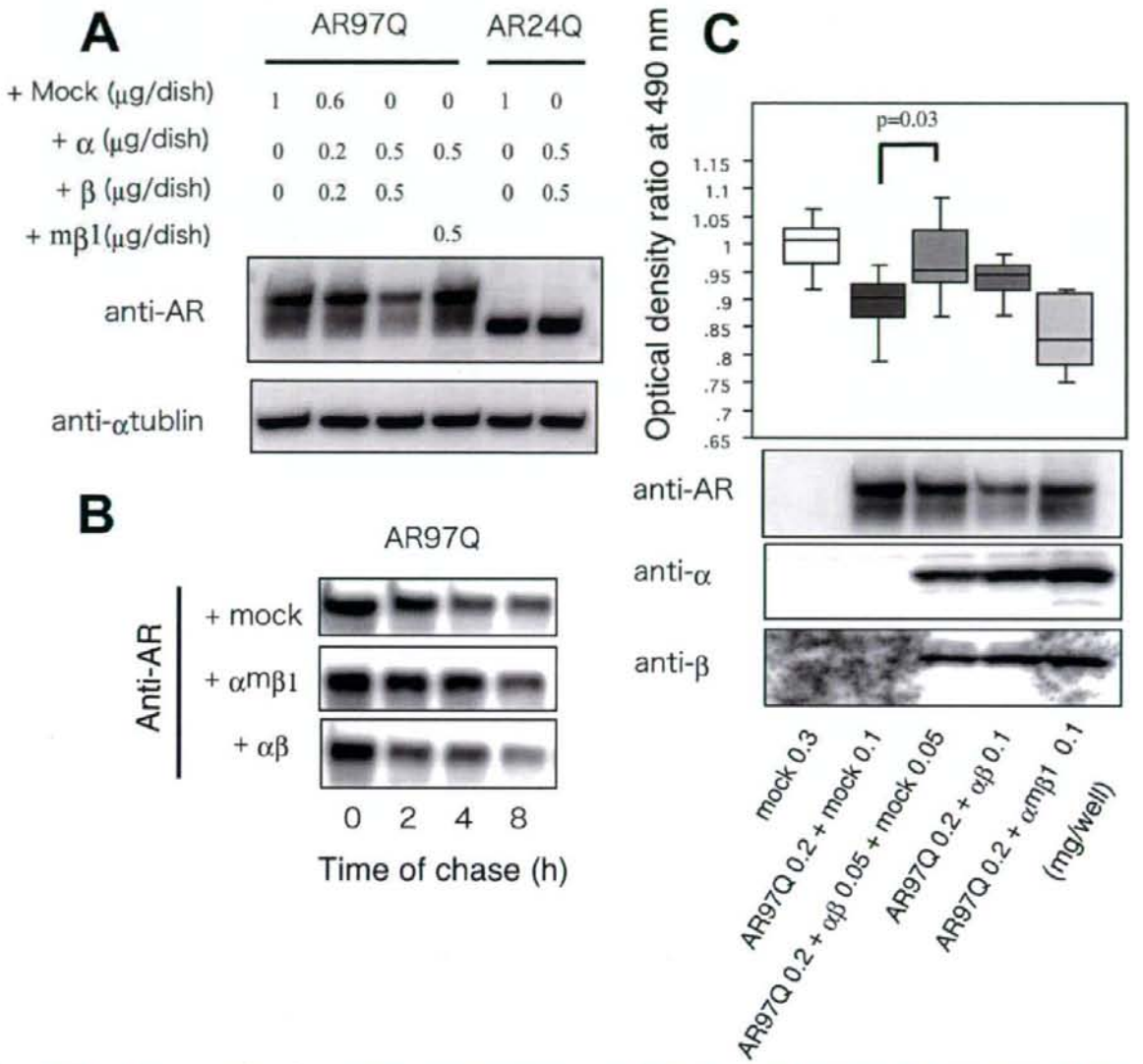

FIGURE 6. ***M. mazei* proteasome degrades mutant androgen receptor with expanded polyglutamine tract and reduces its cellular toxicity.** *A*, Neuro2a cells grown on 6-cm dishes and co-transfected with 1 μg of AR containing either normal (24Q) or expanded (97Q) polyglutamine tract vectors and increasing doses of Mm proteasome subunits were analyzed. The levels of AR^{97Q} proteins were reduced as Mm proteasome αβ increased. *B*, cycloheximide chase analysis (see "Experimental Procedures") showing that the half-lives of AR^{97Q} proteins were decreased in the presence of Mm 20 S proteasome αβ. Transfected DNA dose/6-cm dish was as follows: AR^{97Q} (1 μg), α-subunit (0.5 μg), β-subunit (0.5 μg). *C*, the rescue effect of Mm proteasome αβ expression on cell viability in AR^{97Q}-transfected HEK293 cells as shown in an MTS assay. The *box plots* show the median values (*center line* of *box*), the 25th (*lower line* of *box*), 75th (*upper line* of *box*), 10th (*lower T bar*), and 90th (*upper T bar*) percentiles in each group ($n = 3 \times 6$ wells). The *numbers* indicate transfected DNA dose in a well of a 96-well plate (αβ, 0.1 μg; α, 0.05 μg; β, 0.05 μg). The expression levels of AR, α-subunit, and β-subunit at analyzed points are shown.

GFP-positive $SOD1^{G93A}$ aggregates are also anti-His positive, whereas the cells expressing wild-type SOD1-GFP are diffusely stained with anti-His antibody. There were no GFP-negative inclusion bodies stained with anti-His antibody, indicating that Mm proteasome co-localizes with the inclusion bodies consisting of mutant SOD1 in the vicinity of the nucleus. The percentages of aggregate-positive cells among the GFP-positive cells were determined in Fig. 5*B*. $SOD1^{G93A}$ aggregates were significantly reduced when co-expressed with Mm proteasome αβ.

M. mazei Proteasome Degrades Specifically Mutant Androgen Receptor with Expanded Polyglutamine Tract and Reduces Its Cellular Toxicity—To demonstrate the ability of the Mm proteasome to degrade aggregation-prone proteins, we examined the AR with expanded polyglutamine tract (97-repeated glutamine; 97Q) protein, the causative protein of spinal and bulbar muscular atrophy. Similar to the results obtained with SOD1 proteins, Fig. 6*A* shows that in Neuro2a cells, the levels of mutant AR (97Q) were markedly reduced as the expression of

