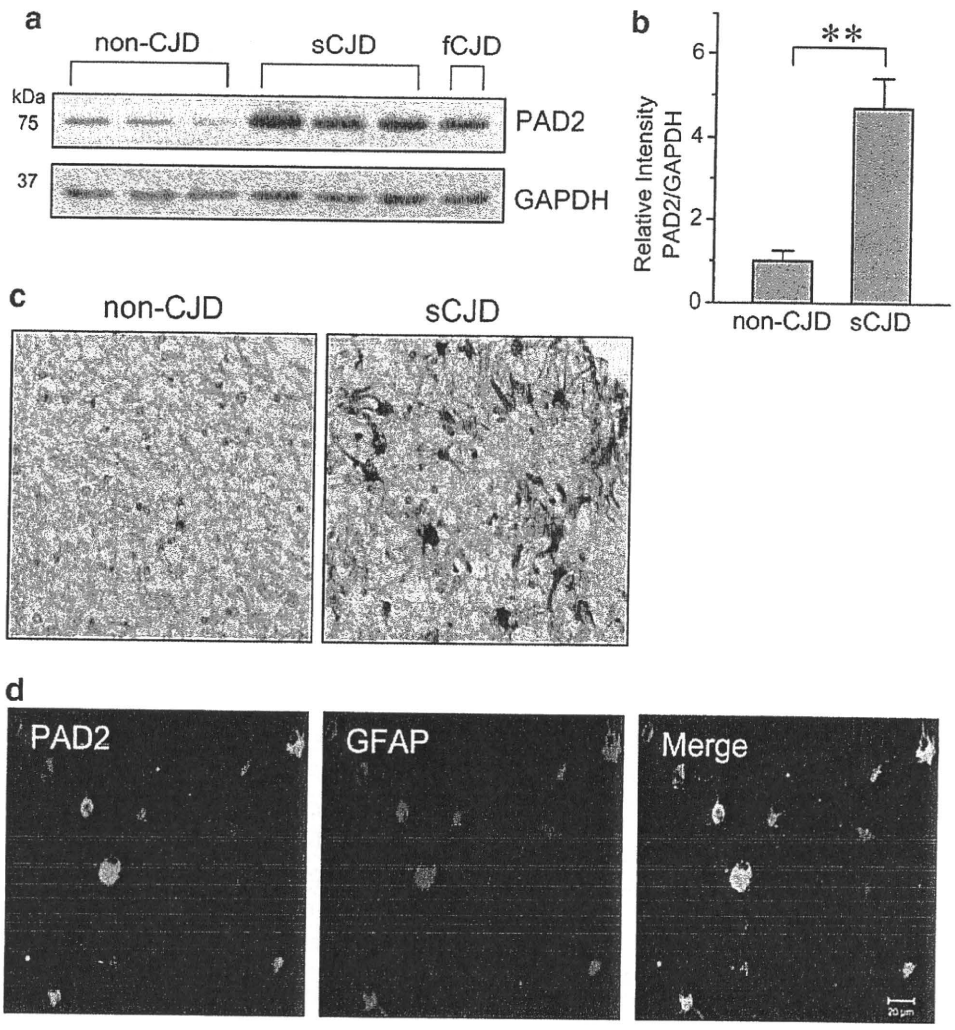

Fig. 2 Expression level of PAD2 and its cellular localization. **a** PAD2 protein was detected in frontal cortex of non-CJD and sCJD groups by Western blot analysis. GAPDH was used as a loading control. **b** PAD2 expression was normalized with GAPDH by Image J software (http://rsb.info.nih.gov/ij/). $^{**}P < 0.01$. **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

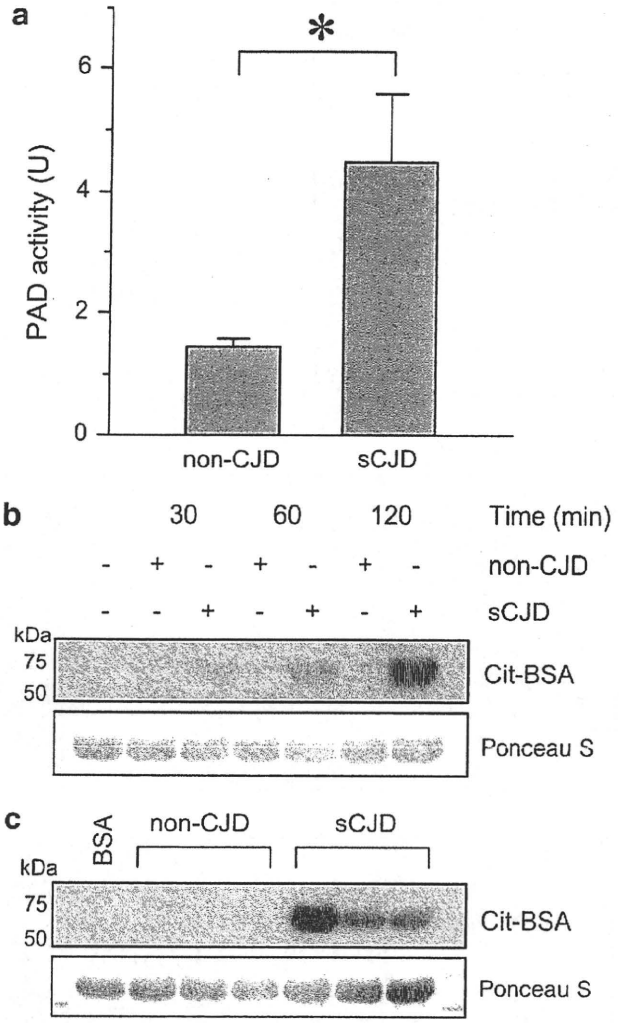

Fig. 3 Enzymatic activity of brain-derived PAD. **a** Comparison of PAD activity using BAEE as an arginine derivative in non-CJD and sCJD groups ($n = 3$/each group, $^*P < 0.05$); **b**, **c** Brain PAD-mediated deimination of BSA, which was used as a natural protein substrate. Time-dependent increase 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,

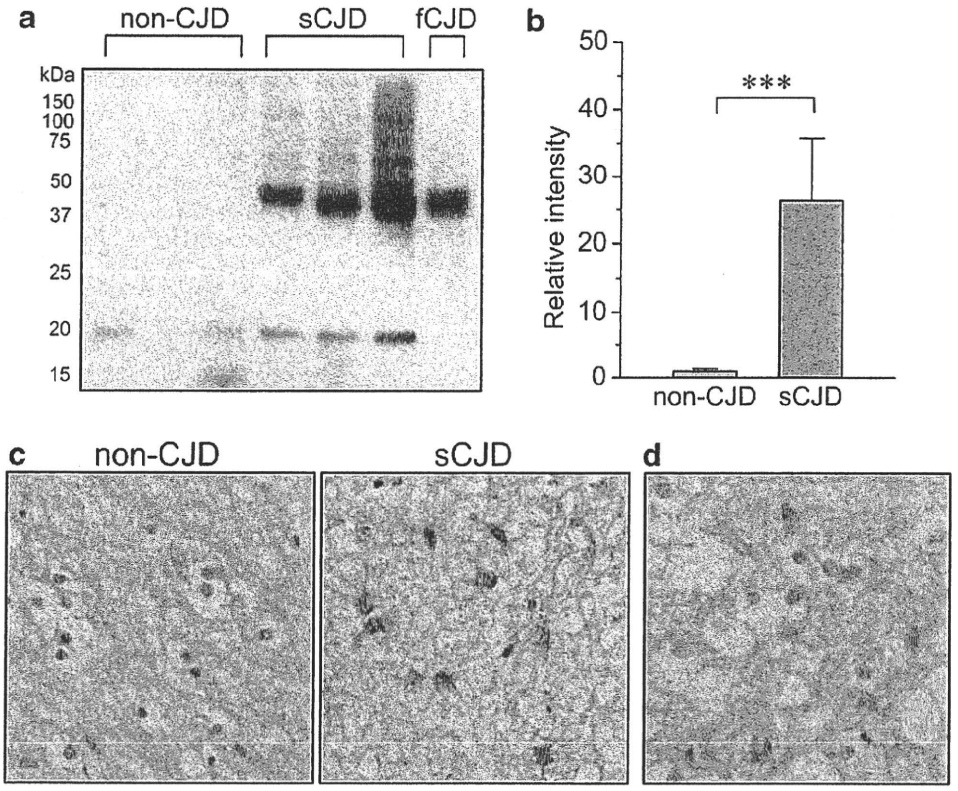

Fig. 4 Accumulation of citrullinated proteins and their cellular localization. **a** Detection of citrullinated proteins using chemically modified membrane labeled with anti-MC antibody. **b** Relative density in non-CJD and sCJD brain samples after normalization with GAPDH. Immunohistochemical staining 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

