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Caspase-cleaved actin (fractin) immunolabelling of Hirano bodies

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Hirano bodies are eosinophilic rod-like inclusions that are found predominantly in neuronal processes in the hippocampal CA1 sector with increasing age and are particularly numerous in Alzheimer's disease. They contain a variety of cytoskeletal epitopes, especially actin and actin-binding proteins. Actin cleavage by cysteinyl aspartate-specific proteases (caspases) is a feature of apoptosis. Cleavage at aspartate 244 generates N-terminal 32 kDa and C-terminal 15 kDa actin fragments. This has led to the development of a rabbit polyclonal antibody specific for caspase-cleaved actin, directed to the last five C-terminal amino acids of the 32 kDa fragment of actin ('fractin'). Fractin immunohistochemistry was performed on hippocampal sections from Alzheimer's disease and control cases containing numerous Hirano bodies, in addition to immunolabelling with CM1 antiserum which recognizes activated caspase-3. The Hirano bodies showed strong diffuse fractin immunoreactivity. They did not label with CM1 antiserum, perhaps reflecting too low a level of activated caspase-3 for immunodetection, or involvement of a different member of the caspase family. The finding of fractin immunoreactivity of Hirano bodies suggests that caspase-like cleavage of actin may play a role in their formation and further supports caspase-like activity in neuronal processes, distinct from that associated with acute perikaryal apoptosis.

Keywords: actins, Alzheimer's disease, caspases, hippocampus, Hirano bodies, immunohistochemistry

Introduction

Hirano bodies are rod-shaped, eosinophilic, cytoplasmic inclusions (Figure 1**a**) consisting of lattice-like aggregates of parallel filaments approximately 10 nm in diameter [12,34,38]. They are found preferentially in neuronal processes in the hippocampal CA1 stratum pyramidale with increasing age and in a variety of neurodegenerative disorders, including Alzheimer's disease [10]. They can also be found in the CA1 stratum lacunosum, where their numbers peak during middle-age and subsequently decline [15,25,33]. Hirano bodies consist mostly of F-actin and actin-associated proteins [6,9]. They also contain microtubule associated proteins [7,27], neurofilament epitopes [33], C-terminal fragments of β-amyloid [24], hippocampal cholinergic neurostimulating peptide [22], advanced glycation endproducts [23] and FAC1 protein [13]. Hirano bodies likely represent alteration of the microfilamentous cytoskeleton [10,13], perhaps related to changes in the septo-hippocampal cholinergic system [20,22].

Activation of a family of cysteinyl aspartate-specific proteases (caspases) has recently been identified as a central feature of apoptotic cell death [37]. Caspase activation results in proteolytic cleavage of multiple intracellular structural and functional substrates at specific aspartate residues, including actin microfila-

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ments [4,16,19]. Caspase-3, which is activated by proteolysis of its zymogen into p18 and p12 subunits, appears to be an especially important apoptosis effector molecule [4,28]. A rabbit polyclonal antiserum, CM1, which specifically recognizes the p18 subunit of activated caspase-3, but not the inactive zymogen, has recently been characterized [35]. Furthermore, identification of caspase cleavage of actin into N-terminal ~32 kDa and C-terminal ~15 kDa fragments in apoptotic cell extracts [14,18] has led to the development of an end-specific rabbit polyclonal antibody directed to β-actin residues 240–244 (YELPD), representing the five C-terminal residues of the ~32 kDa fragment generated by cleavage at aspartate 244 (Figure 1**b**) [36,39]. This fractin (*fra*gment of ac*tin*) antibody specifically recognizes caspase-cleaved, but not intact actin. It labels apoptotic, but not necrotic, neuroblastoma cells *in vitro* and also the cell bodies and processes of plaque-associated neurones and microglia in Alzheimer's disease brain [39].

In the present study, fractin and CM1 immunohistochemistry were performed on hippocampal sections from Alzheimer's and non-demented control cases containing numerous Hirano bodies. Part of this work has previously been presented in abstract form [31].

Materials and methods

Paraffin-embedded hippocampal sections (7 μm) containing numerous Hirano bodies in the CA1 pyramidal layer were used in this study. This material was prepared from formalin-fixed *post-mortem* brain tissue from five neuropathologically confirmed Alzheimer's disease cases (70–88-year-old) and two non-demented controls (70 and 76 years).

