Short Communication

Nonneural Nuclear Inclusions of Androgen Receptor Protein in Spinal and Bulbar Muscular Atrophy

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Spinal and bulbar muscular atrophy is an X-linked motor neuronopathy caused by the expansion of an unstable CAG repeat in the coding region of the androgen receptor (AR) gene. Nuclear inclusions of the mutant AR protein have been shown to occur in the spinal motor neurons of spinal and bulbar muscular atrophy (Li M, Kobayashi Y, Merry D, Tanaka F, Doyu M, Hashizume Y, Fischbeck KH, Sobue G: Nuclear inclusions in spinal and bulbar muscular atrophy. *Ann Neurol* 1998 (in press)). In this study, we demonstrate the tissue-specific distribution, immunohistochemical features, and fine structure of nuclear inclusions of spinal and bulbar muscular atrophy. Nuclear inclusions were observed in affected spinal and brainstem motor neurons, but not in other, nonaffected neural tissues. Similar nuclear inclusions occurred in nonneural tissues including scrotal skin, dermis, kidney, heart, and testis, but not in the spleen, liver, and muscle. These inclusions had similar epitope features detectable by antibodies that recognize a small portion of the N-terminus of the AR protein only, and they were ubiquitinated. Electron microscopic immunohistochemistry showed dense aggregates of AR-positive granular material without limiting membrane, both in the neural and nonneural inclusions. These findings indicate that nuclear inclusions of AR protein are present in selected nonneural tissues as well as in neurons that degenerate in spinal and bulbar muscular atrophy, suggesting that a common mechanism underlies in the formation of neural and

nonneural nuclear inclusions. (*Am J Pathol* 1998, 153:695–701)

Spinal and bulbar muscular atrophy (SBMA) is an X-linked motor neuronopathy characterized by the adult onset of chronic progressive proximal limb and bulbar muscular weakness and atrophy with fasciculations, mild sensory involvement, and signs of androgen insufficiency such as testicular atrophy, gynecomastia, and feminized skin changes.^{1–3} CAG repeat expansion in the androgen receptor (AR) gene is the mutation responsible for SBMA.⁴ SBMA patients have CAG repeats in the AR gene ranging from 40 to 62 CAGs, whereas normal individuals have 10 to 36 CAGs. The number of CAGs is inversely correlated with the age at onset of the disease.^{5–7} Inter-generational CAG repeat expansion is observed, predominantly in paternal rather than maternal transmission, suggesting that the particular instability of the CAG repeat occurs in spermatogenesis.^{7,8}

Other disorders caused by CAG repeat expansion include Huntington's disease (HD),⁹ dentatorubral-pallidoluysian atrophy (DRPLA),^{10,11} Machado-Joseph disease (MJD),¹² and spinocerebellar ataxia type 1 (SCA1),¹³ type 2 (SCA2)^{14–16}, type 6 (SCA6),¹⁷ and type 7 (SCA7).¹⁸ These disorders share several characteristics that are likely relevant to a common pathological mechanism leading to selective neuronal loss. The mechanism is thought to be a toxic gain of function of the mutant gene products^{19,20} involving cell-specific protein-protein or protein-nucleic acid interactions with the products of the mutant genes.^{21–26} Intranuclear inclusions of the mutant proteins have recently been documented in the neurons of HD,^{27,28} MJD,^{29,30} SCA1,^{26,31} DRPLA,³ and SBMA motor neurons,³³ as well as in the transgenic

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Figure 1. Locations of the epitopes of Abs in the human AR.

models of HD^{34,35} and SCA1.²⁶ In all of these disorders, the inclusions can be labeled with antibodies (Abs) to the disease protein product and to ubiquitin. The inclusions are so far detected in the neurons of the affected brain areas of each disease, and rarely in other brain regions,^{27–32} despite the ubiquitous expression of the disease gene product.³⁶ Furthermore, in HD, there is a correlation between increasing CAG repeat length and increasing density of the inclusions.³⁶ Thus, intranuclear inclusions of mutant protein mediated by polyglutamine-directed aggregation are thought to have a primary pathogenic role in neuronal loss for these CAG repeat diseases.^{26–35}

In this study, we demonstrate that nuclear inclusions of mutant AR protein occur in selected nonneural tissues as well as neural tissues in SBMA.