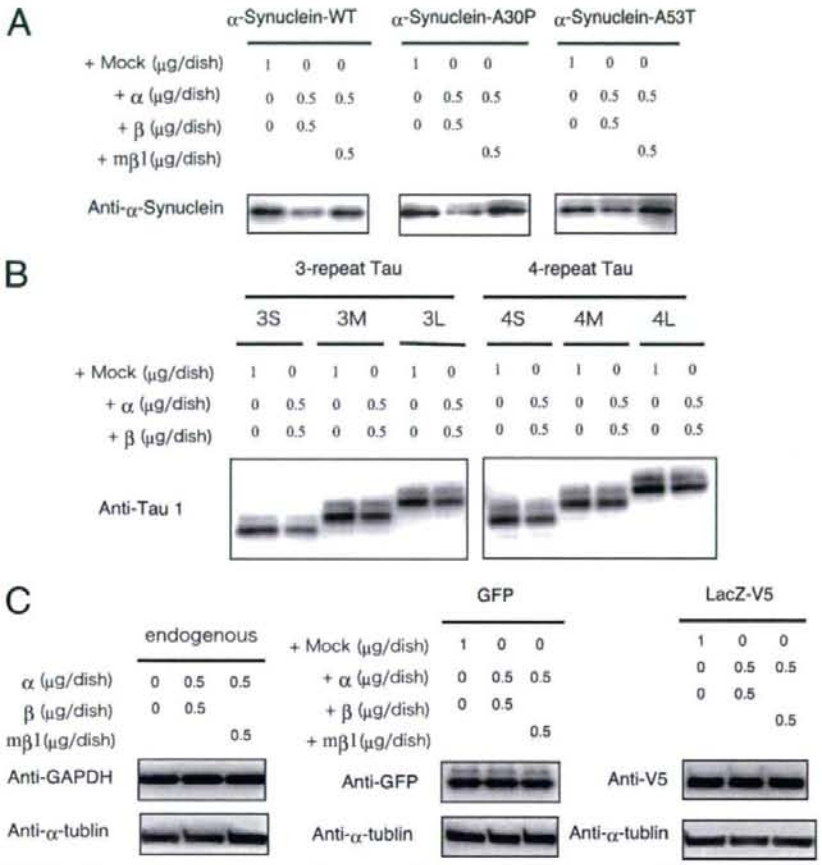

FIGURE 7. ***M. mazei* proteasome degrades aggregation-prone but not non-aggregation-prone proteins.** Neuro2a cells grown on 6-cm dishes and co-transfected with Mm proteasome subunits vectors or mock and 1 μg of α-synuclein vectors (wild type, A30P, and A53T) (*A*), Tau vectors (six isoforms: three (3L, 3M, and 3S) or four (4L, 4M, and 4S) tubulin binding domains in the C-terminal portion and two (3L and 4L), one (3M and 4M), or no (3S and 4S) inserts of 29 amino acids each in the N-terminal portion) (*B*), or empty GFP vector or LacZ-V5 vector (*C*). *A* and *B*, the expression levels of all of α-synuclein and tau proteins were reduced when co-transfected with the Mm proteasome αβ. *C*, the expression levels of endogenous glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), GFP, and LacZ-V5 proteins were not changed in the presence of the Mm proteasome αβ.

Mm proteasome αβ increased, but they were unaffected by the expression of the Mm proteasome αmβ1. On the other hand, wild-type AR (24-repeated glutamine; 24Q) levels were not affected by the expression of Mm proteasome αβ. Cycloheximide-chasing analysis demonstrated that the half-life of mutant AR (97Q) was reduced in the presence of the Mm proteasome but not in the presence of the mutant Mm proteasome (Fig. 6*B*). The viability of cells expressing mutant AR (97Q) was reduced compared with wild-type AR (24Q), and this reduction was attenuated by the co-transfection with Mm proteasome αβ (Fig. 6*C*). These results show that Mm proteasome αβ can accelerate the degradation of the aggregation-prone mutant AR with expanded polyglutamine tract and possibly protect the cells from its toxicities.

M. mazei Proteasome Degrades Other Aggregation-prone Proteins but Not Non-aggregation-prone Proteins—To determine whether the Mm proteasome degrades other aggregation-prone proteins as well, we examined its effects on α-synuclein (wild-type, A53T, and A30P) and six isoforms of wild-type tau protein in Neuro2a cells. The six tau isoforms contained either three (3L, 3M, and 3S) or four (4L, 4M, and 4S) microtubule binding domains in the C-terminal portion and two (3L, 4L), one (3M, 4M), or no (3S, 4S) inserts of 29 amino acids each in the N-terminal portion. Similar to the results obtained with the mutant SOD1 and AR with an expanded polyglutamine tract, the expression levels of all α-synuclein and tau proteins were reduced in the presence of Mm proteasome αβ (Fig. 7, *A* and *B*). Although the degradations of wild-type SOD1 and AR proteins were not accelerated by Mm proteasome, the expression levels of α-synuclein including wild-type and all of the six forms of wild-type tau were reduced.

We also examined whether Mm proteasomes degrade non-aggregation-prone proteins such as GFP or LacZ. Fig. 7*C* shows that the Mm proteasome does not affect the degradation of the exogenously expressed proteins, GFP and LacZ.

DISCUSSION

In this study, we showed that the archaeal Mm proteasome α- and β-subunits properly assembled to have proteolytic activity and accelerate the degradation of aggregation-prone, neurodegeneration-associated proteins in mammalian cells. Archaeal proteasomes contain 14 identical active sites that, although originally classified as chymotrypsin-like, were later shown to cleave after acidic and basic residues (22), and they consist of only one type of each of the α- and β-subunits (6). A comparison between archaeal and eukaryotic proteasomes *in vitro* showed that archaeal proteasomes are far more active in degrading poly(Q) peptides than are eukaryotic proteasomes (9). We utilized this potential power and manageability of archaeal proteasomes to degrade abnormal proteins that could not be effectively degraded by eukaryotic proteasomes. This is the first report showing that archaeal proteasomes can work to accelerate degradation of aggregation-prone proteins in mammalian cells.