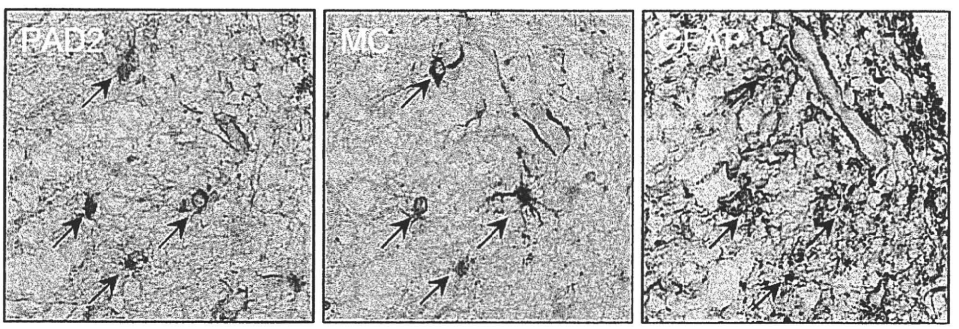

Fig. 5 Immunostaining of PAD2, citrullinated proteins, and GFAP using brain serial sections of sCJD patient. *Arrows* indicate co-localization of PAD2, citrullinated proteins (MC), and reactive astrocytes (GFAP). Original magnification ×20

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

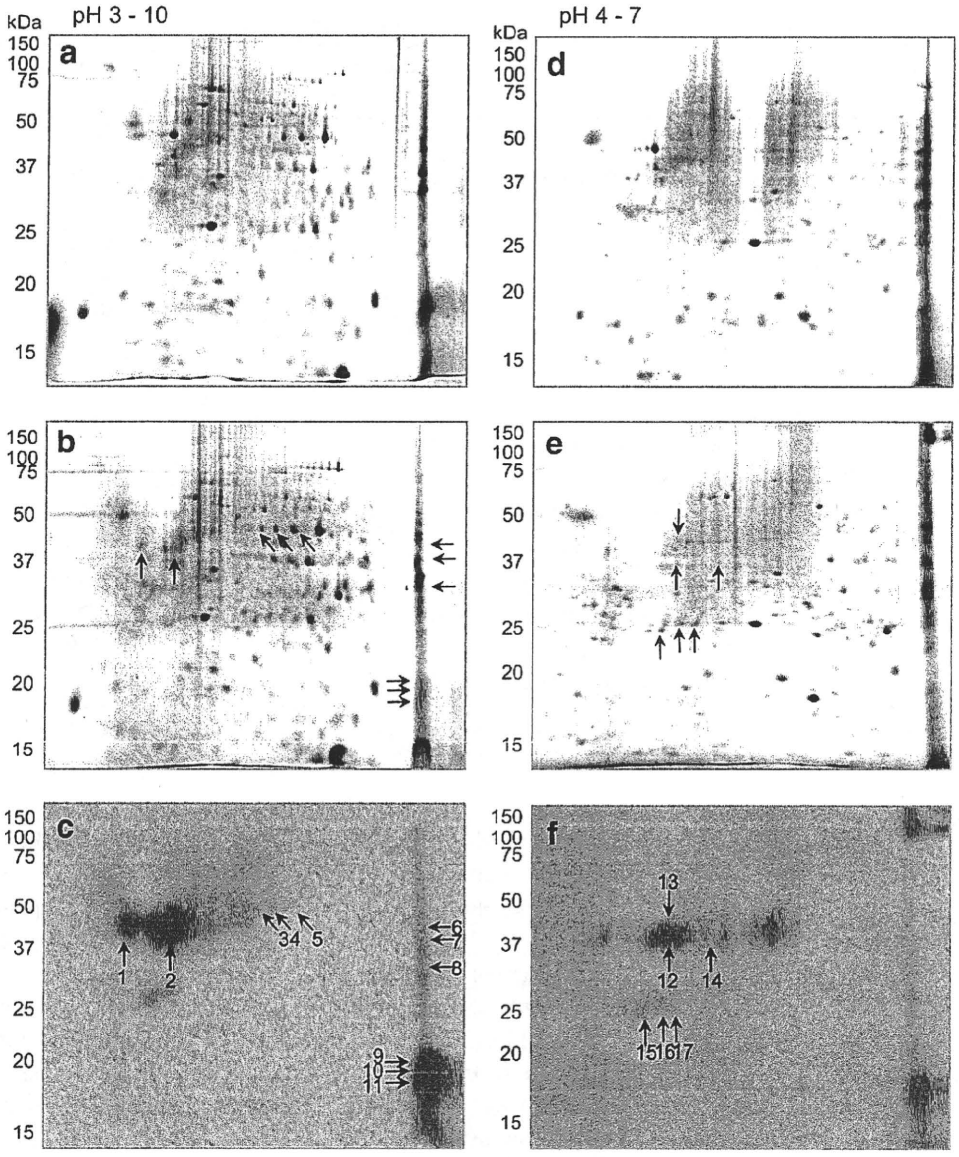

Fig. 6 2-DE analysis of brain proteins from non-CJD and sCJD. Proteins were separated on pH 3–10 (**a–c**) and pH 4–7 (**d–f**) IPG strips. **a**, **b**, **d**, **e** Coomassie staining of 2-DE gels. **c**, **f** Anti-MC antibody-labeled brain samples from patients with sCJD. **a**, **d** Control. **b**, **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.

Acknowledgments This study was supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare and Family Affairs, Republic of Korea (A085082).

References

1. Aguzzi A, Sigurdson C, Heikenwaelder M (2008) Molecular mechanisms of prion pathogenesis. Annu Rev Pathol 3:11–40
2. Arita K, Hashimoto H, Shimizu T, Nakashima K, Yamada M, Sato M (2004) Structural basis for Ca(2+)-induced activation of human PAD4. Nat Struct Mol Biol 11:777–783
3. Aronica E, Yankaya B, Jansen GH et al (2001) Ionotropic and metabotropic glutamate receptor protein expression in glioneuronal tumours from patients with intractable epilepsy. Neuropathol Appl Neurobiol 27:223–237
4. Asaga H, Akiyama K, Ohsawa T, Ishigami A (2002) Increased and type II-specific expression of peptidylarginine deiminase in activated microglia but not hyperplastic astrocytes following kainic acid-evoked neurodegeneration in the rat brain. Neurosci Lett 326:129–132
5. Asaga H, Ishigami A (2001) Protein deimination in the rat brain after kainate administration: citrulline-containing proteins as a novel marker of neurodegeneration. Neurosci Lett 299:5–8
6. Bhattacharya SK, Bhat MB, Takahara H (2006) Modulation of peptidyl arginine deiminase 2 and implication for neurodegeneration. Curr Eye Res 31:1063–1071
7. Bhattacharya SK, Crabb JS, Bonilha VL, Gu X, Takahara H, Crabb JW (2006) Proteomics implicates peptidyl arginine deiminase 2 and optic nerve citrullination in glaucoma pathogenesis. Invest Ophthalmol Vis Sci 47:2508–2514
8. Brown AM, Ransom BR (2007) Astrocyte glycogen and brain energy metabolism. Glia 55:1263–1271
9. Castilla J, Saá P, Soto C (2005) Detection of prions in blood. Nat Med 11:982–985
10. Chang X, Han J (2006) Expression of peptidylarginine deiminase type 4 (PAD4) in various tumors. Mol Carcinog 45:183–196
11. Chang X, Han J, Pang L, Zhao Y, Yang Y, Shen Z (2009) Increased PADI4 expression in blood and tissues of patients with malignant tumors. BMC Cancer 9:40
12. Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science 247:470–473
13. DeArmond SJ, Fajardo M, Naughton SA, Eng LF (1983) Degradation of glial fibrillary acidic protein by a calcium dependent proteinase: an electroblot study. Brain Res 262:275–282
14. Eddleston M, Mucke L (1993) Molecular profile of reactive astrocytes—implications for their role in neurologic disease. Neuroscience 54:15–36
15. Evers S, Droste DW, Lüdemann P, Oberwittler C (1998) Early elevation of cerebrospinal fluid neuron-specific enolase in Creutzfeldt-Jakob disease. J Neurol 245:52–53
16. Fiacco TA, McCarthy KD (2006) Astrocyte calcium elevations: properties, propagation, and effects on brain signaling. Glia 54:676–690