Materials and Methods

Postmortem Tissues from SBMA and Control Subjects

Various portions of brain, spinal cord, peripheral nerve, muscle, and nonneural visceral organs were sampled from five autopsied patients with SBMA (three of whom were processed for frozen samples and all five fixed in formalin). These patients were 54 to 82 years of age at death, and each showed a typical clinical phenotype of SBMA, with dysphagia, bulbar and extremity muscle weakness, and atrophy with fasciculation. Gynecomastia and diabetes mellitus were present in four patients. Duration from onset to death was 9 to 23 years, and the causes of death were empyema, bronchiectasis, and gastric cancer. The CAG repeat lengths of the *AR* gene determined in blood samples were 40 to 52. Tissue samples for immunohistochemical analysis were obtained at autopsy, frozen in liquid nitrogen, and stored at -80°C or were fixed in 10% buffered formalin and processed for paraffin section. The pathological features of these cases were also typical for SBMA,^{2,3} with minimal variation in extent among the patients; the spinal, bulbar and pontine motor neurons were extensively depleted, with mild gliosis; sensory neurons were mildly affected, with occasional Nageotte's nodules; the posterior column of the spinal cord was depleted in a rostrally accentuated manner; the muscles were chronically denervated, and the sural nerve myelinated fibers were moderately depleted. Testicular atrophy and fatty liver changes were also

present. Other portions of the central nervous system and visceral organs were normal except for pulmonary infection in all patients and gastric cancer in one patient.

Control tissue samples were obtained from four male autopsied patients ages 54 to 71 years, who died of nonneurological diseases. The AR CAG repeat lengths of the controls were 19 to 24.

All autopsies were performed within 6 hours post-mortem.

Abs to AR Protein Used in This Study

Several polyclonal and monoclonal Abs that specifically recognize the AR protein were used in this study (Figure 1): 2F12 (mouse monoclonal Ab (immunoglobulin (Ig) G), NovoCastra, Newcastle, UK), generated against a recombinant protein of 321 amino acids from the N terminus of the human AR; PG-21 (rabbit polyclonal Ab (IgG), Affinity BioReagents, Golden, CO) and AR(N-20) (rabbit polyclonal Ab (IgG), Santa Cruz Biotechnology, Santa Cruz, CA), which recognize 21 and 20 amino acid residues of the N terminus of the AR, respectively; AR52 (rabbit polyclonal Ab (IgG), kindly provided by Dr. E. Wilson, University of North Carolina, Chapel Hill, NC), which recognizes the DNA-binding domain of the AR; and 5F4 (mouse monoclonal Ab (IgM), kindly provided by Dr. T. Demura, Department of Urology, Hokkaido Univer-

Figure 2. Immunohistochemistry of the AR protein in nuclear inclusions in the central nervous system of SBMA patients. AR staining is observed in motor neurons of the medulla oblongata (A and B) and pons (C and D). A and B are stained with PG-21, and C and D are stained with AR(N-20). Magnification: A to D, 600 $\times$.

Table 1. Distribution of the Nuclear Inclusions in SBMA

Region	PG-21/AR(N-20)	Ubiquitin	Frequency of nuclear inclusion (%)*
Neural tissues			
Cerebral cortex			
Frontal	—	—	
Temporal	—	—	
Cerebral white matter	—	—	
Caudate	—	—	
Thalamus	—	—	
Pallidum	—	—	
Pons			
Motor nuclei of the V nerve	+	+	ND
Pontine nuclei	—	—	
Medulla			
Hypoglossal nuclei	+	ND	ND
Cerebellum	—	—	
Spinal cord			
Intermediolateral nuclei	—	—	
Clarke's nuclei	—	—	
Motor neurons	+	+	8.39 ± 5.43
Dorsal root ganglia	—	—	
Sciatic nerve	—	—	
Sural nerve	—	—	
Nonneural tissues			
Muscle	—	—	
Kidney	+	+	0.59 ± 0.02
Spleen	—	—	
Heart	+	ND	Rare
Intestine	—	—	
Testis	+	+	Rare
Prostate	+	ND	ND
Scrotal skin	+	+	0.93 ± 0.50
Dermal skin	+	+	0.40

Nuclear inclusions were determined by immunohistochemistry on frozen and formalin-fixed paraffin-embedded tissues. +, nuclear inclusions are present; —, nuclear inclusions are absent. ND, not determined; Rare, only occasionally detected.