The sections, mounted on charged slides, were deparaffinized in toluene and endogenous peroxidase activity was quenched in 0.35% H_2O_2/methanol for 1 h at room temperature (RT). Microwave antigen retrieval [3] was performed in 0.01 M citric acid (pH 6.0) maintained at close to boiling point for 30 min (it has been previously established that such antigen retrieval is essential for detection of cleaved actin in apoptotic cells in paraffin embedded human [30,36] and rodent tissues, but not in frozen tissue sections [32,39]. This is also the case for immunodetection of activated caspase-3 with the CM1 polyclonal antiserum). Following cooling and brief water and phosphate-buffered saline (PBS) washes, the sections were incubated in a blocking solution containing 2% normal goat serum, 0.5% bovine serum albumin (BSA) and 0.01% Triton X-100 in PBS for 1 h at RT in a humidified chamber.

The sections were then incubated for 24 h at 4°C with (a) affinity-purified rabbit polyclonal fractin antibody or (b) fractin antiserum or (c) CM1 antiserum, diluted (i) 1:50 (ii) 1:1000 and (iii) 1:500, in the above blocking solution, washed in 0.05% Tween-20 in PBS and then incubated for 3 h at RT in biotinylated anti-rabbit secondary antibody (Vector, Burlingame, CA, USA; 1:500). Following further washes, the sections were incubated with Vectastain Elite ABC reagents (Vector; 1:100) for 2 h at RT, washed and reacted with 0.025% diaminobenzidine (DAB) and 0.05% H_2O_2 in Tris-buffered saline for 12 min. The sections were lightly counterstained with haematoxylin.

As negative controls (i) the primary fractin or CM1 antibody was omitted from the incubation for some sections, or (ii) in the case of fractin, the affinity-purified primary antibody or antiserum was pre-absorbed with a 10-fold excess by weight of the free YELPD peptide antigen. As positive controls, paraffin-embedded brainstem sections containing apoptotic pontine neurones from *post-mortem* cases of acute perinatal hypoxic–ischaemic brain injury [30] were used.

Results

Numerous fractin-positive Hirano bodies were seen in the CA1 stratum pyramidale of all the Alzheimer's disease and non-demented control cases, on sections immunolabelled with either the polyclonal fractin antiserum (Figure 1**c**), or the affinity-purified polyclonal fractin antibody (Figure 1**d**). There was no labelling on adjacent negative control sections where the primary antiserum/antibody had been omitted, or preabsorbed with the free YELPD peptide antigen (Figure 1**e**). On positive control sections of neonatal brain stem, there was strong fractin labelling of apoptotic, but not intact, nuclei pontis neurons (Figure 1**f**). At higher power magnification (Figure 1**g**–**j**), the Hirano bodies exhibited moderate to intense diffuse fractin immunoreactivity. Fractin labelled Hirano bodies were also seen in the CA1 stratum lacunosum of several cases. On sections immunostained with the CM1 antibody to activated caspase-3 there was no evidence of labelling of Hirano bodies, although apoptotic neurones in the control brainstem sections showed strong immunoreactivity (not shown).