17. Freixes M, Rodríguez A, Dalfó E, Ferrer I (2006) Oxidation, glycoxidation, lipoxidation, nitration, and responses to oxidative stress in the cerebral cortex in Creutzfeldt-Jakob disease. Neurobiol Aging 27:1807–1815
18. Gottlieb M, Matute C (1997) Expression of ionotropic glutamate receptor subunits in glial cells of the hippocampal CA1 area following transient forebrain ischemia. J Cereb Blood Flow Metab 17:290–300
19. Gray BC, Skipp P, O'Connor VM, Perry VH (2006) Increased expression of glial fibrillary acidic protein fragments and mucalpain activation within the hippocampus of prion-infected mice. Biochem Soc Trans 34:51–54
20. Guentchev M, Voigtländer T, Haberler C, Groschup MH, Budka H (2000) Evidence for oxidative stress in experimental prion disease. Neurobiol Dis 7:270–273
21. Hernandez MR, Agapova OA, Yang P, Salvador-Silva M, Ricard CS, Aoi S (2002) Differential gene expression in astrocytes from human normal and glaucomatous optic nerve head analyzed by cDNA microarray. Glia 38:45–64
22. Hsich G, Kenney K, Gibbs CJ, Lee KH, Harrington MG (1996) The 14-3-3 brain protein in cerebrospinal fluid as a marker for transmissible spongiform encephalopathies. N Engl J Med 335:924–930
23. Imboden JB (2009) The immunopathogenesis of rheumatoid arthritis. Annu Rev Pathol 4:417–434
24. Ishigami A, Ohsawa T, Hiratsuka M et al (2005) Abnormal accumulation of citrullinated proteins catalyzed by peptidylarginine deiminase in hippocampal extracts from patients with Alzheimer's disease. J Neurosci Res 80:120–128
25. Jang B, Kim E, Choi JK et al (2008) Accumulation of citrullinated proteins by up-regulated peptidylarginine deiminase 2 in brains of scrapie-infected mice: a possible role in pathogenesis. Am J Pathol 173:1129–1142
26. Jimi T, Wakayama Y, Shibuya S et al (1992) High levels of nervous system-specific proteins in cerebrospinal fluid in patients with early stage Creutzfeldt-Jakob disease. Clin Chim Acta 211:37–46
27. Jin JK, Choi JK, Lee HG, Kim YS, Carp RI, Choi EK (1999) Increased expression of CaM kinase II alpha in the brains of scrapie-infected mice. Neurosci Lett 273:37–40
28. Johnston AR, Black C, Fraser J, MacLeod N (1997) Scrapie infection alters the membrane and synaptic properties of mouse hippocampal CA1 pyramidal neurones. J Physiol 500:1–15
29. Kascsak RJ, Rubenstein R, Merz PA et al (1987) Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. J Virol 61:3688–3693
30. Kim JI, Ju WK, Choi JH et al (1999) Expression of cytokine genes and increased nuclear factor-kappa B activity in the brains of scrapie-infected mice. Mol Brain Res 73:17–27
31. Kizawa K, Takahara H, Troxler H, Kleinert P, Mochida U, Heizmann CW (2008) Specific citrullination causes assembly of a globular S100A3 homotetramer: a putative Ca2+ modulator matures human hair cuticle. J Biol Chem 283:5004–5013
32. Klareskog L, Rönnelid J, Lundberg K, Padyukov L, Alfredsson L (2008) Immunity to citrullinated proteins in rheumatoid arthritis. Annu Rev Immunol 26:651–675
33. Kordek R, Nerurkar VR, Liberski PP et al (1996) Heightened expression of tumor necrosis factor a, interleukin 1a, and glial fibrillary acidic protein in experimental Creutzfeldt-Jakob disease in mice. Proc Natl Acad Sci USA 93:9754–9758
34. Ladogana A, Puopolo M, Croes EA et al (2005) Mortality from Creutzfeldt-Jakob disease and related disorders in Europe, Australia, and Canada. Neurology 64:1586–1591
35. Loos T, Mortier A, Gouwy M et al (2008) Citrullination of CXCL10 and CXCL11 by peptidylarginine deiminase: a naturally occurring posttranslational modification of chemokines and new dimension of immunoregulation. Blood 112:2648–2656
36. Mastronardi FG, Wood DD, Mei J et al (2006) Increased citrullination of histone H3 in multiple sclerosis brain and animal models of demyelination: a role for tumor necrosis factor-induced peptidylarginine deiminase 4 translocation. J Neurosci 26:11387–11396
37. Méchin MC, Sebbag M, Arnaud J et al (2007) Update on peptidylarginine deiminases and deimination in skin physiology and severe human diseases. Int J Cosmet Sci 29:147–168
38. Moscarello MA, Mastronardi FG, Wood DD (2007) The role of citrullinated proteins suggests a novel mechanism in the pathogenesis of multiple sclerosis. Neurochem Res 32:251–256
39. Mouser PE, Head E, Ha KH, Rohn TT (2006) Caspase-mediated cleavage of glial fibrillary acidic protein within degenerating astrocytes of the Alzheimer's disease brain. Am J Pathol 168:936–946
40. Musse AA, Li Z, Ackerley CA et al (2008) Peptidylarginine deiminase 2 (PAD2) overexpression in transgenic mice leads to myelin loss in the central nervous system. Dis Model Mech 1:229–240
41. Nakashima K, Hagiwara T, Yamada M (2002) Nuclear localization of peptidylarginine deiminase V and histone deimination in granulocytes. J Biol Chem 277:49562–49568
42. Nicholas AP, King JL, Sambandam T et al (2003) Immunohistochemical localization of citrullinated proteins in adult rat brain. J Comp Neurol 459:251–266
43. Nicholas AP, Sambandam T, Echols JD, Tourtellotte WW (2004) Increased citrullinated glial fibrillary acidic protein in secondary progressive multiple sclerosis. J Comp Neurol 473:128–136
44. Newcombe J, Woodroofe MN, Cuzner ML (1986) Distribution of glial fibrillary acidic protein in gliosed human white matter. J Neurochem 47:1713–1719
45. Otvos L Jr, Cudic M (2002) Post-translational modifications in prion proteins. Curr Protein Pept Sci 3:643–652
46. Pamplona R, Naudí A, Gavín R et al (2008) Increased oxidation, glycoxidation, and lipoxidation of brain proteins in prion disease. Free Radic Biol Med 45:1159–1166
47. Pekny M, Nilsson M (2005) Astrocyte activation and reactive gliosis. Glia 50:427–434
48. Peoc'h K, Manivet P, Beaudry P et al (2000) Identification of three novel mutations (E196K, V203I, E211Q) in the prion protein gene (PRNP) in inherited prion diseases with Creutzfeldt-Jakob disease phenotype. Hum Mutat 15:482
49. Pritzker LB, Joshi S, Gowan JJ, Harauz G, Moscarello MA (2000) Deimination of myelin basic protein. 1. Effect of deimination of arginyl residues of myelin basic protein on its structure and susceptibility to digestion by cathepsin D. Biochemistry 39:5374–5381
50. Proost P, Loos T, Mortier A et al (2008) Citrullination of CXCL8 by peptidylarginine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation. J Exp Med 205:2085–2097
51. Prusiner SB (1998) Prions. Proc Natl Acad Sci USA 95:13363–13383
52. Raijmakers R, Vogelzangs J, Raats J et al (2006) Experimental autoimmune encephalomyelitis induction in peptidylarginine deiminase 2 knockout mice. J Comp Neurol 498:217–226
53. Sandberg MK, Wallén P, Wikström MA, Kristensson K (2004) Scrapie-infected GT1-1 cells show impaired function of voltage-gated N-type calcium channels (Ca(v) 2.2) which is ameliorated by quinacrine treatment. Neurobiol Dis 15:143–151
54. Senshu T, Sato T, Inoue T, Akiyama K, Asaga H (1992) Detection of citrulline residues in deiminated proteins on polyvinylidene difluoride membrane. Anal Biochem 203:94–100

55. Shaked GM, Shaked Y, Kariv-Inbal Z, Halimi M, Avraham I, Gabizon R (2001) A protease-resistant prion protein isoform is present in urine of animals and humans affected with prion diseases. J Biol Chem 276:31479–31482
56. Shimada N, Handa S, Uchida Y et al (2009) Developmental and age-related changes of peptidylarginine deiminase 2 in the mouse brain. J Neurosci Res (in press)
57. Sorgato MC, Bertoli A (2009) From cell protection to death: may Ca2+ signals explain the chameleonic attributes of the mammalian prion protein? Biochem Biophys Res Commun 379:171–174
58. Tarcsa E, Marekov LN, Mei G, Melino G, Lee SC, Steinert PM (1996) Protein unfolding by peptidylarginine deiminase. Substrate specificity and structural relationships of the natural substrates trichohyalin and filaggrin. J Biol Chem 271:30709–30716
59. Vossenaar ER, Radstake TR, van der Heijden A et al (2004) Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages. Ann Rheum Dis 63:373–381
60. Vossenaar ER, Zendman AJ, van Venrooij WJ, Pruijn GJ (2003) PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. Bioessays 25:1106–1118
61. Wang Y, Wysocka J, Sayegh J et al (2004) Human PAD4 regulates histone arginine methylation levels via demethylimination. Science 306:279–283
62. Wood DD, Ackerley CA, Brand B et al (2008) Myelin localization of peptidylarginine deiminases 2 and 4: comparison of PAD2 and PAD4 activities. Lab Invest 88:354–364
63. Wood DD, Moscarello MA (1989) The isolation, characterization, and lipid-aggregating properties of a citrulline containing myelin basic protein. J Biol Chem 264:5121–5127
64. Young DS, Meersman F, Oxley D et al (2009) Effect of enzymatic deimination on the conformation of recombinant prion protein. Biochim Biophys Acta 1794:1123–1133
65. Zonta M, Angulo MC, Gobbo S et al (2003) Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. Nat Neurosci 6:43–50

Geriatr Gerontol Int 2010; **10 (Suppl. 1):** *S53–S58*

REVIEW ARTICLE

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

Akihito Ishigami[1] and Naoki Maruyama[2]

[1]*Department of Biochemistry, Faculty of Pharmaceutical Sciences, Toho University, Chiba, and* [2]*Aging Regulation, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan*

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.

Accepted for publication 20 November 2009.

