

- 464 7. Baker, C. A., Z. Y. Lu, and L. Manuelidis. 2004. Early induction of
 465 interferon-responsive mRNAs in Creutzfeldt-Jakob disease. *J*
 466 *Neurovirol* 10:29-40.
- 467 8. Beutler, B., Z. Jiang, P. Georgel, K. Crozat, B. Croker, S. Rutschmann,
 468 X. Du, and K. Hoebe. 2006. Genetic analysis of host resistance:
 469 Toll-like receptor signaling and immunity at large. *Annu Rev*
 470 *Immunol* 24:353-389.
- 471 9. Brown, K. L., D. L. Ritchie, P. A. McBride, and M. E. Bruce. 2000.
 472 Detection of PrP in extraneural tissues. *Microsc Res Tech* 50:40-45.
- 473 10. Daffis, S., M. A. Samuel, B. C. Keller, M. Gale, Jr., and M. S. Diamond.
 474 2007. Cell-specific IRF-3 responses protect against West Nile virus
 475 infection by interferon-dependent and -independent mechanisms.
 476 *PLoS pathogens* 3:e106.
- 477 11. Delhaye, S., S. Paul, G. Blakqori, M. Minet, F. Weber, P. Staeheli, and
 478 T. Michiels. 2006. Neurons produce type I interferon during viral
 479 encephalitis. *Proc Natl Acad Sci U S A* 103:7835-7840.
- 480 12. Doehle, B. P., F. Hladik, J. P. McNevin, M. J. McElrath, and M. Gale,
 481 Jr. 2009. Human immunodeficiency virus type 1 mediates global
 482 disruption of innate antiviral signaling and immune defenses within
 483 infected cells. *J Virol* 83:10395-10405.
- 484 13. Field, E. J., G. Joyce, and A. Keith. 1969. Failure of interferon to
 485 modify scrapie in the mouse. *J Gen Virol* 5:149-150.
- 486 14. Field, R., S. Champion, C. Warren, C. Murray, and C. Cunningham.
 487 Systemic challenge with the TLR3 agonist poly I:C induces amplified
 488 IFNalpha/beta and IL-1beta responses in the diseased brain and
 489 exacerbates chronic neurodegeneration. *Brain Behav Immun*
 490 24:996-1007.
- 491 15. Goodbourn, S., L. Didcock, and R. E. Randall. 2000. Interferons: cell
 492 signalling, immune modulation, antiviral response and virus
 493 countermeasures. *J Gen Virol* 81:2341-2364.
- 494 16. Gresser, I., C. Maury, and R. L. Chandler. 1983. Failure to modify
 495 scrapie in mice by administration of interferon or anti-interferon
 496 globulin. *J Gen Virol* 64 (Pt 6):1387-1389.

- 497 17. Haritani, M., Y. I. Spencer, and G. A. Wells. 1994. Hydrated autoclave
 498 pretreatment enhancement of prion protein immunoreactivity in
 499 formalin-fixed bovine spongiform encephalopathy-affected brain. *Acta*
 500 *Neuropathol* 87:86-90.
- 501 18. Hidmark, A. S., G. M. McInerney, E. K. Nordstrom, I. Douagi, K. M.
 502 Werner, P. Liljestrom, and G. B. Karlsson Hedestam. 2005. Early
 503 alpha/beta interferon production by myeloid dendritic cells in response
 504 to UV-inactivated virus requires viral entry and interferon regulatory
 505 factor 3 but not MyD88. *Journal of virology* 79:10376-10385.
- 506 19. Hiscott, J. 2007. Triggering the innate antiviral response through
 507 IRF-3 activation. *The Journal of biological chemistry*
 508 282:15325-15329.
- 509 20. Honda, K., and T. Taniguchi. 2006. IRFs: master regulators of
 510 signalling by Toll-like receptors and cytosolic pattern-recognition
 511 receptors. *Nat Rev Immunol* 6:644-658.
- 512 21. Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N.
 513 Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi. 2005.
 514 IRF-7 is the master regulator of type-I interferon-dependent immune
 515 responses. *Nature* 434:772-777.
- 516 22. Karpova, A. Y., P. M. Howley, and L. V. Ronco. 2000. Dual utilization of
 517 an acceptor/donor splice site governs the alternative splicing of the
 518 IRF-3 gene. *Genes Dev* 14:2813-2818.
- 519 23. Katze, M. G., Y. He, and M. Gale, Jr. 2002. Viruses and interferon: a
 520 fight for supremacy. *Nat Rev Immunol* 2:675-687.
- 521 24. Kim, T., T. Y. Kim, Y. H. Song, I. M. Min, J. Yim, and T. K. Kim. 1999.
 522 Activation of interferon regulatory factor 3 in response to
 523 DNA-damaging agents. *The Journal of biological chemistry*
 524 274:30686-30689.
- 525 25. Kitamoto, T., K. Ogomori, J. Tateishi, and S. B. Prusiner. 1987. Formic
 526 acid pretreatment enhances immunostaining of cerebral and systemic
 527 amyloids. *Lab Invest* 57:230-236.
- 528 26. Liu, W. G., D. A. Brown, and J. R. Fraser. 2003. Immunohistochemical
 529 comparison of anti-prion protein (PrP) antibodies in the CNS of mice

- 530 infected with scrapie. *J Histochem Cytochem* 51:1065-1071.
- 531 27. Nishida, N., D. A. Harris, D. Vilette, H. Laude, Y. Frobert, J. Grassi, D.
 532 Casanova, O. Milhavet, and S. Lehmann. 2000. Successful
 533 transmission of three mouse-adapted scrapie strains to murine
 534 neuroblastoma cell lines overexpressing wild-type mouse prion protein.
 535 *J Virol* 74:320-325.
- 536 28. Nishida, N., S. Katamine, and L. Manuelidis. 2005. Reciprocal
 537 interference between specific CJD and scrapie agents in neural cell
 538 cultures. *Science* 310:493-496.
- 539 29. Prinz, M., M. Heikenwalder, P. Schwarz, K. Takeda, S. Akira, and A.
 540 Aguzzi. 2003. Prion pathogenesis in the absence of Toll-like receptor
 541 signalling. *EMBO Rep* 4:195-199.
- 542 30. Prusiner, S. B. 1998. Prions. *Proc Natl Acad Sci U S A* 95:13363-13383.
- 543 31. Raymond, I., T. Al Saati, J. Tkaczuk, S. Chittal, and G. Delsol. 1997.
 544 CNA.42, a new monoclonal antibody directed against a
 545 fixative-resistant antigen of follicular dendritic reticulum cells. *Am J*
 546 *Pathol* 151:1577-1585.
- 547 32. Rosicarelli, B., B. Serafini, M. Sbriccoli, M. Lu, F. Cardone, M.
 548 Pocchiari, and F. Aloisi. 2005. Migration of dendritic cells into the
 549 brain in a mouse model of prion disease. *J Neuroimmunol*
 550 165:114-120.
- 551 33. Sato, M., H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, K. Nakao, T.
 552 Nakaya, M. Katsuki, S. Noguchi, N. Tanaka, and T. Taniguchi. 2000.
 553 Distinct and essential roles of transcription factors IRF-3 and IRF-7 in
 554 response to viruses for IFN-alpha/beta gene induction. *Immunity*
 555 13:539-548.
- 556 34. Servant, M. J., N. Grandvaux, and J. Hiscott. 2002. Multiple signaling
 557 pathways leading to the activation of interferon regulatory factor 3.
 558 *Biochem Pharmacol* 64:985-992.
- 559 35. Sethi, S., G. Lipford, H. Wagner, and H. Kretzschmar. 2002.
 560 Postexposure prophylaxis against prion disease with a stimulator of
 561 innate immunity. *Lancet* 360:229-230.
- 562 36. Spinner, D. S., I. S. Cho, S. Y. Park, J. I. Kim, H. C. Meeker, X. Ye, G.