Mm proteasomes promoted degradation of mutant SOD1, AR with an expanded polyglutamine tract, wild-type and mutant α-synuclein, and six isoforms of wild-type tau. The first two proteins, mutant SOD1 and AR with an expanded polyglutamine tract, exhibit toxicity in cell culture models. Mice overexpressing these mutant proteins display abnormal aggrega-

tions in their motor neurons and significant loss of motor functions, and they have been used as disease models (23, 24). Mm proteasomes accelerated the degradation of only the mutant forms of these two proteins and not that of the nonaggregating wild-type forms. Furthermore, chasing studies (Fig. 3, *A* and *B*) confirmed our belief that Mm proteasomes directly accelerate the degradation of mutant proteins.

However, both the wild-type and two mutants of α-synuclein as well as six isoforms of wild-type tau were also degraded by Mm proteasomes (Fig. 7). α-Synuclein and tau are pathogenically different proteins from SOD1 and AR, since they are known to accumulate as wild-type proteins in the affected lesions of PD and AD, respectively. Aggregation of the presynaptic protein, α-synuclein, has been implicated in synucleinopathies, such as sporadic and familial PD, diffuse Lewy body disease, and multiple-system atrophy (25). In sporadic PD patients, wild-type α-synuclein is accumulated, and increased expression of wild-type α-synuclein is also observed (26). Proteasomal dysfunction has been thought to impair α-synuclein degradation and thereby to facilitate its aggregation (27). Three- and four-repeat wild-type tau are among the proteins characteristically detected in neurofibrillary tangles formed by paired helical filaments in sporadic AD (28). Decreased proteasomal activity has been also reported in the AD brain (29). α-Synuclein and tau are both relatively easily misfolded, which leads to the formation of aggregates, even in their wild-type forms (30, 31), thus possibly explaining why the Mm proteasomes degraded wild-type α-synuclein and tau. Mm proteasomes might be able to recognize a wide range of aggregation-prone proteins, whereas they do not affect the degradation of exogenously expressed nonaggregating proteins, such as GFP and LacZ, or abundant endogenous proteins, such as α-tubulin and glyceraldehyde-3-phosphate dehydrogenase (Fig. 7).

The question raised here is what is the molecular mechanism of such selective, mutant species-dependant degradation. Archaeal 20 S proteasomes contain proteasome-activating nucleotidase, PAN, enabling substrates to enter the proteasomes easily and effectively (8). PAN has a chaperone-like activity to unfold aggregated proteins (32) and is thought to be an evolutionary precursor to the 19 S base in eukaryotic cells (8). Archaeal recognition tags (like ubiquitin tags in eukaryotic cells) have not been identified yet. However, archaeal 20 S proteasomes have been reported to rapidly degrade polyglutamine aggregates *in vitro*, without the help of PAN (9). Here we confirmed that this PAN-independent degradation by Mm 20 S proteasomes could occur in mammalian cells. Since the pore diameter of the closed gate in 20 S proteasomes is estimated to be much smaller than that of aggregated proteins (33), the question is, how do the unfolded substrate proteins enter the 20 S proteasomes? One hypothesis might be that the α-ring in Mm proteasomes has chaperone-like activity to recognize and unfold the aggregation-prone proteins or misfolded proteins. The gated channel in the α-ring of the archaeal 20 S proteasomes is thought to regulate substrate entry into the proteasomes and is assumed to be in either an open (34) or a closed state (2, 33) *in vitro*. In our experiments, the gate-free Mm 20 S proteasome $\Delta\alpha\beta$ substantially reduced cell viability, but the Mm proteasome $\alpha\beta$, with the "gate," had little toxic effect on the cells and, furthermore, accelerated the degradation of mutant proteins. This would be hard to explain if the gate is always in the closed state. There is a possibility that when Mm proteasomes gather, actively or passively, near aggregation-prone proteins, the α-ring opens its gate and unfolds the aggregated proteins, enabling them to enter the proteasomes to be degraded.