Correspondence: Dr Akihito Ishigami, PhD, Department of Biochemistry, Faculty of Pharmaceutical Sciences, Toho University, Miyama 2-2-1, Funabashi, Chiba 274-8510, Japan. Email: ishigami@phar.toho-u.ac.jp

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

doi: 10.1111/j.1447-0594.2010.00593.x

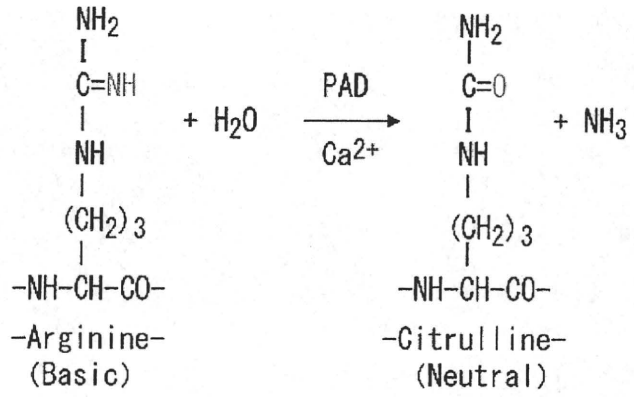

Figure 1 Conversion of arginine residues in proteins to citrulline residues catalyzed by peptidylarginine deiminase. Calcium ion is essential for enzyme activation.

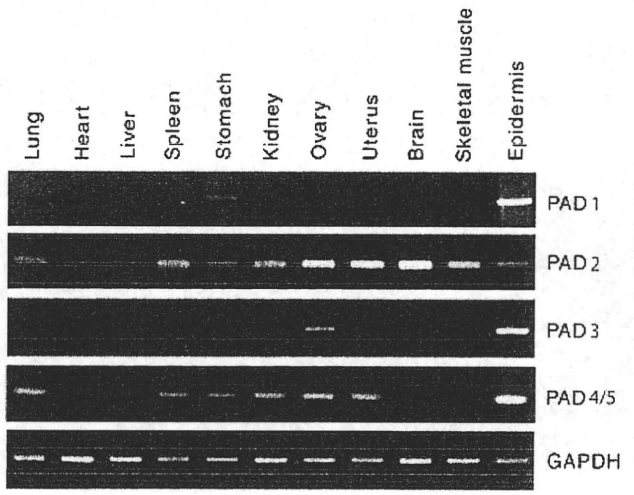

Figure 2 Expression of PAD1, PAD2, PAD3 and PAD4/5 transcripts in various rat tissues analyzed by RT-PCR.[18] Expected sizes were 631 bp for rat PAD1, 428 bp for rat PAD2, 648 bp for PAD3, 205 bp for PAD4/5 and 788 bp for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

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

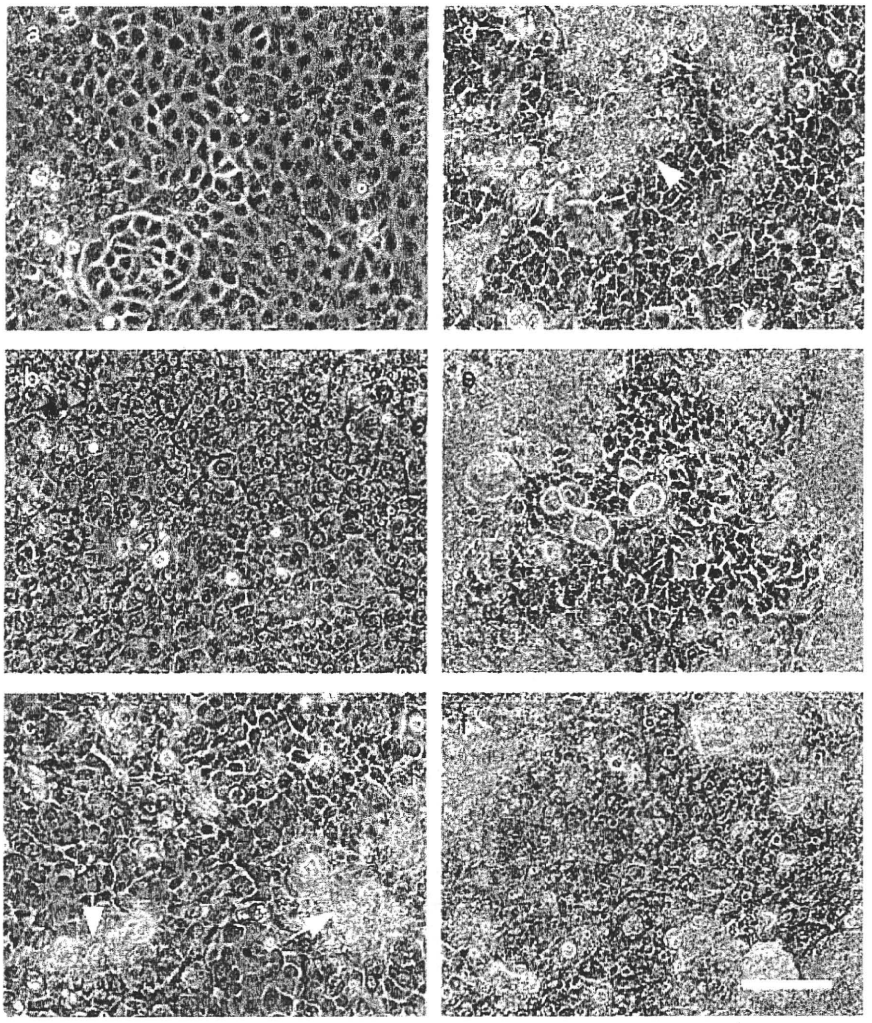

Figure 3 Morphological changes during cultivation of an epidermal keratinocyte line originating from a newborn rat.[26] The initiating inoculum contained 1.3×10^4 cells/cm^2 and cultivation followed for (a) 4 days; (b) 6 days; (c) 8 days; (d) 11 days; (e) 15 days; and (f) 20 days. The arrow indicates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

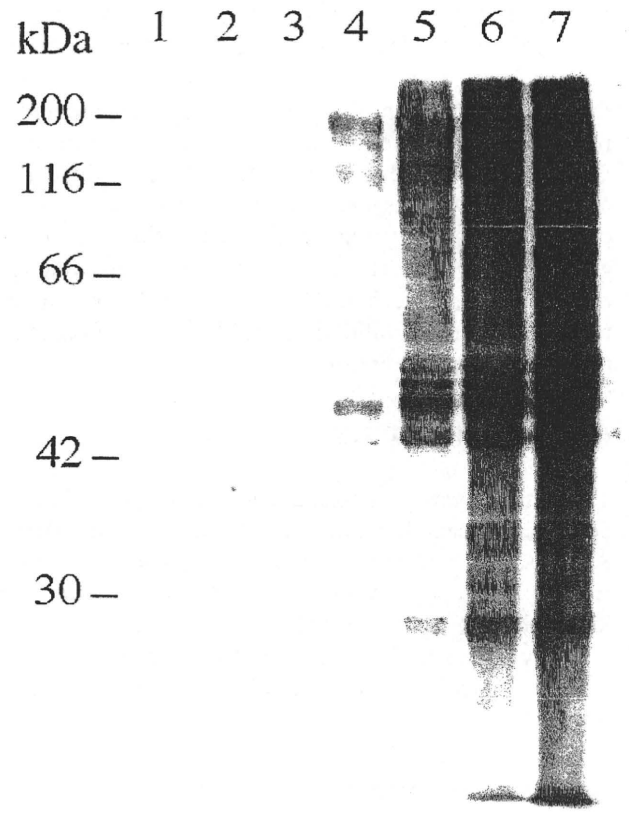

Figure 4 Western blot analysis of citrullinated proteins in keratinocytes during cultivation.[26,35] Lane 1. 4 days; lane 2. 6 days; lane 3, 8 days; lane 4. 11 days; lane 5, 15 days; lane 6, 20 days; and lane 7, 30 days.

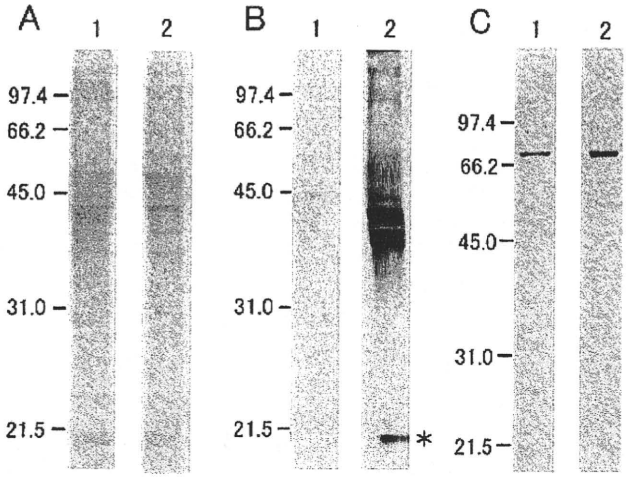

Figure 5 Western blot analysis of citrullinated proteins and PAD2 in hippocampi from the brains of Alzheimer's disease patients and normal controls. (a) Typical protein profiles detected by Amido black staining. (b) Citrullinated protein profiles. (c) Immunoreactive PAD2 profiles. Lane 1, age-matched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

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.