- 563 Lafauci, D. J. Kerr, M. J. Flory, B. S. Kim, R. B. Kasczak, T.
 564 Wisniewski, W. R. Levis, G. B. Schuller-Levis, R. I. Carp, E. Park, and
 565 R. J. Kasczak. 2008. Accelerated prion disease pathogenesis in
 566 Toll-like receptor 4 signaling-mutant mice. *J Virol* **82**:10701-10708.
- 567 37. Suh, H. S., M. L. Zhao, N. Choi, T. J. Belbin, C. F. Brosnan, and S. C.
 568 Lee. 2009. TLR3 and TLR4 are innate antiviral immune receptors in
 569 human microglia: role of IRF3 in modulating antiviral and
 570 inflammatory response in the CNS. *Virology* **392**:246-259.
- 571 38. Takaoka, A., and T. Taniguchi. 2003. New aspects of IFN-alpha/beta
 572 signalling in immunity, oncogenesis and bone metabolism. *Cancer Sci*
 573 **94**:405-411.
- 574 39. Tal, Y., L. Souan, I. R. Cohen, Z. Meiner, A. Taraboulos, and F. Mor.
 575 2003. Complete Freund's adjuvant immunization prolongs survival in
 576 experimental prion disease in mice. *J Neurosci Res* **71**:286-290.
- 577 40. Taniguchi, T., and A. Takaoka. 2001. A weak signal for strong
 578 responses: interferon-alpha/beta revisited. *Nat Rev Mol Cell Biol*
 579 **2**:378-386.
- 580 41. Thackray, A. M., A. N. McKenzie, M. A. Klein, A. Lauder, and R.
 581 Bujdoso. 2004. Accelerated prion disease in the absence of
 582 interleukin-10. *J Virol* **78**:13697-13707.
- 583 42. Tribouillard-Tanvier, D., J. F. Striebel, K. E. Peterson, and B.
 584 Chesebro. 2009. Analysis of protein levels of 24 cytokines in scrapie
 585 agent-infected brain and glial cell cultures from mice differing in prion
 586 protein expression levels. *J Virol* **83**:11244-11253.
- 587 43. Weissmann, C., M. Enari, P. C. Klohn, D. Rossi, and E. Flechsig. 2002.
 588 Molecular biology of prions. *Acta Neurobiol Exp (Wars)* **62**:153-166.
- 589 44. Worthington, M. 1972. Interferon system in mice infected with the
 590 scrapie agent. *Infect Immun* **6**:643-645.
- 591 45. Zhai, J., D. Gao, W. Liu, R. Hong, Y. Qin, H. Ouyang, Y. Kong, Y. Wang,
 592 Y. Xie, and J. Liu. 2008. Characterization of a novel isoform of murine
 593 interferon regulatory factor 3. *Biochemical and biophysical research*
 594 *communications* **377**:384-388.
- 595

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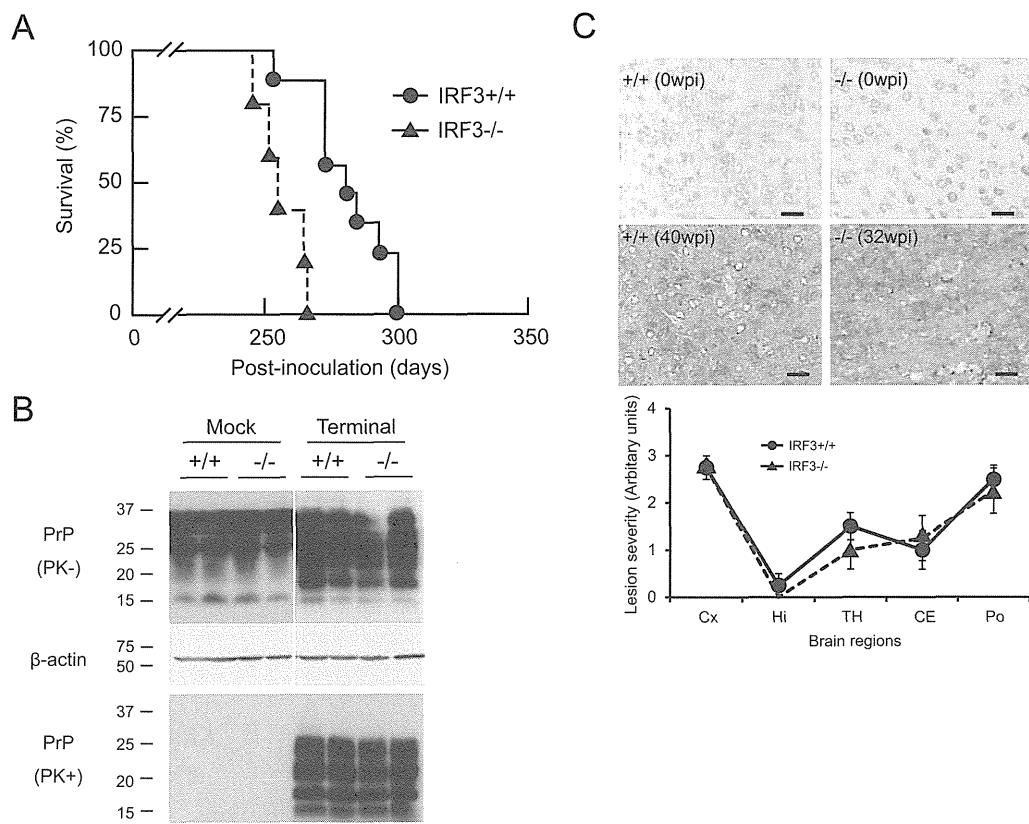