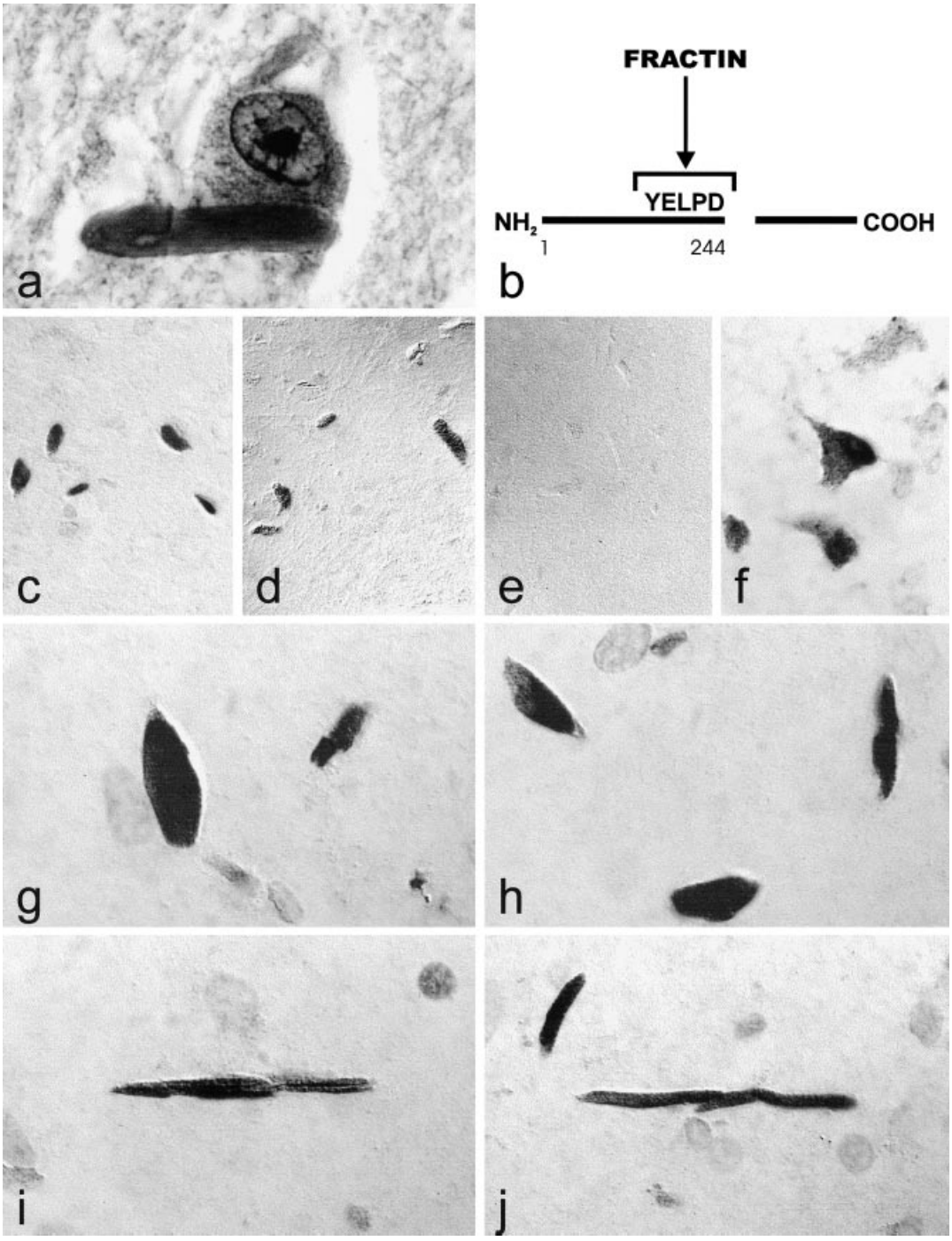

Figure 1. (**a**) Hirano body in the CA1 stratum pyramidale; Solochrome cyanine R stain. (**b**) Schematic diagram of β-actin showing caspase cleavage site at aspartate 244. The end-specific fractin polyclonal antibody was generated by injecting rabbits with synthetic β-actin residues 240–244, coupled via a K residue (K-YELPD) to a carrier protein (purified derivative of tuberculin) [39]. (**c**) Hirano bodies immunolabelled with fractin antiserum (1:1000) and (**d**) affinity-purified polyclonal fractin antibody (1:50). (**e**) Negative control; absence of fractin labelling on adjacent section following pre-absorbtion of the primary fractin antibody with the YELPD antigen. (**f**) Positive control; fractin-immunolabelled apoptotic neurons in the nuclei pontis from a *post-mortem* case of acute perinatal hypoxic–ischaemic brain injury. (**g–j**) Representative high-power views of fractin-immunolabelled Hirano bodies (fractin antiserum, 1:1000). **a** × 1400; **c–e** × 220; **f** × 900; **g–i** × 1000; **j** × 800. Photographed with Nomarski optics.

Discussion

The lattice-like filamentous aggregates comprising Hirano bodies likely consist predominantly of actin and actin-associated proteins [7,9,10,15]. The diffuse immunoreactivity of Hirano bodies with the fractin polyclonal antibody, specific for a neo-epitope at a caspase-cleavage site, shows that the actin is, at least in part, proteolytically altered. This finding suggests that caspase-like cleavage of the actin microfilamentous network may play a role in the formation of Hirano bodies. Interestingly, low level expression of the caspase generated C-terminal 15 kDa portion of actin has recently been shown to induce marked condensation and partial fragmentation of the endogenous actin network in cultured 293T embryonic kidney cells [19]. Alternatively, a proteolytic process directed at degrading pre-existing Hirano bodies may generate fractin.