*Frequency was determined on three to five cases for motor neurons, kidney, and scrotal skin and one case for the dermal skin and was expressed as a percentage of the inclusion-positive cells over whole counted cells, as indicated in the text.

sity, Hokkaido, Japan) and AR(C-19) (rabbit polyclonal Ab (IgG), Santa Cruz Biotechnology), which recognizes the C terminus of the human AR. Characterization and binding specificities of all of these Abs to human AR were previously described.^{33,37–39} Anti-ubiquitin Ab (rabbit polyclonal Ab (IgG), Dakopatts, Glostrup, Denmark) was also used.

Light and Electron Microscopic Immunohistochemistry

Cryostat sections of 8 μ m were prepared from the frozen tissues of SBMA patients and controls, quickly dried, and lightly fixed with Zamboni fixative for 10 minutes. Then the tissue sections were washed, blocked with normal horse serum (1:20), and incubated with Abs against AR, ubiquitin, or affinity-purified mouse IgG1 at concentrations of 0.5 to 4 μ g/ml. Endogenous peroxidase was blocked by preincubation of tissue sections with 0.3% H₂O₂ in methanol for 30 minutes. Endogenous biotin was also blocked by incubation with an avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA). Immune complexes were visualized using the avidin-biotinylated horseradish peroxidase system (Elite Vector kit from Vector Laboratories) and 3,3'-diaminobenzidine (Dakopatts) substrate. Sections were counterstained with methyl green. For paraffin-

embedded samples, the 4- μ m tissue sections were deparaffinized in xylene, hydrated with alcohol, and then heated in a microwave oven for 5 minutes. The tissue sections were then processed in the same way as those for the frozen tissue sections. Immune complexes were visualized using the tyramide signal amplification system (New England Nuclear, Boston, MA) following the manufacturer's protocol.

For electron microscopic immunohistochemistry, buffered formalin-fixed, paraffin-embedded tissue sections were immunostained with Abs against AR and then incubated with horseradish peroxidase-labeled second Ab (Amersham, Poole, UK). The tissue sections were then visualized with 3,3'-diaminobenzidine (Dakopatts), fixed with 2% osmium tetroxide in 0.1 mol/L phosphate buffer, pH 7.4, and dehydrated in an alcohol gradient and embedded in epoxy resin, from which ultrathin sections were obtained and then observed under an electron microscope (Hitachi H-7000).

CAG Repeat Length Assessment for Normal and Mutant AR Genes

CAG repeat length of the AR gene was determined on the autopsied tissue samples using methods described previously.^{5,40}

Figure 3. Nuclear inclusions in nonneural tissues of SBMA patients. AR staining is observed in epithelial cells in the proximal tubules of the kidney (A), scrotal skin epidermal cells (C), and testis (E). PG-21 was used as the first Ab. These inclusions all stained positively for ubiquitin (B, D and F). Magnification: A to F, 840X.