Fig. 1

Fig. 2

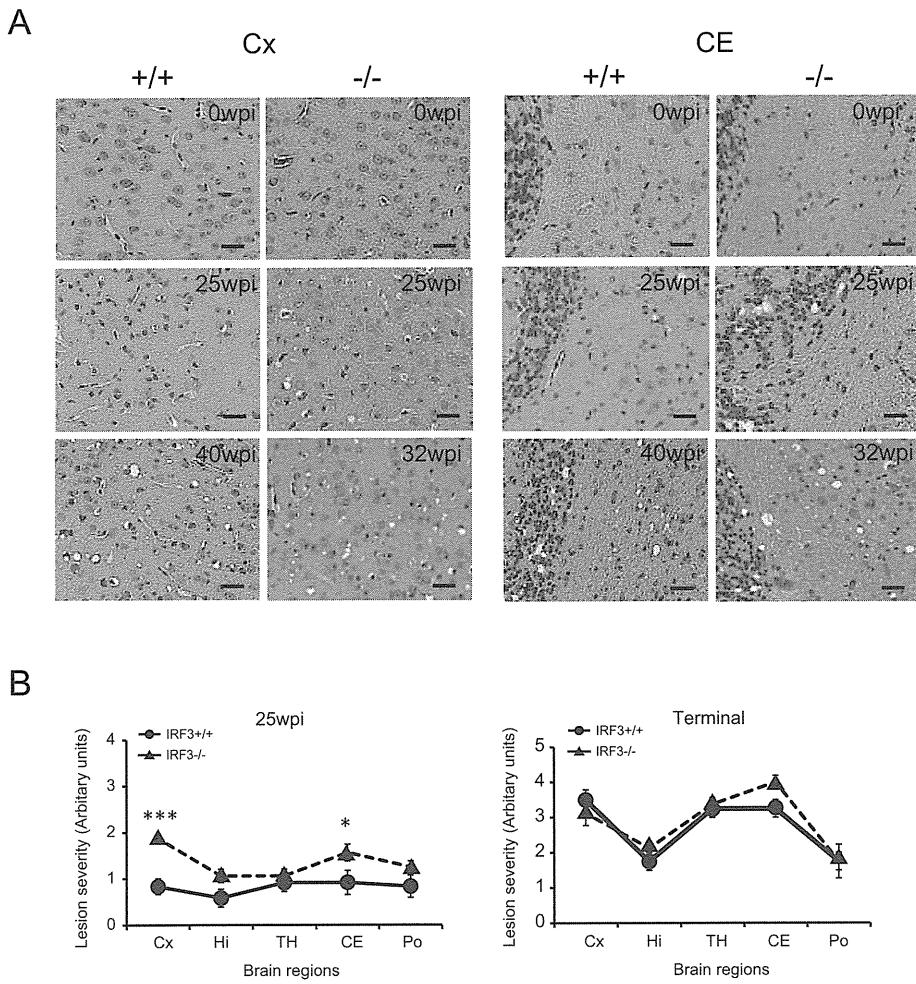
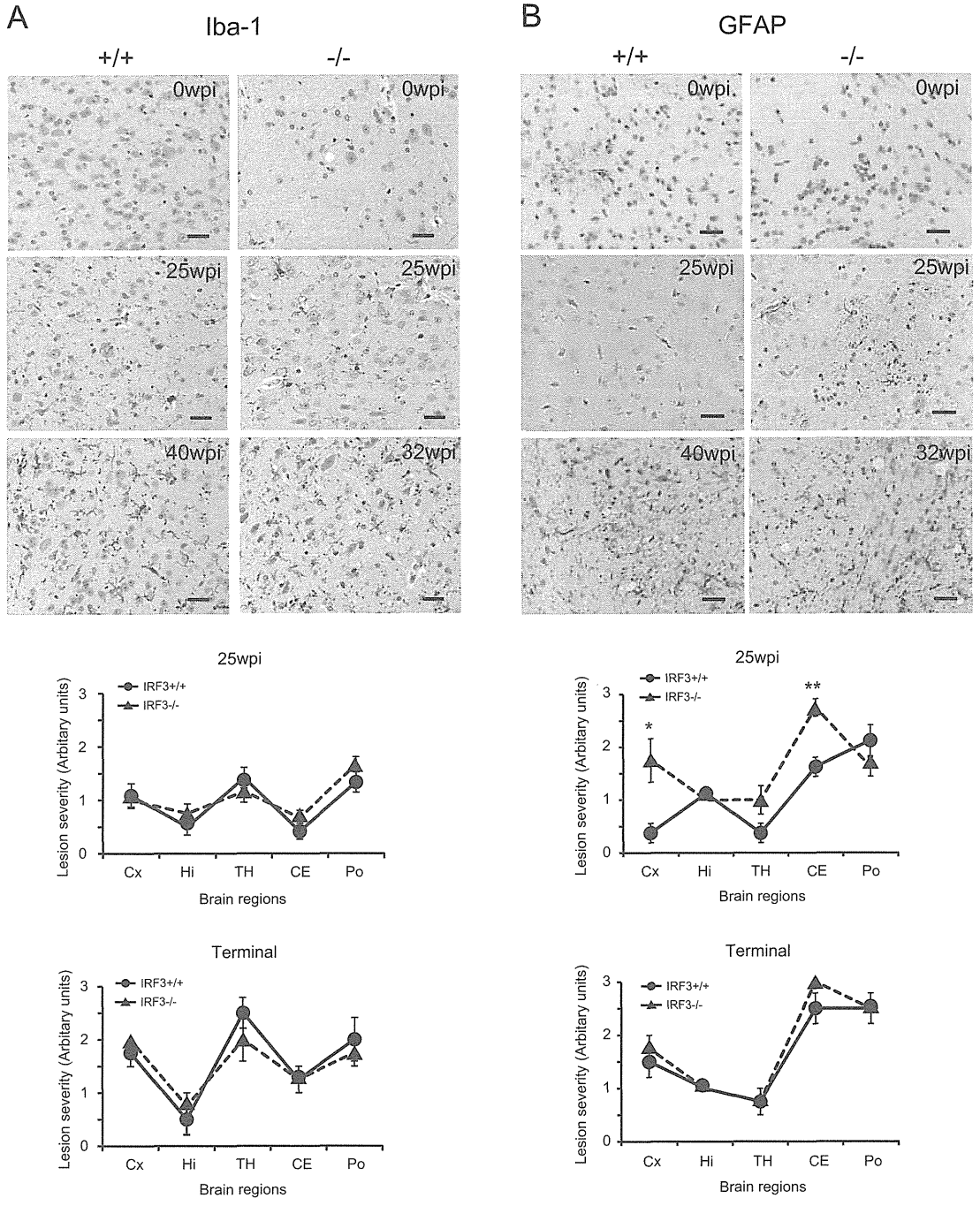


Fig. 3



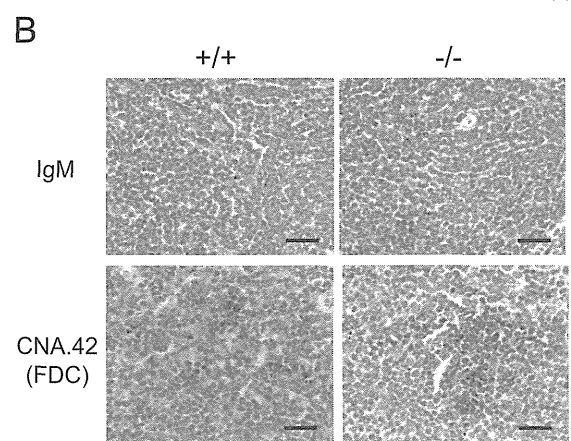
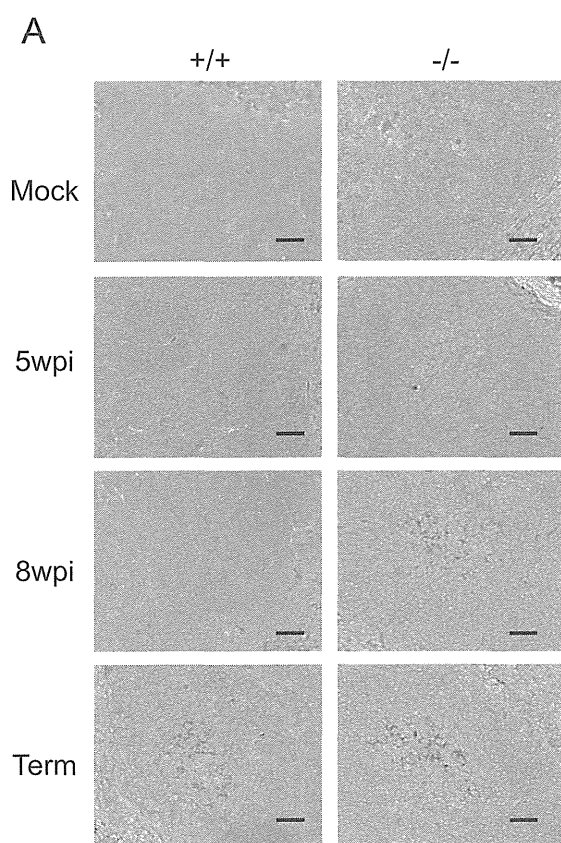