Some kinds of molecular chaperones, such as Hsp90, -70, and -27, have been reported to assist in the selective degradation of mutant SOD1 and AR proteins in proteasome degradation pathways (35, 17). However, neither the protein levels of molecular chaperones (Hsp90, -70, -40, and -27) nor the ubiquitylation levels of mutant SOD1 and AR were changed in the presence of Mm proteasome $\alpha\beta$ expression (data not shown), thus supporting the idea that endogenous ubiquitin-proteasome degradation pathways possibly did not play an important role in the accelerated degradation of mutant proteins. Further study is needed to elucidate the molecular mechanisms of selective recognition of misfolded aggregation-prone proteins by Mm proteasomes.

In this paper, we demonstrated that Mm proteasomes could effectively degrade neurodegenerative disease-related aggregation-prone proteins *in vivo*. Further studies are needed to determine whether archaeal proteasomes can be available to treat diseases in which toxic gain of proteins is causative.

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REVIEW

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Modulation of Hsp90 function in neurodegenerative disorders: a molecular-targeted therapy against disease-causing protein

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Abstract Abnormal accumulation of disease-causing protein is a commonly observed characteristic in chronic neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and polyglutamine (polyQ) diseases. A therapeutic approach that could selectively eliminate would be a promising remedy for neurodegenerative disorders. Spinal and bulbar muscular atrophy (SBMA), one of the polyQ diseases, is a late-onset motor neuron disease characterized by proximal muscle atrophy, weakness, contraction fasciculations, and bulbar involvement. The pathogenic gene product is polyQ-expanded androgen receptor (AR), which belongs to the heat shock protein (Hsp) 90 client protein family. 17-Allylamino-17-demethoxygeldanamycin (17-AAG), a novel Hsp90 inhibitor, is a new derivative of geldanamycin that shares its important biological activities but shows less toxicity. 17-AAG is now in phase II clinical trials as a potential anticancer agent because of its ability to selectively degrade several oncoproteins. We have recently demonstrated the efficacy and safety of 17-AAG in a mouse model of SBMA. The administration of 17-AAG significantly ameliorated polyQ-mediated motor neuron degeneration by reducing the total amount of mutant AR. 17-AAG accomplished the preferential reduction of mutant AR mainly through Hsp90 chaperone complex formation and subsequent proteasome-dependent degradation. 17-AAG induced Hsp70 and Hsp40 in vivo as previously reported; however, its ability to induce HSPs was limited, suggesting that the HSP induction might support the degradation of mutant protein. The ability of 17-AAG to preferentially degrade mutant protein would be directly applicable to SBMA and other neurodegenerative diseases in which the disease-causing proteins also belong to the Hsp90 client protein family. Our proposed therapeutic approach, modulation of Hsp90 function by 17-AAG treatment, has emerged as a candidate for molecular-targeted therapies for neurodegenerative diseases. This review will consider our research findings and discuss the possibility of a clinical application of 17-AAG to SBMA and other neurodegenerative diseases.

Keywords Hsp90 inhibitor · Hsp90–client protein complex · Proteasomal degradation · Polyglutamine · Neurodegenerative diseases

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Introduction

Polyglutamine (polyQ) diseases are caused by the expansion of a trinucleotide CAG repeat encoding glutamine in the causative genes and, to date, nine disorders have been identified as polyQ diseases [1]. Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease, was the first polyQ disease to be identified [2] and is characterized by premature muscular exhaustion, slow progressive muscular weakness, atrophy, and fasciculation in bulbar and limb muscles [3]. In SBMA, the pathogenic gene product is the androgen receptor (AR), which contains an abnormally expanded polyQ. The number of polymorphic CAG repeats in the *AR* gene is normally 14 to 32, but it is expanded to 40 to 62 CAGs in SBMA patients [4]. A correlation exists between the number of CAG repeats and disease severity [5]. The pathologic features of SBMA are motor neuron loss in the spinal cord and brainstem [3], and diffuse nuclear accumulations and nuclear inclusions (NIs) containing the mutant AR in the residual motor neurons and certain visceral organs [6].