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

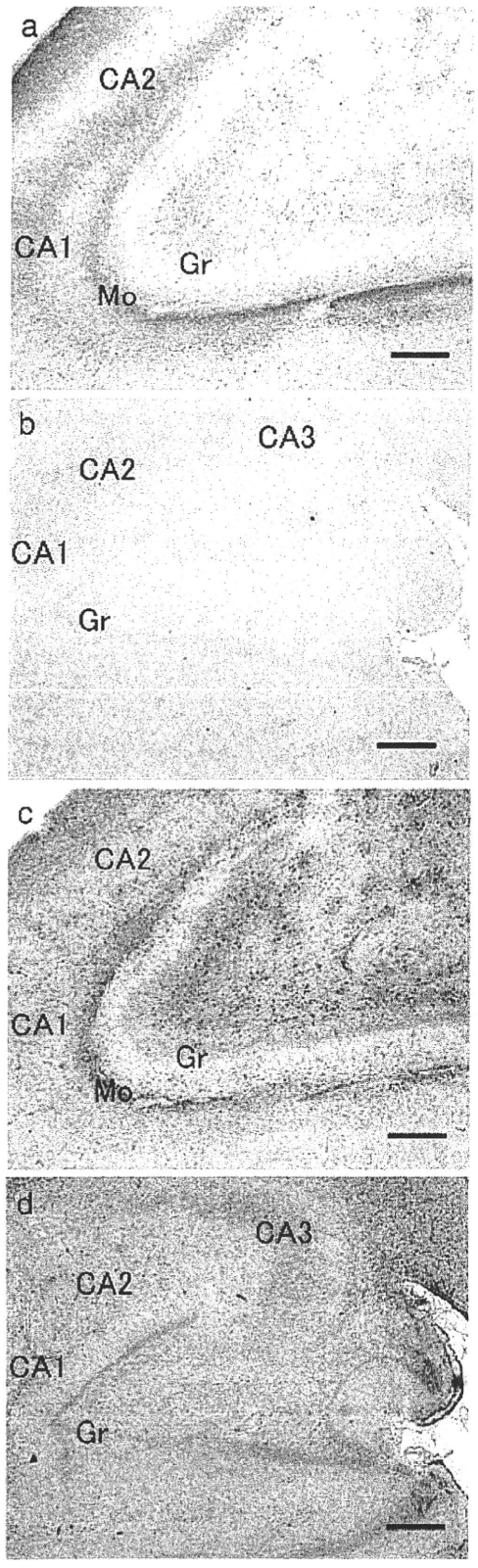

Figure 6 Immunohistochemical staining of citrullinated proteins and PAD2. Hippocampal sections from brains of (a,c) Alzheimer's disease patients and (b,d) controls were stained for (a,b) citrullinated proteins and (c,d) PAD2 (bar, 500 μm). Gr, granule cell layer; Mo, molecular cell layer.

proposed to play a major role in hypoxic and ischemic brain injury.[39,40] Haun *et al.*[41] suggested that an influx of extra cellular calcium contributes to astroglial injury during ischemia on the basis of their experimental results with simulated ischemia in a primary culture of astrocytes. Our reports showed that PAD2 activated and citrullinated various cerebral proteins under hypoxic conditions[30] and during kainic acid-evoked neurodegeneration.[32,33] Clearly, from the weight of evidence now available, abnormal PAD activation that results in random protein citrullination could trigger the onset of neurodegenerative disease.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan (A.I.) and a grant from Health Science Research Grants for Comprehensive Research on Aging and Health supported by the Ministry of Health, Labor and Welfare, Japan (A.I.). The excellent editorial assistance of Ms P Minick is gratefully acknowledged.

Conflicts of interest

None.

References

1 Kubilus J, Waitkus RF, Baden HP. Partial purification and specificity of an arginine-converting enzyme from bovine epidermis. *Biochim Biophys Acta* 1980; **615**: 246–251.
2 Rogers GE, Simmonds DH. Content of citrulline and other amino acids in a protein of hair follicles. *Nature* 1958; **182**: 186–187.
3 Kubilus J, Baden HP. Purification and properties of a brain enzyme which deiminates proteins. *Biochim Biophys Acta* 1983; **745**: 285–291.
4 Ishigami A, Ohsawa T, Watanabe K, Senshu T. All-trans retinoic acid increases peptidylarginine deiminases in a newborn rat keratinocyte cell line. *Biochem Biophys Res Commun* 1996; **223**: 299–303.
5 Imparl JM, Senshu T, Graves DJ. Studies of calcineurin-calmodulin interaction: probing the role of arginine residues using peptidylarginine deiminase. *Arch Biochem Biophys* 1995; **318**: 370–377.
6 Lamensa JW, Moscarello MA. Deimination of human myelin basic protein by a peptidylarginine deiminase from bovine brain. *J Neurochem* 1993; **61**: 987–996.
7 Tarcsa E, Marekov LN, Mei G, Melino G, Lee S-C, Steinert PM. Protein Unfolding by Peptidylarginine Deiminase. Substrate specificity and structural relationships of the natural substrates trichohyalin and filaggrin. *J Biol Chem* 1996; **271**: 30709–30716.
8 Senshu T, Akiyama K, Ishigami A, Nomura K. Studies on specificity of peptidylarginine deiminase reactions using an immunochemical probe that recognizes an enzymatically deiminated partial sequence of mouse keratin K1. *J Dermatol Sci* 1999; **21**: 113–126.

9 Senshu T, Akiyama K, Kan S, Asaga H, Ishigami A, Manabe M. Detection of deiminated proteins in rat skin: probing with a monospecific antibody after modification of citrulline residues. *J Invest Dermatol* 1995; **105**: 163–169.

10 Ishigami A, Ohsawa T, Hiratsuka M *et al.* Abnormal accumulation of citrullinated proteins catalyzed by peptidylarginine deiminase in hippocampal extracts from patients with Alzheimer's disease. *J Neurosci Res* 2005; **80**: 120–128.

11 Watanabe K, Akiyama K, Hikichi K, Ohtsuka R, Okuyama A, Senshu T. Combined biochemical and immunochemical comparison of peptidylarginine deiminases present in various tissues. *Biochim Biophys Acta* 1988; **966**: 375–383.

12 Terakawa H, Takahara H, Sugawara K. Three types of mouse peptidylarginine deiminase: characterization and tissue distribution. *J Biochem (Tokyo)* 1991; **110**: 661–666.

13 Ishigami A, Kuramoto M, Yamada M, Watanabe K, Senshu T. Molecular cloning of two novel types of peptidylarginine deiminase cDNAs from retinoic acid-treated culture of a newborn rat keratinocyte cell line. *FEBS Lett* 1998; **433**: 113–118.

14 Tsuchida M, Takahara H, Minami N *et al.* cDNA nucleotide sequence and primary structure of mouse uterine peptidylarginine deiminase. Detection of a 3'-untranslated nucleotide sequence common to the mRNA of transiently expressed genes and rapid turnover of this enzyme's mRNA in the estrous cycle. *Eur J Biochem* 1993; **215**: 677–685.

15 Watanabe K, Senshu T. Isolation and characterization of cDNA clones encoding rat skeletal muscle peptidylarginine deiminase. *J Biol Chem* 1989; **264**: 15255–15260.

16 Nishijyo T, Kawada A, Kanno T, Shiraiwa M, Takahara H. Isolation and molecular cloning of epidermal- and hair follicle-specific peptidylarginine deiminase (type III) from rat. *J Biochem (Tokyo)* 1997; **121**: 868–875.

17 Rus'd AA, Ikejiri Y, Ono H *et al.* Molecular cloning of cDNAs of mouse peptidylarginine deiminase type I, type III and type IV, and the expression pattern of type I in mouse. *Eur J Biochem* 1999; **259**: 660–669.

18 Ishigami A, Asaga H, Ohsawa T, Akiyama K, Maruyama N. Peptidylarginine deiminase type I, type II, type III and type IV are expressed in rat epidermis. *Biomed Res* 2001; **22**: 63–65.

19 Guerrin M, Ishigami A, Mechin MC *et al.* cDNA cloning, gene organization and expression analysis of human peptidylarginine deiminase type I. *Biochem J* 2003; **370**: 167–174.

20 Ishigami A, Ohsawa T, Asaga H, Akiyama K, Kuramoto M, Maruyama N. Human peptidylarginine deiminase type II: molecular cloning, gene organization, and expression in human skin. *Arch Biochem Biophys* 2002; **407**: 25–31.

21 Kanno T, Kawada A, Yamanouchi J *et al.* Human peptidylarginine deiminase type III: molecular cloning and nucleotide sequence of the cDNA, properties of the recombinant enzyme, and immunohistochemical localization in human skin. *J Invest Dermatol* 2000; **115**: 813–823.

22 Nakashima K, Hagiwara T, Ishigami A *et al.* Molecular characterization of peptidylarginine deiminase in HL-60 cells induced by retinoic acid and 1alpha,25-dihydroxyvitamin D(3). *J Biol Chem* 1999; **274**: 27786–27792.

23 Chavanas S, Mechin MC, Takahara H *et al.* Comparative analysis of the mouse and human peptidylarginine deiminase gene clusters reveals highly conserved non-coding segments and a new human gene, PADI6. *Gene* 2004; **330**: 19–27.

24 Asaga H, Nakashima K, Senshu T, Ishigami A, Yamada M. Immunocytochemical localization of peptidylarginine deiminase in human eosinophils and neutrophils. *J Leukocyte Biol* 2001; **70**: 46–51.

25 Baden HP, Kubilus J. The growth and differentiation of cultured newborn rat keratinocytes. *J Invest Dermatol* 1983; **80**: 124–130.

26 Ishigami A, Asaga H, Ohsawa T, Akiyama K, Maruyama N. Protein deimination and peptidylarginine deiminase expression during cornification of rat epidermal keratinocytes. *Biomed Res* 2002; **23**: 145–151.

27 Resing KA, al-Alawi N, Blomquist C, Fleckman P, Dale BA. Independent regulation of two cytoplasmic processing stages of the intermediate filament-associated protein filaggrin and role of Ca2+ in the second stage. *J Biol Chem* 1993; **268**: 25139–25145.