Fig. 4

Fig. 5

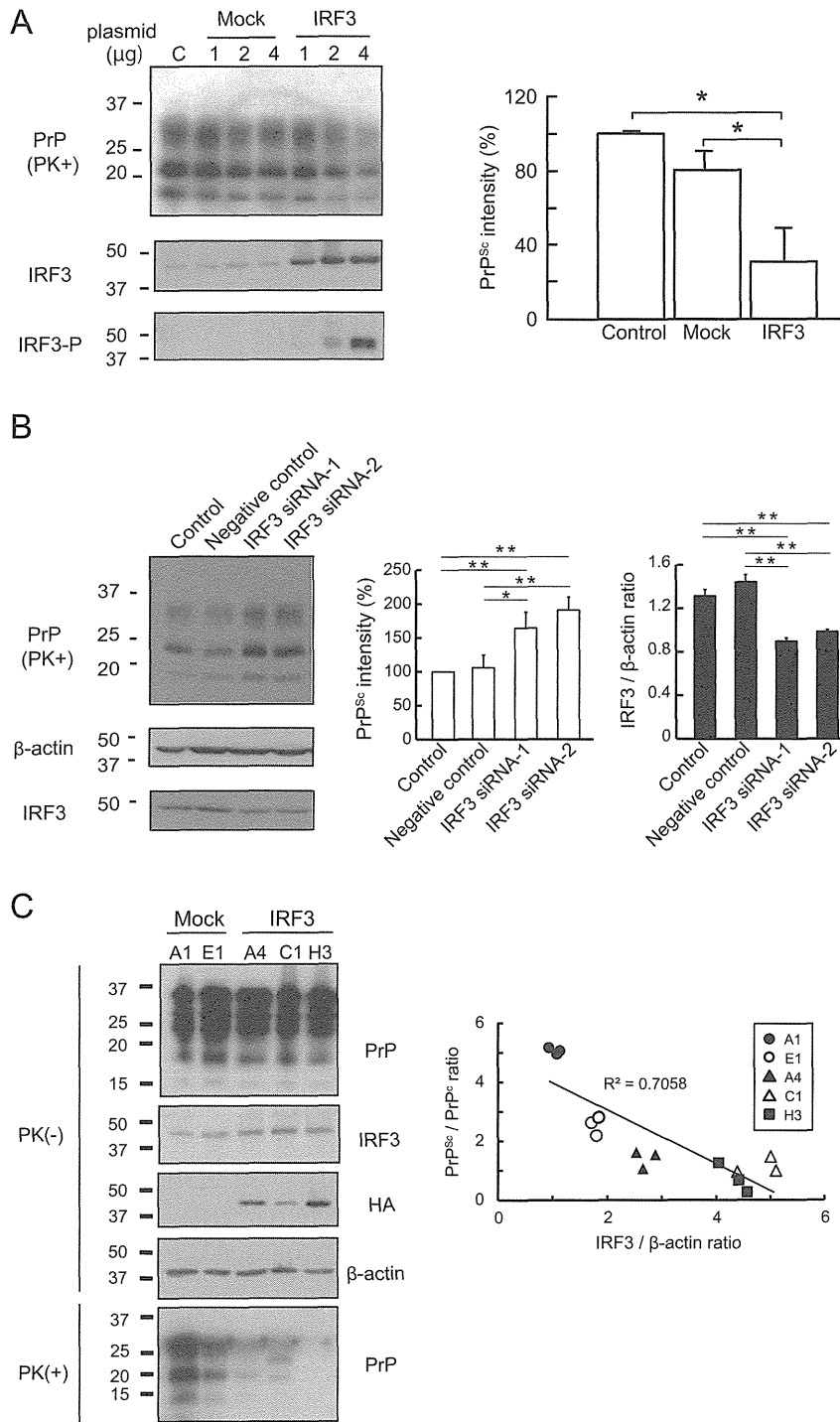


Table 1. Survival periods of prion-infected wild-type (+/+) and IRF3 knockout (-/-) mice.

Strains	Survival periods (days) ^a	
	+/+ (n) ^b	-/- (n) ^b
22L	281 ± 15 (9)	257 ± 8 (5) ***
BSE	380 ± 33 (7)	335 ± 37 (7) *
FK-1	321 ± 24 (4)	251 ± 31 (5) **

^a Survival periods were shown as "Average ± SD (days)".

^b (n), number of mice.

Animals were intraperitoneally administrated with 10⁻³ dilution of brain homogenate from prion-infected terminal mice. *p < 0.05, **p < 0.01, ***p < 0.001 by student's t-test.

Table 2. PrP^{Sc} positive rate in the spleens in mice with i.p. inoculation of 22L at the indicated time points.

Weeks post inoculation	PrP ^{Sc} positive/total number of mice ^a	
	+/+	-/-
1	0/5	0/1
2	0/5	1/5
5	0/5	4/5
8	0/5	5/5
Terminal	1/1	1/1

^aThe presence of PrP^{Sc} accumulation in the spleen of prion-infected mice was determined by immunohistochemical staining.

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Enhanced Antigen Retrieval of Amyloid β Immunohistochemistry : Re-evaluation of Amyloid β Pathology in Alzheimer Disease and Its Mouse Model

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What is This?

Enhanced Antigen Retrieval of Amyloid β Immunohistochemistry: Re-evaluation of Amyloid β Pathology in Alzheimer Disease and Its Mouse Model

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Summary

Senile plaques, extracellular deposits of amyloid β peptide ($A\beta$), are one of the pathological hallmarks of Alzheimer disease (AD). As the standard immunohistochemical detection method for $A\beta$ deposits, anti- $A\beta$ immunohistochemistry combined with antigen retrieval (AR) by formic acid (FA) has been generally used. Here, we present a more efficient AR for $A\beta$ antigen. On brain sections of AD and its mouse model, a double combination of either autoclave heating in EDTA buffer or digestion with proteinase K plus FA treatment reinforced $A\beta$ immunoreactivity. A further triple combination of digestion with proteinase K (P), autoclave heating in EDTA buffer (A), and FA treatment (F), when employed in this order, gave a more enhanced immunoreactivity. Our PAF method prominently visualized various forms of $A\beta$ deposits in AD that have not been clearly detected previously and revealed numerous minute-sized plaques both in AD and the mouse model. Quantification of $A\beta$ loads showed that the AR effect by the PAF method was 1.86-fold (in the aged human brain) and 4.64-fold (in the mouse brain) higher than that by the FA method. Thus, the PAF method could have the potential to be the most sensitive tool so far to study $A\beta$ pathology in AD and its mouse model. (*J Histochem Cytochem* 60:761–769, 2012)

Keywords

Alzheimer disease, amyloid β , antigen retrieval, APP-SL mouse, autoclave heating, formic acid, immunohistochemistry, minute plaque, PAF method, proteinase K

Senile plaques (SPs) and neurofibrillary tangles (NFTs) are two pathological hallmarks that characterize brains afflicted with Alzheimer disease (AD). SPs are extracellular deposits of amyloid β peptide ($A\beta$) mainly consisting of 40 and 42 residues, which are cleavage products of the amyloid precursor proteins (APPs) (Masters et al. 1985; Kang et al. 1987; Iwatsubo et al. 1994). $A\beta$ is a hydrophobic self-aggregating peptide, and the aggregation of soluble $A\beta$ monomers leads to the composition of insoluble fibrillar polymers, $A\beta$ fibrils. NFTs are intracellular aggregated bundles of a hyperphosphorylated form of the microtubule-associated protein tau (Lee et al. 1991; Ballatore et al. 2007).

Although it is not yet completely elucidated whether SPs and NFTs are the causes or the results of AD onset, the aggregation of $A\beta$ is believed to be implicated in the upper stream

of the cascade of AD pathogenesis as a pivotal player in the development of dementia: the amyloid hypothesis (Selkoe 1991; Hardy and Higgins 1992; Hardy and Selkoe 2002). Therefore, the detection of SPs or $A\beta$ deposits with high specificity and sensitivity is essential for elucidating the roles of

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parenchymal A β deposition and its implication for the pathogenesis of AD as well as its pathological diagnosis. In 1987, our attempt to attain sensitive A β immunohistochemistry (IHC) results on formalin-fixed paraffin-embedded (FFPE) tissue sections led to the development of A β antigen retrieval (AR) by formic acid (FA) (Kitamoto et al. 1987). This straightforward method dramatically enhances the detection level of A β deposits in the AD brain, and since then, anti-A β IHC coupled with FA treatment has been the standard method in the field of A β pathology. There is no guarantee, however, that this method can expose all of the existing A β deposits without the remains, and we consider that there might be room for improvement of the AR technique. In fact, we had a chance to find irregular and larger forms of A β staining, known as fleecy amyloid deposits (Thal et al. 1999), which appear different from the usual SPs in the entorhinal cortex of some AD cases. These structures of A β aggregates stained too faintly to be recognized clearly, and we thought that the AR mediated by FA was not efficient enough to detect these A β structures. Thus, we tackled the development of a new AR method with a higher efficiency than the conventional FA method. We could substantially improve A β IHC by applying two other AR procedures prior to FA treatment. This new AR method enhanced the detection level of numerous SPs and various A β deposits that have not been clearly detected by the conventional method and provides a tool to uncover new aspects of A β pathology in AD and its mouse models.