Hirano bodies are likely located predominantly within neurites [10]. Consequently, their fractin immunoreactivity supports caspase-like activity within neuronal processes, as distinct from that associated with acute perikaryal apoptosis. This is consistent with increasing evidence for a role of localized caspase activation in neuritic degeneration ('neuritic/synaptic apoptosis') in Alzheimer's disease [8,11,21,39].

There are several potential explanations for the lack of immunolabelling of Hirano bodies with the CM1 antibody to activated caspase-3: the enzyme cleavage product of an abundant substrate such as actin is likely to be much more prevalent than the enzyme itself, so the concentration of caspase-3 may be too low to detect by immunohistochemistry; the actin cleavage may all have occurred at an earlier time and/or at a different location, such as the neuronal perikaryon, or the cleavage may be mediated by a different member of the caspase family. Alternatively, the presence of a cleaved-actin epitope in Hirano bodies may reflect the activity of a non-caspase enzyme. The lysosomal aspartyl protease cathepsin D is upregulated in Alzheimer's disease [1,2] and could potentially generate the fractin epitope by directly cleaving actin, or indirectly by activating caspase cascades, as there is increasing evidence of it having a causal role in apoptotic pathways [5,17,26,29]. In conclusion, the present findings indicate that Hirano bodies are composed, at least in part, of proteolytically cleaved actin.

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References

1 Callahan LM, Vaules WA, Coleman PD. Quantitative decrease in synaptophysin message expression and increase in cathepsin D message expression in Alzheimer disease neurons containing neurofibrillary tangles. *J Neuropathol Exp Neurol* 1999; **58**: 275–87
2 Cataldo AM, Barnett JL, Berman SA *et al.* Gene expression and cellular content of cathepsin D in Alzheimer's disease brain: evidence for early up-regulation of the endosomal–lysosomal system. *Neuron* 1995; **14**: 671–80
3 Cattoretti G, Pileri S, Parravicini C *et al.* Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *J Pathol* 1993; **171**: 83–98
4 Cohen GM. Caspases: the executioners of apoptosis. *Biochem J* 1997; **326**: 1–16
5 Deiss LP, Galinka H, Berissi H, Cohen O, Kimchi A. Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. *EMBO J* 1996; **15**: 3861–70
6 Galloway PG, Perry G, Gambetti P. Hirano body filaments contain actin and actin-associated proteins. *J Neuropathol Exp Neurol* 1987; **46**: 185–99
7 Galloway PG, Perry G, Kosik KS, Gambetti P. Hirano bodies contain tau protein. *Brain Res* 1987; **403**: 337–40
8 Gervais FG, Xu D, Robertson GS *et al.* Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic A beta peptide formation. *Cell* 1999; **97**: 395–406
9 Goldman JE. The association of actin with Hirano bodies. *J Neuropathol Exp Neurol* 1983; **42**: 146–52
10 Hirano A. Hirano bodies and related neuronal inclusions. *Neuropathol Appl Neurobiol* 1994; **20**: 3–11
11 Ivins KJ, Bui ET, Cotman CW. Beta-amyloid induces local neurite degeneration in cultured hippocampal neurons: evidence for neuritic apoptosis. *Neurobiol Dis* 1998; **5**: 365–78
12 Izumiyama N, Ohtsubo K, Tachikawa T, Nakamura H. Elucidation of three-dimensional ultrastructure of Hirano bodies by the quick-freeze, deep-etch and replica method. *Acta Neuropathol (Berl)* 1991; **81**: 248–54
13 Jordan-Sciutto K, Dragich J, Walcott D, Bowser R. The presence of FAC1 protein in Hirano bodies. *Neuropathol Appl Neurobiol* 1998; **24**: 359–66
14 Kayalar C, Ord T, Testa MP, Zhong LT, Bredesen DE. Cleavage of actin by interleukin 1 beta-converting enzyme to reverse DNAse i inhibition. *Proc Natl Acad Sci USA* 1996; **93**: 2234–8