28 Keller JN, Hanni KB, Markesbery WR. Impaired proteasome function in Alzheimer's disease. *J Neurochem* 2000; **75**: 436–439.

29 Maccioni RB, Munoz JP, Barbeito L. The molecular bases of Alzheimer's disease and other neurodegenerative disorders. *Arch Med Res* 2001; **32**: 367–381.

30 Asaga H, Ishigami A. Protein deimination in the rat brain: generation of citrulline-containing proteins in cerebrum perfused with oxygen-deprived media. *Biomed Res* 2000; **21**: 197–205.

31 Vincent SR, Leung E, Watanabe K. Immunohistochemical localization of peptidylarginine deiminase in the rat brain. *J Chem Neuroanat* 1992; **5**: 159–168.

32 Asaga H, Ishigami A. Protein deimination in the rat brain after kainate administration: citrulline-containing proteins as a novel marker of neurodegeneration. *Neurosci Lett* 2001; **299**: 5–8.

33 Asaga H, Akiyama K, Ohsawa T, Ishigami A. Increased and type II-specific expression of peptidylarginine deiminase in activated microglia but not hyperplastic astrocytes following kainic acid-evoked neurodegeneration in the rat brain. *Neurosci Lett* 2002; **326**: 129–132.

34 Akiyama K, Sakurai Y, Asou H, Senshu T. Localization of peptidylarginine deiminase type II in a stage-specific immature oligodendrocyte from rat cerebral hemisphere. *Neurosci Lett* 1999; **274**: 53–55.

35 Senshu T, Sato T, Inoue T, Akiyama K, Asaga H. Detection of citrulline residues in deiminated proteins on polyvinylidene difluoride membrane. *Anal Biochem* 1992; **203**: 94–100.

36 Asaga H, Senshu T. Combined biochemical and immunocytochemical analyses of postmortem protein deimination in the rat spinal cord. *Cell Biol Int* 1993; **17**: 525–532.

37 Katzman R. Alzheimer's disease. *N Engl J Med* 1986; **314**: 964–973.

38 Smith MA. Alzheimer disease. *Int Rev Neurobiol* 1998; **42**: 1–54.

39 Hossmann KA. The hypoxic brain. Insights from ischemia research. *Adv Exp Med Biol* 1999; **474**: 155–169.

40 Choi DW. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci* 1988; **11**: 465–469.

41 Haun SE, Murphy EJ, Bates CM, Horrocks LA. Extracellular calcium is a mediator of astroglial injury during combined glucose-oxygen deprivation. *Brain Res* 1992; **593**: 45–50.

REFERENCES

1. Barr RG, Levine MD, Wilkinson RH et al. Chronic and occult stool retention: A clinical tool for its evaluation in school-aged children. Clin Pediatr 1979;18:674–686.
2. Leech SC, McHugh K, Sullivan PB. Evaluation of a method of assessing faecal loading on plain abdominal radiographs in children. Pediatr Radiol 1999;29:255–258.
3. McKay LF, Smith RG, Eastwood MA et al. An investigation of colonic function in the elderly. Age Ageing 1983;12:105–110.
4. Donald IP, Smith RG, Cruikshank JG et al. A study of constipation in the elderly living at home. Gerontology 1985;31:112–118.
5. Smith RG, Lewis S. The relationship between digital rectal examination and abdominal radiographs in elderly patients. Age Ageing 1990;19:142–143.
6. Talley NJ, O'Keefe EA, Zinsmeister AR et al. Prevalence of gastrointestinal symptoms in the elderly: A population-based study. Gastroenterology 1992;102:895–901.
7. Rockney RM, McQuade WH, Days AL. The plain abdominal roentgenogram in the management of encopresis. Arch Pediatr Adolesc Med 1995;149:623–667.
8. Gau JT, Acharya U, Marx T et al. Megacolon and stercoral proctitis after frequent fecal impactions: Report of an unusual case and review of the literature. J Am Med Dir Assoc 2007;8:338–341.
9. Gupta KL. Intestinal obstruction due to constipation in the elderly. Br J Clin Pract 1983;37:155–156.
10. Binkovitz LA, Allen E, Bloom D et al. Atypical presentation of Clostridium difficile colitis in patients with cystic fibrosis. Am J Roentgenol 1999;172: 517–521.

MOOD DISORDERS IN COMMUNITY-DWELLING OLDER ADULTS IN ASIA

To the Editor: The article entitled, "Achieving effective antidepressant pharmacotherapy in primary care: the role of depression care management in treating late-life depression," by Bao and colleagues[1] deeply impressed us and led us to think back to an article entitled, "Reducing suicidal ideation in depressed older primary care patients," by Unützer and colleagues.[2] In Japan, 1998 marks the beginning of the third wave of increased suicides (>30,000 per year), which was preceded by a second (1980–1983) and first (1947–1951) wave.[3] Each wave in Japan was closely associated with periods of economic depression.[4] The third wave of suicides in Japan began soon after the Asian economic crisis of 1997, which was reported to be closely associated with depression and suicide in some Asian countries.[5] The Japanese government has reported that suicides in Japan in 2007 were associated with old age (37%), health concerns (44%), and depression (18%).[3] A screening-based investigation revealed that depression in community-dwelling older adults was closely associated with more difficulty in performing activities of daily living (ADLs) and lower quality of life (QOL) both in Japan[6] and in other Asian communities.[7] Although old age, health problems, and depression may be commingled, the reported prevalence of depression in older adults in Asian countries varied.[8–10]

The findings of a study using screenings and interviews to investigate the prevalence of depression in community-dwelling older adults (aged ≥ 60) in six Asian communities: Urausu, Japan (n = 729); Hong Chong, Korea (n = 329); Phuto district, Vietnam (n = 387); Savannakhet, Laos (n = 294); Maubin, Myanmar (n = 336); and Khon Kaen, Thailand (n = 407) are reported here (Table 1). The surveys were conducted from 2004 to 2007. All participants were first screened using a 15-item Geriatric Depression Scale (GDS) translated into the local language. Japanese psychiatrists interviewed participants with GDS scores of 6 or higher and diagnosed them based on Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria. Statistical analysis was performed using StatView version 5 for Macintosh (SAS institute, Inc., Cary, NC). Analysis of variance was used for continuous variables in six groups, and the chi-square test was used for categorical variables. $P < .05$ was used to indicate statistical significance (analysis of variance comparing multiple variance).

Table 1 shows mean GDS scores, prevalence of participants with GDS scores of 6 and higher and of 10 and higher and prevalence of mood disorders diagnosed based on DSM-IV criteria. Although mean GDS score, the prevalence of participants with GDS scores of 6 and higher, and prevalence with GDS scores of 10 and higher varied between communities, the prevalence of elderly participants diagnosed with mood disorders was similar (6.2–9.6%). Many elderly people with depression had subjective experiences of illness, but few elderly participants in the surveyed population, including Japan, consulted psychiatrists or took antidepressant medications.

Table 1. Comparison of Geriatric Depression Scale Scores and Prevalence of Mood Disorders of Community-Dwelling Older Adults in Six Asian Communities

Variable	Urausu Japan (N = 728)	Hong Chong Korea (N = 329)	Phuto Vietnam (N = 387)	Savannakhet Laos (N = 294)	Maubin Myanmar (N = 336)	Khon Kaen Thailand (N = 407)	P-# value
Age, mean ± SD	74.4 ± 7.1	72.3 ± 6.1	70.8 ± 8.1	69.6 ± 7.6	70.3 ± 7.0	68.4 ± 6.7	<.001
Male/female, n/n	325/403	180/149	175/212	121/173	175/161	265/141	<.001
Geriatric Depression Scale (GDS) score (range 0–15)							
Mean ± SD	4.4 ± 3.2	5.4 ± 4.2	3.4 ± 2.7	5.5 ± 2.8	3.7 ± 3.1	4.1 ± 3.1	<.001
≥ 6, %	42	40	17	36	23	27	<.001
≥ 10, %	8	20.5	7.6	6.5	7.6	9	<.001
Prevalence of mood disorders diagnosed by psychiatrists, %*	9.0	9.6	7.2	7.8	6.7	6.2	<.001

*Based on Diagnostic and Statistical Manual of Mental Disorders Fourth Edition.

SD = standard deviation.

#*P*-values were calculated by using analysis of variance (ANOVA) for continuous variables and chi-square tests for categorical variables. ANOVA gave a statistical test of whether the means (mean age or score in GDS) of several groups were all equal, and therefore generalized Student's two-sample t-test to more than two groups.

Although a study population that is not necessarily representative enough to compare the prevalence of depression cross-culturally between communities limits these data, 6.2% to 9.6% of elderly participants in this study were diagnosed with mood disorders based on DSM-IV criteria. More attention should be given to the practical application of standard criteria for screening and diagnosis of depression, which will be beneficial for early detection and consideration of follow-up and intervention in elderly patients with mood disorders in primary care and community settings in Asia.