Results

Nuclear inclusions were detected in the spinal motor neurons, neurons in the motor nucleus of the trigeminal nuclei in the pons, and neurons of the hypoglossal nuclei of the medulla oblongata (Figure 2 (see also Figure 4), Table 1). Nuclear inclusions were not detected in the neurons of cerebral cortex, caudate, thalamus, pallidum, cerebellar cortex, dentate nucleus nuclei other than motor nuclei in the brain stem, intermediolateral and Clarke's nuclei of the spinal cord, or dorsal root ganglion neurons. Nuclear inclusions similar to those in the motor neurons were detected in the scrotal skin, dermal skin, kidney, heart, and testis (Figures 3 and 4, Table 1). The inclusions were not seen in the liver, spleen, or muscle. Both neural and nonneural nuclear inclusions were strongly stained by PG-21 and AR(N-20), but not by 2F12, AR52, 5F4, or AR(C-19) (Table 2). The inclusions were spherical structures of 1 to 5 μm in diameter, and those in the nonneural tissues were generally smaller in size than those in the neurons. They were ubiquitinated (Figure 3, Tables 1 and 2). There were no detectable inclusions in cytoplasm of the neural or nonneural tissues. Most commonly we saw one inclusion in the nucleus of the individual cells, but two or three inclusions per cell were also observed (Figures 2 to 4) in both the neural and nonneural tissues. The frequency of the nuclear inclusions in the spinal motor neurons was $8.39 \pm 5.43\%$ of total remaining motor neurons. The frequencies of nuclear inclusions in nonneural tissues were $0.93 \pm 0.50\%$ in scrotal skin

epidermal cells, 0.40% in nonscrotal skin epidermal cells, and $0.59 \pm 0.02\%$ in kidney tubular cells, and nuclear inclusions were observed only occasionally in the heart and testis.

Electron microscopically, these inclusions consisted of granular dense aggregates of AR-positive materials without limiting membrane (Figure 4), and the morphological appearance was quite similar among the inclusions in the spinal motor neurons, scrotal skin, and kidney. There was no evidence of filamentous structures as reported in HD²⁷ and MJD³⁰ (Figure 4).

The morphological appearance of the motor neurons and nonneural cells with nuclear inclusions was indistinguishable from those without inclusions.

Neural and nonneural tissues from four control cases were also examined in the same manner as that for SBMA cases, but the inclusions were not seen in the control individuals.

Discussion

The present study demonstrates that nuclear inclusions of the mutant AR protein are present in the motor neurons of the spinal cord and the brain stem; moreover, similar inclusions are also detected in certain nonneural tissues of the SBMA patients, indicating that the occurrence of the nuclear inclusions is not restricted to the affected neural tissues, but is also found in certain nonneural tissues.

The electron microscopic appearance of the AR-positive dense aggregates with similar granular size without limiting membrane was common to both neural and nonneural tissues. Furthermore, a characteristic selective staining pattern of the nuclear inclusions seen with only Abs recognizing N-terminal 20 and 21 amino acids of the AR protein was also common among the neural and nonneural tissues. These observations strongly suggest that same mechanism is involved in formation of AR-positive components in the nuclear inclusions in both the neural and nonneural tissues. The immunostaining pattern suggests that only a small portion of the N terminus of the AR protein is available as an epitope in the nuclear inclusions, and other portions of the AR may be masked within the inclusions of the mutant protein, or alternatively, the AR protein may be cleaved by proteolytic activity resulting in N-terminal fragments that participate in the aggregation, as was suggested in other polyglutamine diseases.^{26-36,41} In addition, these AR nuclear inclusions are ubiquitinated in the nonneural as well as neural tissues, indicating that the nuclear inclusion is a pathological structure of the mutant AR, even in the nonneural tissues, where the pathological involvement is not apparent. Our electron microscopic and light microscopic immunohistochemical data indicate that nuclear inclusions in both motor neurons and nonneural tissues are identical in morphological and immunochemical features. Furthermore, absence of filamentous structures in the nuclear inclusion of SBMA was different from observations in HD²⁷ and MJD.³⁰ This difference may suggest that the pathway of the nuclear aggregation is different among

Figure 4. Electron microscopic immunohistochemical features of the nuclear inclusions in motor neurons and scrotal skin epidermal cells of SBMA patients. Dense granular aggregation of AR-immunoreactive material without a limiting membrane was found in both motor neurons (A to C) and scrotal skin epidermal cells (D to F). Nucleoli, which are larger than the inclusions, are clearly distinguished. The same inclusions observed light microscopically (A and D) are demonstrated electron microscopically (B, C, E, and F). AR(N-20) was used as the first Ab. Magnification: A, 720 $\times$; D, 1,000 $\times$; B and E, 2,000 $\times$; C, 10,000 $\times$; F, 15,000 $\times$.

the different protein products or, alternatively, may represent variances in sample preparation.