Materials and Methods

Brain Specimens

Human brain specimens were derived from patients with AD ($n=11$; age range = 63–79 years), non-AD aged individuals with A β plaques ($n=4$; age range = 63–77 years), and negative controls for A β IHC, who have no family history of AD, including non-AD aged individuals without A β plaques ($n=10$; age range = 64–94 years) and healthy young individuals ($n=6$; age range = 21–38 years). For quantification of the A β loads, we examined a series of aged human individuals with varying degrees of the A β burden ($n=54$; age range = 69–94 years). As transgenic AD mouse models, we used the APP–Swedish/London (SL) lines 7–5 and 7–9, which overexpress human APP^{Sw/Lon} harboring both the Swedish- and London-type mutations. The levels of APP, A β 40, and A β 42 in the brain tissues of the mice of line 7–5 are higher than the corresponding levels in the mice of line 7–9 (Shin et al. 2007). The outline of the ages of each of them is as follows: line 7–5 of APP-SL mice aged 6 months ($n=3$), 8 months ($n=8$), 9 months ($n=2$), 10 months ($n=2$), 11 months ($n=1$), 12 months ($n=4$), 13 months ($n=6$), 15 months ($n=1$), 16 months ($n=5$), and 18 months ($n=1$); line 7–9 of APP-SL mice aged 3 months ($n=3$), 6 months ($n=3$), 9 months ($n=3$), 12 months ($n=2$), 13 months ($n=3$), 15

months ($n=1$), 16 months ($n=2$), 18 months ($n=2$), 19 months ($n=1$), and 36 months ($n=1$). The fixation time of brains was 7–13 days with 20% buffered formalin in humans and 3–4 days with 10% buffered formalin in mice. The use of human brains for this work was approved by the Institutional Review Board of Tohoku University Graduate School of Medicine and Tokyo Metropolitan Geriatric Hospital & Institute of Gerontology, and all the animal experiments were done according to the Guidelines for Animal Care and Use at Otsuka Pharmaceutical Co. Ltd.

AR Procedures

FA pretreatment has been the standard AR method for A β IHC. In FA pretreatment, brain tissue sections were incubated in 98% FA (Wako Pure Chemical Industries; Osaka, Japan) for 5 min at room temperature. A challenging trial to largely improve the FA method was performed by combining and applying other AR methods prior to FA treatment. The other AR methods used in this study include heating that employs immersion of tissue sections in 10 mM EDTA (pH 3.0, pH 6.0, and pH 10.0) (Murayama et al. 1999), 0.05% citraconic anhydride (pH 3.0, pH 7.4, and pH 10.0) (Namimatsu et al. 2005), and 0.1 M sodium citrate (pH 3.0, pH 7.2, and pH 10.0) (Bataille et al. 2006) solutions, and distilled water (DW) (pH 3.0 adjusted with hydrochloric acid, pH 7.1, and pH 10.0 adjusted with sodium hydroxide), using an autoclave at 105C or 121C for 10 min (Shin et al. 1991) or using a microwave oven at 90C intermittently but for a total of about 10 min; the proteolytic digestion of tissue sections was performed at 37C for 30 min with 1.0 μ g/ml of proteinase K (PK) (Wako Pure Chemical Industries) and 100.0 μ g/ml trypsin (Wako Pure Chemical Industries) dissolved in 1.0 mM CaCl₂/50 mM Tris buffer (pH 7.6). After each AR treatment, these sections were washed with tap water for at least 5 min and then incubated in DW for at least 5 min.

Immunostaining

With pretreatment of various combinations of the AR methods, immunostaining was performed as described (Murayama et al. 1999; Shin et al. 2007) using the polyclonal A β antibody 4702 (1:1500) (Shin et al. 2007) and monoclonal A β antibodies 6E10 (1:2000–4000; Senetek, Maryland Heights, MO) and 4G8 (1:20,000; Senetek). The concentrations of these antibodies were optimized in consideration of both the immunoreactivity and backgrounds of IHC. Brain sections were incubated with primary antibodies in 0.1% Tween-20/Tris-buffered saline (Tris, 50 mM; NaCl, 500 mM; pH 7.6) containing 5% nonfat dried milk for about 15 hr at room temperature. To exclude non-specific staining unrelated to these polyclonal and monoclonal antibodies, immunostaining was performed with

omission of the antibodies but with all other procedures unchanged in some experiments. Secondary antibodies of EnVision+ system HRP-labeled polymer (Dako; Glostrup, Denmark) were used for the detection of antigen primary mouse or rabbit antibody complexes by diaminobenzidine (DAB). The incubation of the secondary antibodies was for about 1 hr at room temperature. The immunostained brain sections were counterstained with hematoxylin.

Microscopes

The photomicrographs of human and murine samples were captured by an Axiophot2 microscope (Carl Zeiss; Oberkochen, Germany) with Axio Vision version 4.6.3.0 software (Carl Zeiss). In quantification of the A β load in the hippocampus of the murine samples, the same system was used in order to assemble sequential micrographs into a single larger one. For measuring the A β load, aged human sample photomicrographs were captured by C9600 NanoZoomer (Hamamatsu Photonics; Hamamatsu, Japan) with NDP.view software (Hamamatsu Photonics) because this system is conveniently applicable for capturing and comparing the same regions from serial sections.

Measurement of the Area of A β Deposits

In the human brains, we selected three microscopic fields in the fusiform gyrus, which are adequately separated from each other and contain relatively higher A β loads. We photographed precisely the same fields in each serial section immunostained following the FA method or PK digestion (P), EDTA autoclaving (A), and FA treatment (F) (in that order; referred to as "PAF") method. All images were from regions of 1408 $\mu\text{m} \times 1874 \mu\text{m}$. In the mouse brains, we selected the whole hippocampus, and its image was constructed from the photomicrographs of 872 $\mu\text{m} \times 1100 \mu\text{m}$ by using panorama module of AxioVision version 4.6.3 (Carl Zeiss). All the images were analyzed by ImageJ version 1.43 m (National Institutes of Health; Bethesda, MD) as follows: 1) each raw image was resolved into three images by the color deconvolution setting in hematoxylin and eosin and DAB; 2) the DAB color image among the three resolved images was selected for analysis; 3) the threshold value of the selected image was set to zero as the minimum value and at the optically optimum value set as the maximum value; 4) the thresholded areas of cerebral amyloid angiopathy and artifacts were excluded by selecting and filling them; 5) areas of the A β loads (%) to be measured were within a circle (diameter = 1408 μm), the center of which being in the middle of each image in the aged human brains, and within the circumscribed edge of the hippocampus drawn using the selection tools in the mouse brains; and 6) area fractions of the residual thresholded objects within these selections were measured.

Statistical Analyses

All the statistical analyses were performed with SPSS version 17.0 (SPSS; Chicago, IL).

Results

Development of the Enhanced AR Method for A β IHC

For the development of a more efficient A β AR method, our strategy was to modify and reinforce the retrieving effects of FA by applying other AR procedures prior to FA treatment. Such AR procedures included autoclave heating in EDTA buffer (the chelating autoclave method) (Murayama et al. 1999) and digestion with PK. With each of these AR procedures followed by FA treatment or with FA treatment alone, immunostaining using the polyclonal anti-A β 4702 antibody was performed on brain tissue sections derived from AD patients and APP-SL line 7–5 mice. In immunostaining using the 4702 antibody with no AR, almost no plaques were detected in the AD brains or only a few in the mouse brains (Suppl. Fig. S1). Thus, this 4702 antibody was conveniently used to easily evaluate the effectiveness of the A β AR methods.