Kozo Matsubayashi, MD, PhD
Center for Southeast Asian Studies
Kyoto University
Kyoto, Japan

Teiji Sakagami, MD
Department of Psychiatry
Otsu Red-Cross Hospital
Otsu, Japan

Taizo Wada, MD, PhD
Center for Southeast Asian Studies
Kyoto University
Kyoto, Japan

Masayuki Ishine, MD
Ryota Sakamoto, MD, PhD
Research Institute for Humanity and Nature
Kyoto, Japan

Gaku Yamanaka, MD, PhD
Kuniaki Otsuka, MD, PhD
Medical Center East
Tokyo Women's Medical University
Tokyo, Japan

Michiko Fujisawa, MD, PhD
Wildlife Research Center
Kyoto University
Kumamoto, Japan

Kiyohito Okumiya, MD, PhD
Research Institute for Humanity and Nature
Kyoto, Japan

ACKNOWLEDGMENTS

We cordially thank all of the elderly participants in each community. Field medical work and findings in each community were partly supported by the Grant-in-Aid of Japan Society of the Promotion of Science Global Centers of Excellence Program (E-04, In Search of Sustainable Humanosphere in Asia and Africa) and by a research project of Human Life, Aging, Disease in High Altitude Environments: Physiological-medical, ecological and cultural adaptation in great "highland civilizations" in Research Institute for the Humanity and Nature.

Conflict of Interest: The editor in chief has reviewed the conflict of interest checklist provided by the authors and has determined that the authors have no financial or any other kind of personal conflicts with this paper.

Author Contributions: KM, TS: study design and preparation of the article. KM, TS, TW, MI, RS, GY, KO, MF, KO: participation in field studies and analysis, interpretation, and discussion of the data.

Sponsor's Role: None.

REFERENCES

1. Bao Y, Post EP, Ten TR et al. Achieving effective antidepressant pharmacotherapy in primary care: The role of depression care management in treating late-life depression. J Am Geriatr Soc 2009;57:895–900.
2. Unützer J, Tang L, Oishi S et al. for the IMPACT Investigators. Reducing suicidal ideation in depressed older primary care patients. J Am Geriatr Soc 2006;54:1550–1556.
3. Japanese Ministry of Health Labour and Welfare. 2009 [on-line]. Available at http://www.mhlw.go.jp/ (in Japanese Accessed June 20–23, 2009).
4. Ikeda K, Ito K Precise analysis of suicide in Japan. 1999 [on-line]. Available at http://www.tokyo-eiken.go.jp/SAGE/SAGE99/jisatsu.pdf (in Japanese Accessed June 20–23, 2009).
5. Chang SS, Gunnell D, Sterne JA et al. Was the economic crisis 1997–1998 responsible for rising suicide rates in East/Southeast Asia? A time-trend analysis for Japan, Hong Kong, South Korea, Taiwan, Singapore and Thailand. Soc Sci Med 2009;68:1322–1331.
6. Wada T, Ishine M, Kita T et al. Depression screening of elderly community-dwelling Japanese. J Am Geriatr Soc 2003;51:1328–1329.
7. Wada T, Ishine M, Sakagami T et al. Depression, activities of daily living, and quality of life of community-dwelling elderly in three Asian countries: Indonesia, Vietnam, and Japan. Arch Gerontol Geriatr 2005;41:271–280.
8. Shin KR, Jung D, Jo I et al. Depression among community-dwelling older adults in Korea: A prediction model of depression. Arch Psychiatr Nurs 2009;23:50–57.
9. Rajkumar AP, Thangadurai P, Senthilkumar P et al. Nature, prevalence and factors associated with depression among the elderly in a rural south Indian community. Int Psychogeriatr 2009;21:372–378.
10. Phillips MR, Zhang J, Shi Q et al. Prevalence, treatment, and associated disability of mental disorders in four provinces in China during 2001–05: An epidemiologic survey. Lancet 2009;373:2041–2053.

Geriatrics Gerontology International — 1st Impact Factor in June 2010 — Medline Indexed

Geriatr Gerontol Int 2010; **10:** *113–114*

LETTER TO THE EDITOR

Farsightedness (presbyopia) in a wild elderly chimpanzee: The first report

Michiko Fujisawa,[1] Kozo Matsubayashi,[2] Aly Gaspard Soumah,[3] Yoriko Kasahara,[4] Masahiro Nakatsuka[5] and Tetsuro Matsuzawa[6]

[1]Wildlife Research Center, Kyoto University, Kumamoto, [2]Center for Southeast Asian Studies, [4]Department of Field Medicine, Graduate School of Public Health, [5]Human Brain Research Center, Kyoto University, Kyoto, [6]Primates Research Institute, Kyoto University, Aichi, Japan; and [3]Institute of Environmental Research at Bossou, Bossou, Guinea

Dear Editor,

The chimpanzee (*Pan troglodytes*) is one of four genera in the family Hominidae, which also includes humans, gorillas and orangutans. Approximately 340 chimpanzees live in captivity in Japan and of these, only two chimpanzees are estimated to be older than 50 years old while the chimpanzees in the wild live for approximately 50 years.[1] The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) was ratified in 1980 in Japan, so the captive study of chimpanzee elders is limited. Important differences exist between chimpanzees raised in the wild and those raised in captivity. For example, chimpanzees born in the wild acquire social skills and knowledge from their mothers and the elder members of the community, while chimpanzees born in captivity and raised by humans do not have the ability to communicate well with other chimpanzees. As a result, chimpanzees raised by humans frequently abandon their babies when they become mothers. In that sense, it is very important for the chimpanzees to be grown up in a community with elder members. From the standpoint of research on the biology of human aging, it is important to observe aging in chimpanzees, who are the closest evolutionary relatives of humans, especially in a wild setting. Observing research on behavior of elderly chimpanzees in wild settings may be useful not only in the preservation of this endangered species but also in detecting geriatric syndrome in the chimpanzee related to that in human beings.

In the Bossou district in Guinea, West Africa, a group of 13 wild chimpanzees has been studied since 1976. Each individual in the group has been identified and named, and its age has been confirmed or estimated. These chimpanzees coexist with humans in the primary and the secondary forests near the small hills surrounding the village of Bossou.[2] Bossou chimpanzees sometimes raid the farms and orchards to eat cultivated fruits. However, the chimpanzees have no predators in this area and they are not hunted by the villagers. Thanks to the long-term research, Bossou chimpanzees are fully habituated to humans and we have the record of each individual in the past four decades.[2] Six of these chimpanzees are estimated to be more than 40 years old, and three are believed to be at least 50 years old.

From 23–30 December 2008, we carried out the intensive observation focusing on the old female chimpanzee named Jire. She was estimated to be 50 years old. We did the focal animal sampling following the particular individuals at close range, from early morning till the time of sleeping in the beds in the tree, approximately 12 h a day. During the observation, we focused on the grooming behavior, a form of communication in chimpanzees.[3] In terms of the grooming skill, the eye–hand coordination is very important. We carried out a quantitative observational study of behaviors of the chimpanzee every 5 min. Nineteen grooming scenes of Jire were recorded among a total 862 records of her behavior, of which 104 were missed records.

Following the focal observation, we noticed that the female chimpanzee named Jire groomed her daughter Joya with her eyes focusing at a distance of 40–50 cm. This makes a clear contrast to the younger chimpanzee Foaf (aged 27 years) that groomed with his eyes focusing directly on and close to (10–20 cm) the grooming spot (Fig. 1). Distance estimation of grooming eye spot was measured by video-photo analysis. Other old chimpanzees aged approximately 50 years or over also groomed with their eyes focusing at a longer distance than younger ones. Because chimpanzees remove very small

Author contributions: all authors participated in the research in Bossou, Guinea in 2008–2009, and discussed the findings.

doi: 10.1111/j.1447-0594.2009.00575.x

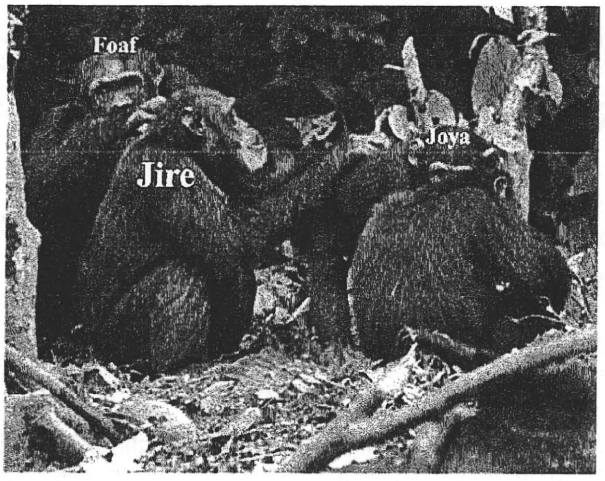

Figure 1 Jire (~50 years old) kept her eyes focused at a longer distance (40–50 cm) with her arm extended while grooming her daughter Joya, on the other hand Foaf (28 years old) kept his eyes focused closely (10–20 cm) while grooming Jire.

insects like lice from fur during grooming, they typically focus their eyes closely.[4] The previous study reported that the visual acuity of the chimpanzee is approximately 1.5, comparable to humans.[5] Thus, we strongly suspected that the grooming behavior of the old chimpanzee must be a symptom of farsightedness (i.e. experienced presbyopia) like an elderly human.