In polyglutamine diseases that have been analyzed to date, the nuclear inclusions have been shown to occur selectively in neurons of the affected brain regions. The selective occurrence of nuclear inclusions in the affected cells of central nervous system in SBMA that we observed in this study agrees well with observations of HD, MJD, SCA1, and DRPLA,²⁷⁻³² as well as our previous

observations in SBMA.³³ However, the appearance of similar nuclear inclusions in the nonneural tissues observed in this study is novel. It is an important question why the neurons are selectively affected despite the presence of nuclear inclusions in both the affected neurons and nonaffected nonneural tissues. The cells of the nonneural tissues are mitotic cells in contrast to motor neurons; the epidermal cells in the scrotal and dermal skin and epithelial cells in the kidney tubules are all

Table 2. Immunoreactivity of Nuclear Inclusions in SBMA

Tissues	PG-21	AR(N-20)	2F12	AR52	5F4	AR(C-19)	Ubiquitin
Spinal motor neuron	+	+	—	—	—	—	+
Kidney	+	+	—	ND	ND	—	+
Scrotal skin	+	+	—	—	—	—	+
Dermal skin	+	+	—	—	—	—	+
Testis	+	+	—	ND	ND	—	+

PG-21, AR(N-20), and 2F12 recognize the 21, 20, and 321 N-terminal amino acids of AR, respectively. AR 52 recognizes the DNA binding domain of AR. 5F4 and AR(C-19) recognize the C terminus of the AR protein. +, positive staining; —, negative staining; ND, not determined.

capable of mitosis in adulthood, and are eventually replaced by newly generated cells. Hence, those cells with toxic effects associated with the nuclear inclusions may be replaced by turnover. It may also be that neurons are particularly susceptible to whatever deleterious effects the inclusions may have. As demonstrated in the HD transgenic model,³⁴ a long latent period is necessary for inclusions to induce neuronal death. Neurons, as postmitotic cells, may be specifically affected because they survive long enough for the inclusions to have effect, whereas nonneural cells with nuclear inclusions turn over before the inclusions have pathological consequences. The significantly lower frequency of nuclear inclusion in nonneural tissues than in neurons may support this view. These differences in cell turnover rates could contribute to selective neuronal degeneration and neuronal loss.

Another interesting observation in this study is that the presence of nuclear inclusions is also selective among the various nonneural tissues, as it is in neural tissues. The inclusions are frequent in scrotal skin, dermal skin, and kidney; only occasionally seen in the testis and heart muscle; and not detected in spleen, liver, and muscle. The distribution of inclusions is not related to the expression level of mutant AR in these tissues.³³ AR protein is highly expressed in the testis, skin, and muscle, whereas it is low in the kidney, spleen, and liver.³³ A similar lack of correlation between the AR protein expression levels and pathological involvement is seen in the nervous system; neurons in Onuf's nuclei and Purkinje cells, for example, express a relatively large amount of AR protein,³³ but these neurons are not affected in SBMA. The underlying mechanism that induces the selective formation of nuclear inclusions in neural as well as nonneural tissues remains undetermined; specific factors may be present only in the tissues with the nuclear inclusions, such as a protease that cleaves the N-terminal portion from the full-length AR protein, or specific proteins that interact with the mutant AR, as demonstrated in SCA1^{23,26} and HD,^{21,22,24,25} may contribute to the cell type-specific formation of the nuclear inclusions. Alternatively, a degenerative process leading to apoptotic cell death with activation of a protease such as caspase 3 may be involved in the formation of nuclear inclusions as proposed in HD, DRPLA, MJD, and SBMA.^{41,42} Furthermore, the tissue-specific inclusion formation could be influenced by the different AR functional activity among the different tissues. The nonneural tissues we found to contain nuclear inclusions have relatively rapid cell turnover, with physiological cell degeneration and cell death.

In summary, cell type-specific nuclear inclusions are present in the nonneural as well as neural tissues in SBMA, but the high physiological rate of cell turnover may contribute to the absence of functional impairment with the formation of nuclear inclusions in the nonneural tissues.

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