Compared to the AR procedures above followed by FA treatment and FA treatment alone, both combinations of AR enhanced the A β immunoreactive intensity and increased the loads of A β plaques, albeit with low to high enhancing effects (Fig. 1A–C, E–G). However, when we applied heating to the EDTA solution, counterstaining of tissue sections with hematoxylin was remarkably thin compared with counterstaining in the FA only method. Notably, the reversed application of the two AR procedures, that is, application of FA treatment and either autoclave heating in EDTA buffer or digestion with PK in this order, showed limited and almost no enhancement, respectively, compared with the single application of FA treatment (data not shown). These results prompted us to try a triple combination of these three AR procedures. The use of the PAF method produced a remarkably stronger enhancement of A β immunoreactivity than the two double combinations above (Fig. 1B–D, F–H). In the triple combinations of the three AR methods in different orders other than that used in the PAF method, varying degrees of tissue damage ensued, especially in human brains. Therefore, we refrained from estimating those triple AR combinations. To confirm that this PAF method is universally applicable to A β IHC, we examined other A β antibodies including 6E10 (Fig. 2A, B, E, F) and 4G8 (Fig. 2C, D, G, H). All of these antibodies showed enhanced A β immunoreactivity following the PAF method compared with the FA method, albeit with varied enhancing effects. Omission of the polyclonal or monoclonal primary antibodies in A β IHC with the PAF method totally abolished positive immunostaining (Suppl. Fig. S2),

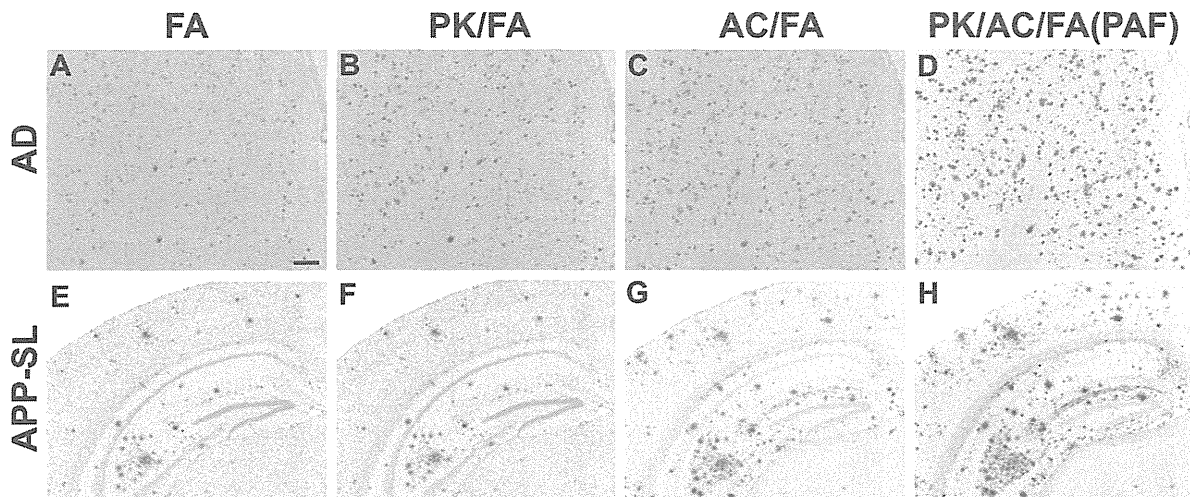


Figure 1. Enhancement of formic acid (FA)-mediated amyloid β peptide ($A\beta$) antigen retrieval. Serial brain tissue sections from a 74-year-old male patient with Alzheimer disease (A–D) and from a 13-month-old amyloid precursor protein–Swedish/London (APP-SL) mouse of line 7–5 (E–H) were immunostained with anti- $A\beta$ antibody 4702 following pretreatment by FA alone (FA) (A, E); combination of digestion with proteinase K and FA (PK/FA) (B, F); combination of autoclave heating in EDTA buffer and FA (AC/FA) (C, G); and triple combination of digestion with proteinase K, autoclave heating in EDTA buffer, and FA (PK/AC/FA) (D, H). Pictures (A–D) are from the temporal cortex. Scale bar = 200 μ m (A–H).

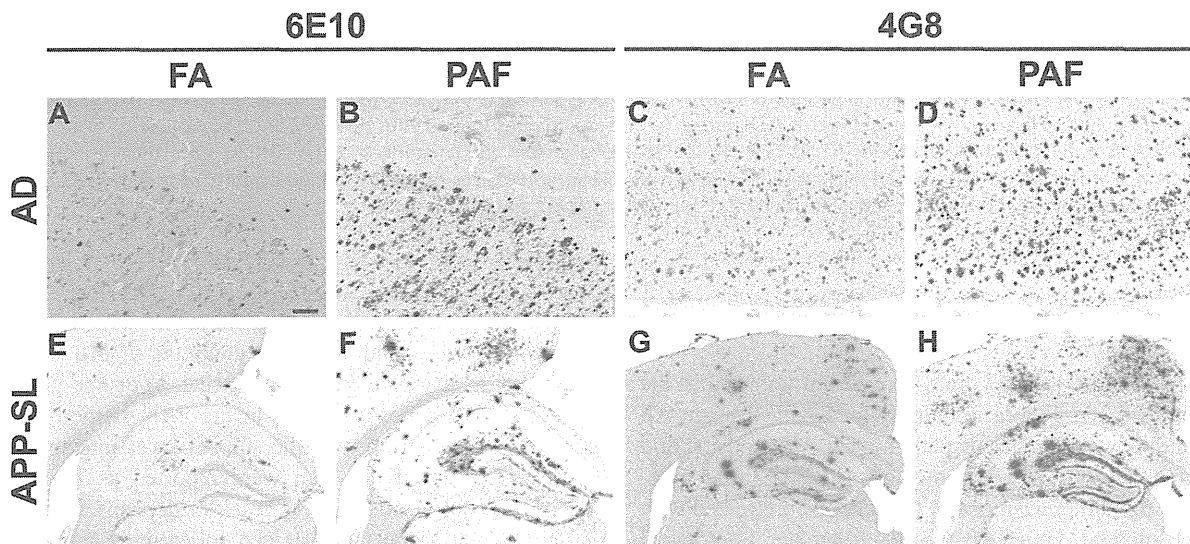


Figure 2. General application of the proteinase K digestion (P), EDTA autoclaving (A), and formic acid (FA) treatment (F) (in that order; referred to as “PAF”) method in amyloid β peptide ($A\beta$) immunohistochemistry. Serial (A–B, C–D, E–F, G–H) brain tissue sections from a 74-year-old male patient with Alzheimer disease (the same patient as shown in Fig. 1) (A–D) and a 16-month-old (E, F) and a 15-month-old (G, H) amyloid precursor protein–Swedish/London (APP-SL) mouse of line 7–5 were immunostained with monoclonal anti- $A\beta$ antibodies 6E10 (A, B, E, F) and 4G8 (C, D, G, H) following pretreatment by the FA (A, C, E, G) and PAF methods (B, D, F, H). Pictures are from the cingulate cortex (A, B) and the frontal cortex (C, D). Scale bar = 200 μ m (A–H).

which excludes the possibility that immunoreactivity augmented and disclosed following the PAF method was due to nonspecific staining. In addition, no artifactual

immunostaining was observed in the brain sections from the normal younger individuals following $A\beta$ IHC assisted by the PAF method (Suppl. Fig. S3).