15 Laas R, Hagel C. Hirano bodies and chronic alcoholism. *Neuropathol Appl Neurobiol* 1994; **20**: 12–21
16 LeBlanc A. Detection of actin cleavage in Alzheimer's disease. *Am J Pathol* 1998; **152**: 329–32
17 Levy-Strumpf N, Kimchi A. Death associated proteins (DAPs): from gene identification to the analysis of their apoptotic and tumor suppressive functions. *Oncogene* 1998; **17**: 3331–40
18 Mashima T, Naito M, Noguchi K, Miller DK, Nicholson DW, Tsuruo T. Actin cleavage by cpp-32/apopain during the development of apoptosis. *Oncogene* 1997; **14**: 1007–12
19 Mashima T, Naito M, Tsuruo T. Caspase-mediated cleavage of cytoskeletal actin plays a positive role in the process of morphological apoptosis. *Oncogene* 1999; **18**: 2423–30
20 Matsukawa N, Tooyama I, Kimura H *et al.* Increased expression of hippocampal cholinergic neurostimulating peptide-related components and their messenger RNAs in the hippocampus of aged senescence-accelerated mice. *Neuroscience* 1999; **88**: 79–92
21 Mattson MP, Partin J, Begley JG. Amyloid beta-peptide induces apoptosis-related events in synapses and dendrites. *Brain Res* 1998; **807**: 167–76
22 Mitake S, Ojika K, Katada E, Otsuka Y, Matsukawa N, Fujimori O. Accumulation of hippocampal cholinergic neurostimulating peptide (HCNP)-related components in Hirano bodies. *Neuropathol Appl Neurobiol* 1995; **21**: 35–40
23 Munch G, Cunningham AM, Riederer P, Braak E. Advanced glycation endproducts are associated with Hirano bodies in Alzheimer's disease. *Brain Res* 1998; **796**: 307–10
24 Munoz DG, Wang D, Greenberg BD. Hirano bodies accumulate c-terminal sequences of beta-amyloid precursor protein (beta-APP) epitopes. *J Neuropathol Exper Neurol* 1993; **52**: 14–21
25 Ogata J, Budzilovich GN, Cravioto H. A study of rod-like structures (Hirano bodies) in 240 normal and pathological brains. *Acta Neuropathol (Berl)* 1972; **21**: 61–7
26 Ohsawa Y, Isahara K, Kanamori S *et al.* An ultrastructural and immunohistochemical study of PC12 cells during apoptosis induced by serum deprivation with special reference to autophagy and lysosomal cathepsins. *Arch Histol Cytol* 1998; **61**: 395–403
27 Peterson C, Kress Y, Vallee R, Goldman JE. High molecular weight microtubule-associated proteins bind to actin lattices (Hirano bodies). *Acta Neuropathol (Berl)* 1988; **77**: 168–74
28 Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ* 1999; **6**: 99–104
29 Roberts LR, Adjei PN, Gores GJ. Cathepsins as effector proteases in hepatocyte apoptosis. *Cell Biochem Biophys* 1999; **30**: 71–88
30 Rossiter JP, Anderson LL, Yang F, Cole GM. Caspase-cleaved actin (fractin) immunolabelling of apoptotic neurons in acute perinatal hypoxic–ischemic brain injury. *C J Neurol Sci* 1998; **25**: 338–9
31 Rossiter JP, Anderson LL, Yang F, Cole GM. Caspase-cleaved actin (fractin) immunolabelling of Hirano bodies. *J Neuropathol Exp Neurol* 1999; **58**: 529
32 Rossiter JP, Vanderluit JL, Anderson LL, Yang F, Cole GM, Tetzlaff W. Caspase-cleaved actin (fractin) immunoreactivity of the cytoplasm and axons of apoptotic rat facial motoneurons. *Soc Neurosci Abstracts* 1998; **24**: 707–15
33 Schmidt ML, Lee VM, Trojanowski JQ. Analysis of epitopes shared by Hirano bodies and neurofilament proteins in normal and alzheimer's disease hippocampus. *Lab Invest* 1989; **60**: 513–22
34 Schochet Ss Jr, McCormick WF. Ultrastructure of Hirano bodies. *Acta Neuropathol (Berl)* 1972; **21**: 50–60
35 Srinivasan A, Roth KA, Sayers RO *et al. In situ* immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. *Cell Death Differ* 1998; **5**: 1004–16
36 Suurmeijer AJ, van der Wijk J, van Veldhuisen DJ, Yang F, Cole GM. Fractin immunostaining for the detection of apoptotic cells and apoptotic bodies in formalin-fixed and paraffin-embedded tissue. *Lab Invest* 1999; **79**: 619–20
37 Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998; **281**: 1312–6
38 Tomonaga M. Ultrastructure of Hirano bodies. *Acta Neuropathol (Berl)* 1974; **28**: 365–6
39 Yang F, Sun X, Beech W *et al.* Antibody to caspase-cleaved actin detects apoptosis in differentiated neuroblastoma and plaque-associated neurons and microglia in Alzheimer's disease. *Am J Pathol* 1998; **152**: 379–89

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