Hearing disturbance[6] and chewing ability[7] are closely associated with lower activities of daily living and with lower quality of life in community-dwelling human elderly. Presbyopia is caused by a decline in the accommodation of the lens. Visual impairment in humans is associated with social isolation and increased risk of depression,[8] and presbyopia correction with an assistive device has been reported to improve human disability and depression.[9] The similarity of accommodative mechanisms between human and monkeys have been reported by some researchers in ophthalmo-kinetic examinations, but not in clinical or field-setting study.[10] Bito *et al.* has reported the use of the rhesus monkey as an animal model for presbyopia.[11] However, the underlying mechanisms of presbyopia development in monkeys are thought to differ from those of humans.[12] In monkeys, presbyopia is recognized only by examining lens thickness, intraocular pressure, accommodative amplitude and other ocular dimensions, but clinical or field-setting symptoms remain unknown. Based on our observations, we believe that old chimpanzees must have developed presbyopia. This may be the first report of the observation of clinical presbyopia in chimpanzees in the wild. Although presbyopia in chimpanzees has only been observed to disrupt grooming behavior until now, the future study on the age-related physiological decline and dealing with a disabled state in chimpanzees may provide a clue for understanding human geriatrics and gerontology.

Acknowledgments

We cordially thank all native researching assistants in Bossou for their helping our close observation. We also thank Ms S Carvalho for her useful advice.

References

1 Emery Thompson M, Jones JH, Pusey AE *et al.* Aging and fertility patterns in wild chimpanzees provide insights into evolution of menopause. *Curr Biol* 2007; **17**: 2150–2156.
2 Hockings KJ, Humle T, Anderson JR *et al.* Chimpanzees share forbidden fruit. *PLoS ONE* 2007; **2**: e886.
3 Sugiyama Y. Grooming interactions among adult chimpanzees at Bossou, Guinea, with special reference to social structure. *Int J Primatol* 1988; **9**: 393–407.
4 Zamma K. Leaf-grooming by a wild chimpanzee in Mahale. *Primates* 2002; **43**: 87–90.
5 Matsuzawa T. Form perception and visual acuity in a chimpanzee. *Folia Priomatol* 1990; **55**: 24–32.
6 Ishine M, Okumiya K, Matsubayashi K. A close association between hearing impairment and activities of daily living, depression, and quality of life in community-dwelling older people in Japan. *J Am Geriatr Soc* 2007; **55**: 316–317.
7 Kimura Y, Wada T, Ishine M *et al.* Community-dwelling elderly with chewing difficulties are more disabled, depressed and have lower quality of life scores. *Geriat Gerontol Int* 2009; **9**: 102–104.
8 Carabellese C, Appollonio I, Rozzini R *et al.* Sensory impairment and quality of life in a community elderly population. *J Am Geriatr Soc* 1993; **41**: 401–407.
9 Horowitz A, Brennan M, Reinhardt JP, Macmillan T. The impact of assistive device use on disability and depression among older adults with age-related vision impairments. *J Gerontol B Psychol Sci Soc Sci* 2006; **61**: S274–S280.
10 Glasser A, Kaufman PL. The mechanism of accommodation in primates. *Ophthalmology* 1999; **106**: 863–872.
11 Bito LZ, DeRousseau CJ, Kaufman PL, Bito JW. Age-dependent loss of accommodative amplitude in rhesus monkeys: an animal model for presbyopia. *Invest Ophthalmol Vis Sci* 1982; **23**: 23–31.
12 Strenk SA, Strenk LM, Koretz JF. The mechanism of presbyopia. *Prog Retin Eye Res* 2005; **24**: 379–393.

cases.[4] The mainstay of management is medical treatment and discontinuing the anticoagulant drugs, bowel rest, correction of PT with intravenous vitamin K with fresh-frozen plasma, and correction of anemia if present.[5,6] Surgical intervention is indicated only if there is significant intramural hemorrhage, bowel perforation, ischemia, or peritonitis.[4–6] Efforts to measure the quality of medication use in elderly patients have traditionally focused on inappropriate medications and doses, although a more-comprehensive approach to measuring the quality of medication use in elderly patients has been recommended.[7] The most important determinant of risk for adverse drug reaction–related hospital admission in older patients is the number of drugs being taken. When considering only severe adverse drug reactions, risk is also related to age and frailty.[8]

In a previous study, poor adherence of elderly patients was responsible for 31% of overanticoagulation cases.[9] These admissions could potentially be avoided with better anticoagulation control. Long-term warfarin use requires close monitoring of the coagulation profile to prevent this complication. Physician and patient awareness of the risk of bleeding when using warfarin is especially important for elderly patients. Clearer advice to older patients on the risk of nonadherence is important in such case.

Min-Po Ho, MD
Kuang-Chau Tsai, MD
Department of Emergency Medicine

Wing-Keung Cheung, MD
Kao-Lun Wang, MD
Department of Medical Imaging
Far Eastern Memorial Hospital, Taipei, Taiwan

ACKNOWLEDGMENTS

Conflict of Interest: The editor in chief has reviewed the conflict of interest checklist provided by the authors and has determined that the authors have no financial or any other kind of personal conflicts with this paper.

Author Contributions: Concept and design: Min-Po Ho, Wing-Keung Cheung. Acquisition of subjects and data: Kuang-Chau Tsai, Min-Po Ho, Wing-Keung Cheung. Analysis and interpretation of data: Min-Po Ho and Kao-Lun Wang. Preparation of manuscript: Min-Po Ho, Wing-Keung Cheung. Critical review and approval: All authors.

Sponsor's Role: None.

REFERENCES

1. Hirsh J. Oral anticoagulant drugs. N Engl J Med 1991;324:1865–1875.
2. Palareti G, Leali N, Coccheri S et al. Bleeding complications of oral anticoagulant treatment: An inception-cohort, prospective collaborative study (ISCOAT). Italian study on complications of anticoagulant therapy. Lancet 1996; 348:423–428.
3. Polat C, Dervisoglu A, Guven H et al. Anticoagulant-induced intramural intestinal hematoma. Am J Emerg Med 2003;21:208–211.
4. Eiland M, Han SY, Hicks GM. Intramural hemorrhage of the small intestine. JAMA 1978;239:139–142.
5. Abbas MA, Collins JM, Olden KW. Spontaneous intramural small bowel hematoma: Clinical presentation and long-term outcome. Arch Surg 2002;137: 306–310.
6. Jones WR, Haridin WJ, Davis JT et al. Intramural hematoma of the duodenum: A review of the literature and case report. Ann Surg 1971;173:534–544.
7. Roth MT, Weinberger M, Campbell WH. Measuring the quality of medication use in older adults. J Am Geriatr Soc 2009;57:1096–1102.
8. Onder G, Pedone C, Landi F et al. Adverse drug reactions as a cause of hospital admissions: Results from Italian Group of Pharmocoepidemiology in the elderly (GIFA). J Am Geriatr Soc 2002;50:1962–1968.
9. Hirri HM, Green PJ. Audit of anticoagulant therapy and acute hospital admissions. Clin Lab Haemotol 2002;24:43–45.

STRONG ASSOCIATION BETWEEN POLYCYTHEMIA AND GLUCOSE INTOLERANCE IN ELDERLY HIGH-ALTITUDE DWELLERS IN ASIA

To the Editor: Human longevity and aging of high-altitude dwellers remains to be fully clarified.[1] A high prevalence of diabetes mellitus (DM) and hypertension in elderly highlanders, particularly those living in rapidly modernized highland areas in Asia, because of the influence of socioeconomic globalization, was previously reported.[2] Highlanders have biologically adapted to hypoxic environments by various genetic mechanisms such as an increase in hemoglobin concentration or increased blood flow.[3] Elderly people living at high altitudes are less likely to adapt, and some of them suffer from chronic mountain sickness, characterized by excessive polycythemia, which is regarded as an exaggeration of the normal adaptation to altitude.[4] In addition, highlanders have culturally adapted to the low availability of natural food resources, although it remains unknown whether elderly highlanders are more vulnerable to lifestyle-related diseases associated with rapidly changing and modernizing environments. To address this important issue in a cross-sectional study, the association between glucose intolerance and polycythemia in elderly people living in two highland areas in Asia was investigated.

The study population comprised consecutive elderly volunteers aged 60 and older who took part in medical camps held in two highland communities. The population included 209 Tibetans (mean age 66.1; 87 men, 122 women) living in Jiegu Town in Yushu County, Qinghai, China (3,700 m) and 117 Ladakhi (mean age 69.3 years, 48 men, 69 women) living in Domkhar Village, Ladakh, India (2,900–3,800 m). Yushu has modernized more rapidly than Ladakh.[2] Participants underwent a 75-g oral glucose tolerance test OGTT to screen for DM or impaired glucose tolerance (IGT) based on World Health Organization criteria.[5] Polycythemia was defined as a hemoglobin level of 18 g/dL or higher for men and 16 g/dL or higher for women. Table 1 shows the baseline characteristics of and markers for lifestyle-related diseases, such as DM and IGT, for elderly subjects with or without polycythemia in both regions. The prevalence of polycythemia was higher in elderly people in Yushu (45%) than in Domkhar (31%) ($P = .009$). Mean hemoglobin concentrations in elderly people without and with polycythemia were 14.1 and 17.6 g/dL, respectively, in Domkhar and 15.3 and 17.6 g/dL, respectively, in Yushu.

The mean age of elderly subjects was higher in Domkhar than in Yushu, but there was no difference in mean age between elderly subjects with and without polycythemia in each area.

There was no difference in saturation of peripheral oxygen (SpO_2) between elderly subjects in Domkhar (88.8%) and those in Yushu (89.2%), because all were examined at the same altitude of 3,700 m. Mean SpO_2 was