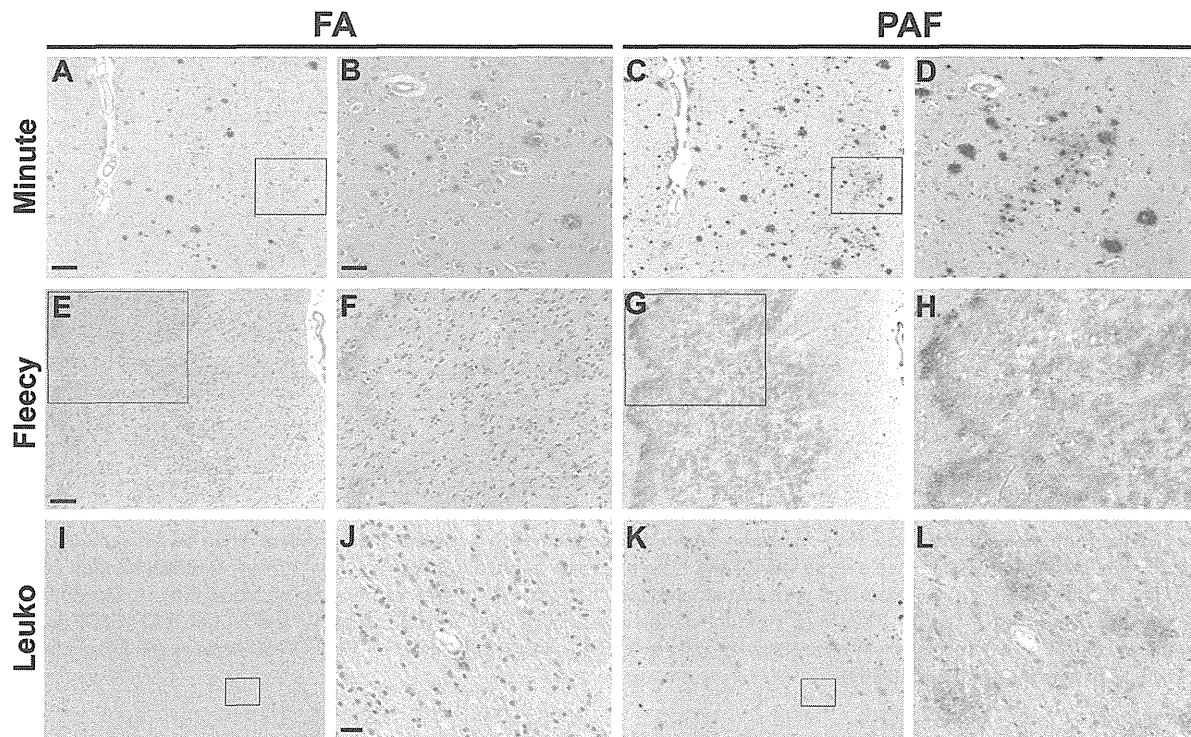


Figure 3. Amyloid β peptide (A β) pathology of Alzheimer disease (AD) brains enhanced by the proteinase K digestion (P), EDTA autoclaving (A), and formic acid (FA) treatment (F) (in that order; referred to as “PAF”) method. Serial brain tissue sections from a 72-year-old female AD patient (the identical patient as shown in Suppl. Fig. S1A,B) (A–D), a 74-year-old male AD patient (the same patient as shown in Fig. 1) (E–H), and a 74-year-old female AD patient (I–L) were immunostained with the 4702 antibody following pretreatment by the FA (A, B, E, F, I, J) and PAF methods (C, D, G, H, K, L). Pictures are from the frontal cortex (A–D), the entorhinal cortex (E–H), and the frontal white matter (I–L). B, D, F, H, J, and L are higher magnification pictures of areas outlined by squares in A, C, E, G, I, and K, respectively. Scale bars = 200 μ m (A, C, I, K); 50 μ m (B, D, F, H); 100 μ m (E, G); and 20 μ m (J, L).

IHC Analysis of AD Brains by the PAF Method

Serial sections of the AD brains pretreated with either the PAF or FA method were immunostained with the anti-A β 4702 antibody, analyzed for pathological A β deposits, and compared between these two methods (Fig. 3). In the cerebral cortex and hippocampus of the sections pretreated with the PAF method, larger A β plaques (diameter > ~15 μ m) showed immunoreactive enhancement with an enlarged robust contour, although there was no apparent increase in the number of these larger A β plaques. The prominent effect given by the PAF method was the disclosure of numerous minute-sized (diameter < ~15 μ m) fine-granular plaques (hereafter referred to as “minute plaques”) in these brain regions, which were not evidently detected by the FA method (Figs. 1A, D and 3A–D). Remarkably, the number of these minute plaques increased with elevation of the total A β load (data not shown). Thus, all of the AD brains examined by the PAF method contained a much higher load of A β plaques in the cerebral cortex and hippocampus than those

by the FA method. In the entorhinal cortex adjacent to the subiculum, large and irregular contours of A β staining appeared, which were composed of fine- to coarse-granular or diffuse A β deposits following the PAF method. These were distributed from near the subpial layer into the deep cortex (Fig. 3G, H), which were reported as fleecy A β deposits (Thal et al. 1999). These A β deposits were only faintly or not appreciably stained following FA treatment (Fig. 3E, F). In the cerebral white matter, small but significant amounts of A β deposits in the diffuse or granular form were previously shown to occur (Wisniewski et al. 1989; Behrouz et al. 1991; Uchihara et al. 1995). The PAF method gave immunoreactive enhancement and revealed larger amounts of granular A β deposits in the cerebral white matter (Fig. 3I–L). The PAF method also enhanced A β immunoreactivity of ribbon-like infiltration in the subpial layer of the cerebral cortex and that of cerebral amyloid angiopathy in the vessels of the brain (data not shown). Thus, the PAF method dramatically enhanced the detection level of a spectrum of all morphological forms of A β deposits.

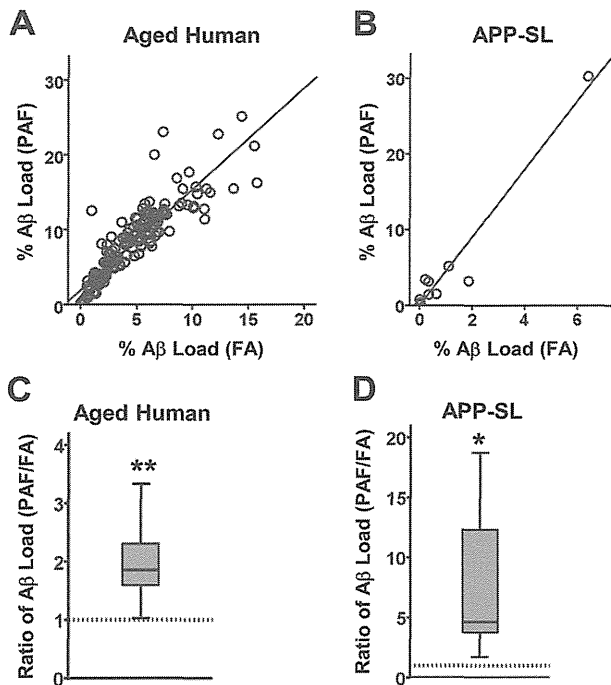


Figure 4. Effect of amyloid β peptide ($A\beta$) antigen retrieval (AR) by the proteinase K digestion (P), EDTA autoclaving (A), and formic acid (FA) treatment (F) (in that order; referred to as “PAF”) method over that by the FA method. (A, B) In immunohistochemistry (IHC) with the 4702 antibody, $A\beta$ loads (%) measured in the fusiform cortex of each case from the aged human brains (A) or in the hippocampus of each from the amyloid precursor protein–Swedish/London (APP-SL) mice (B) following the PAF method were plotted against those following the FA method. Significant correlations between the two AR methods were verified both in the aged human brains (Spearman rank correlation coefficient, $r=0.92$; $p=9 \times 10^{-69}$) and in the mouse brains ($r=0.80$; $p=0.003$). (C, D) The effect of $A\beta$ AR by the PAF method was significantly higher than that by the FA method both in the aged human and in the mouse brains. In IHC with the 4702 antibody, $A\beta$ -loaded areas measured following the PAF method compared with those following the FA method (set at 1.00; the dotted lines) were 1.59-fold at the 25th percentile, 1.86-fold at the 50th percentile, and 2.31-fold at the 75th percentile in the aged human brains (C) and 3.78-fold at the 25th percentile, 4.64-fold at the 50th percentile, and 12.32-fold at the 75th percentile in the mouse brains (D). (C) Ten (≥ 3.39) and (D) one (92.79) outliers are not shown. $**p=2 \times 10^{-28}$, $*p=0.01$; analyzed by Wilcoxon signed-rank test. $n=162$ from 54 individuals where three regions per case examined (A, C), and $n=11$ (B, D).

IHC Analysis of the AD Mouse Model by the PAF Method

We examined the brains of the AD APP-SL mice in the same way as for the AD brains. In our previous (Shin et al. 2007) and present studies using conventional $A\beta$ IHC coupled with the FA method, younger APP-SL mice ($< \sim 9$ months) showed no occurrence of $A\beta$ deposition, and older mice ($\geq \sim 9$ months) exhibited deposition of $A\beta$ plaques that increased

its burden with age. Application of $A\beta$ IHC assisted by the PAF method to those younger mice also failed to reveal $A\beta$ deposition. Therefore, in mice showing no evidence of $A\beta$ deposition as evaluated by the FA method, the PAF method did not create any occurrence of $A\beta$ deposition. Thus, these two methods show no difference in their ability to demonstrate the absence of $A\beta$ deposition. In the older mice showing evidence of $A\beta$ deposition, the PAF method was more efficient than the FA method for AR. The enhanced immunoreactive profile was shown to enlarge the sizes and to increase the numbers and immunointensities of $A\beta$ plaques (Figs. 1E,H and 2E–H). Notably, minute $A\beta$ plaques appeared to have a similar morphology as those seen in the AD brains. These minute plaques occur in brain samples containing significant amounts of $A\beta$ burden and prevail dominantly with severity of $A\beta$ burden, as was shown in the AD brain. In 9-month-old APP-SL mice that show an initial appearance of $A\beta$ deposition, $A\beta$ deposits were indiscernible when evaluated by the FA method. These deposits were prominently visualized as distinct $A\beta$ deposits by the PAF method (data not shown). Thus, the PAF method could have an advantage for the retrieval of antigens in $A\beta$ IHC in comparison with the FA method in the AD mouse model as well as humans.

Efficiency of $A\beta$ AR by the PAF Method

We measured the areas of $A\beta$ deposit loads in the serial sections immunostained with the 4702 antibody following the PAF or FA method, and the total sums of the $A\beta$ -loaded areas per the whole area analyzed were compared between the two methods. In the aged human brains, $A\beta$ deposit loads measured in the fusiform cortex by the PAF method were significantly correlated with those by the FA method ($p=9 \times 10^{-69}$) (Fig. 4A). We compared the ratio of the $A\beta$ deposit area from the PAF method with the ratio from the FA method. The AR effect of the PAF method (Fig. 4C) was significantly higher than that of the FA method (1.86-fold at the median) ($p=2 \times 10^{-28}$). In the APP-SL line 7–9 mice, $A\beta$ deposit loads measured in the whole hippocampus by the PAF method were also significantly correlated with those by the FA method ($p=0.003$) (Fig. 4B). The enhancing effect of the PAF method compared with that of the FA method (the ratio as described above in aged human brains) was significantly higher and 4.64-fold at the median ($p=0.01$) (Fig. 4D). Thus, the PAF method produced $A\beta$ deposit loads that were consistently and significantly larger than those produced by the FA method.

Evaluation of PK versus Trypsin in Enzymatic Digestion and Some Other Solutions in Autoclave Heating

To obtain a more effective enzymatic digestion than PK digestion, we additionally tested trypsin in the double combination of enzymatic digestion and the FA method. Trypsin

digestion, when applied prior to the FA method, produced A β immunostaining slightly higher in its intensity than the FA method only. However, its efficacy was comparable to that of PK digestion (Suppl. Fig. S4). To obtain a more effective autoclave heating than that in the solution of 10 mM EDTA (pH 6.0) at 121C, we additionally tested solutions of EDTA (pH 3.0 and pH 10.0), DW (pH 3.0, pH 7.1, and pH 10.0), citraconic anhydride (pH 3.0, pH 7.4, and pH 10.0), and sodium citrate (pH 3.0, pH 7.2, and pH 10.0) as well as temperatures of 90C, 105C, and 121C. Among these different conditions, DW (pH 10.0 and 105C) and sodium citrate (pH 7.2 and 105C) produced high A β AR effects. A similar effect was observed for EDTA (pH 6.0 and 121C), although sodium citrate slightly damaged the tissue sections (Suppl. Fig. S5). The triple combinations using additional PK or trypsin digestion as the initial step produced higher A β AR effects than each of those double combinations, although heating in sodium citrate solution or basic water damaged the tissue sections. Further nonspecific staining was observed in the brain sections of the APP-SL mice applied by the triple combination of trypsin digestion, EDTA autoclaving, and the FA method (Suppl. Figs. S6 and S7). Thus, the triple combination of 1) PK digestion, 2) autoclave heating in 10 mM EDTA (pH 6.0 and 121C), and 3) FA treatment produced the highest A β AR effects without damaging tissue sections or producing nonspecific staining.

Discussion

The masking of antigens by aldehyde fixatives or by paraffin-embedding procedures is a problem for IHC studies. To overcome this problem, enzymatic digestion, FA treatment, and high-temperature heating have been developed. Among these, FA treatment is the standard method mainly used for A β IHC of FFPE brain tissue sections, although it was originally developed for the immunoreactive enhancement of cerebral amyloids (prion protein and A β) and systemic amyloids (amyloid A and prealbumin) (Kitamoto et al. 1987). The pretreatment of protein digestion with an enzyme such as trypsin had been used for IHC but only in a limited application (Battifora and Kopinski 1986; Huang et al. 1976; Mephram et al. 1979). In 1991, the advent of the heating AR method was a breakthrough in the field of IHC. Shin et al. (1991) reported that the procedure of hydrated autoclaving uncovers the masked epitopes of the microtubule-associated protein tau, showing that high-temperature heating serves as an efficient AR method. Shi et al. (1991) reported that microwave heating also shows an AR effect by testing a variety of antigens and antibodies, establishing the milestone of AR for FFPE tissue sections. Moreover, Shi et al. (1996) devised the test battery approach, which can efficiently determine the optimum protocols of AR for each antigen by comparing the immunostaining results between different kinds of solutions, temperatures, and pH

(O'Leary 2001). As one good example using the test battery approach, it was demonstrated that AR procedures can also be applied to immunoelectron microscopy for amyloid deposits composed of the κ light chain or transthyretin (Rocken and Roessner 1999).

A β AR by FA is proposed because of the unfolding of the conformational amyloid polymers and thereby the exposing of A β antigens through acidic hydrolysis (Kitamoto et al. 1987). Further, FA is suggested to esterify serine residues in A β peptides and to alter the conformation of the amyloid polymers, as nuclear magnetic resonance imaging has revealed (Klunk et al. 1994). On the other hand, the possible mechanisms underlying AR by high-temperature heating are summarized as follows: 1) breaking of aldehyde-induced cross-linkage involving antigenic proteins, 2) extraction of diffusible blocking proteins, 3) precipitation of antigenic proteins, and 4) increased penetration of antibodies with better access to epitopes due to rehydration of the tissue sections (Suurmeijer and Boon 1993). In addition, the application of divalent/trivalent chelators in high-temperature heating removes metal ions that mask antigenic proteins (Murayama et al. 1999; Yamamoto et al. 2002; Shin et al. 2003). If autoclave high-temperature heating with EDTA chelators (Murayama et al. 1999) is then used prior to FA treatment, with the aim of affecting its activity of A β AR, it is likely that FA gains permeability through the tissue sections up to the unmasked A β fibrils. Similarly, if digestion with PK is applied prior to FA treatment, then proteolytic digestion of blocking proteins that surround A β fibrils might occur and unmask and thereby expose them to FA. In short, the procedures of EDTA autoclaving and PK digestion might assist the access of FA to A β fibrils, resulting in reinforcement of A β AR of FA. Indeed, we demonstrated that the combination of preceding EDTA autoclaving or PK digestion with subsequent FA treatment enhanced the AR effects of FA, and the aforementioned hypotheses might indeed be true. In support of this hypothesis, FA treatment prior to PK digestion or EDTA autoclaving gave no or only a minimally discernible enhancement in A β immunoreactivity compared with single FA treatment (data not shown). Our present results, together with other previous studies, show that PK digestion and EDTA autoclaving apparently differ in the mechanisms of the reinforcement of A β AR. If both PK digestion and EDTA autoclaving are combined with the FA method, their effects on A β AR by FA might be complementary rather than equivalent. Indeed, the triple combination of PK digestion and EDTA autoclaving with FA treatment provided a further stronger enhancement of A β AR.

Our results based on the PAF method suggest that previous IHC studies performed by the conventional FA method might have underestimated the quantitative burden of A β pathology and that brains with AD and its mouse models produce much more A β accumulation than previously assumed. Further, the presence of minute plaques revealed