Heat shock protein (Hsp) 90, one of the molecular chaperones, is essential for function and stability of the AR, the C-terminus of which has a high affinity for Hsp90, inducing the conformational change required for its nuclear translocation after ligand activation [7–9]. Hsp90 functions in a multi-component complex of chaperone proteins including Hsp70, Hop (Hsp70 and Hsp90 organizing protein), Cdc37, and p23. In addition, Hsp90 is involved in the folding, activation, and assembly of several proteins, known as Hsp90 client proteins [10]. As numerous oncoproteins belonging to the Hsp90 client protein family are selectively degraded in the ubiquitin–proteasome system (UPS) by Hsp90 inhibitors, 17-allylamino-17-demethoxygeldanamycin (17-AAG), a first-in-class Hsp90 inhibitor, is now under clinical trials as a novel molecular-targeted agent for a wide range of malignancies [11]. AR also belongs to the Hsp90 client protein family and is degraded in the presence of Hsp90 inhibitors [12–14].

In view of this ability of Hsp90 inhibitors to degrade Hsp90 client proteins, we have recently demonstrated that 17-AAG markedly ameliorated polyQ-mediated motor neuron degeneration through degradation of mutant AR [15]. This is apparently different from previous strategies employed against polyQ diseases, which unavoidably allowed abnormal protein to remain and placed much value mainly on the inhibition of protein aggregation. We consider that the ability to facilitate degradation of disease-causing protein by modulation of Hsp90 function would be of value when applied to SBMA and other related neurodegenerative diseases. In this paper, we review our research findings compared with previous studies and discuss the clinical application of Hsp90 inhibitors to neurodegenerative diseases.

Development of Hsp90 inhibitors

The most classical Hsp90 inhibitor is geldanamycin (GA), a natural product that was developed as an antifungal agent [16]. Later, GA was also found to have a potent and selective anti-tumor effect against a wide range of malignancies [17]. Although GA showed potential as a novel anti-cancer agent [18], this agent was also found to have intolerable liver toxicity [19]. To overcome this GA-induced liver toxicity, scientists at the US National Cancer Institute succeeded in developing a new derivative of GA, 17-AAG, that shares its important biological activities [20] but shows less toxicity [21]. Owing to this promising derivative 17-AAG, Hsp90 inhibitors have taken a major developmental leap in their clinical applications, and 17-AAG is now in phase clinical trials with encouraging results as an anti-cancer agent [22–26]. To generate more selective and less toxic derivatives than 17-AAG, further development of Hsp90 inhibitors is also being pursued [27–29].

The anti-tumor effect of Hsp90 inhibitors was previously thought to be due to the inhibition of tyrosine kinase [30]. The mechanism subsequently proved to be based on their ability to specifically bind to the Hsp90 ATP-binding site, thereby modulating Hsp90 function [31, 32] and proteasomal degradation of Hsp90 client proteins. As numerous oncoproteins were shown to belong to the family of Hsp90 client proteins [10]. Hsp90 inhibitors are expected to become a new strategy in anti-tumor therapy [18]. Hsp90 inhibitors including GA and 17-AAG have been shown to have an advantageously higher selectivity for tumor cells compared with general anti-tumor agents [17]. Studies by Kamal et al. suggest a mechanism for this selectivity; Hsp90 in tumor cells is more likely to be incorporated in the Hsp90 multi-chaperone complex than the Hsp90 in normal cells is, thereby increasing their binding affinity to 17-AAG by more than 100-fold [33, 34].

We thought that this selectivity of Hsp90 inhibitors would also be advantageous for the treatment of neurodegenerative diseases. However, as neurodegenerative diseases generally follow a chronic progression and the medical treatment is long compared with that for malignancy, the toxic side effects of the treatments would need to be extensively suppressed. Therefore, we decided to explore the possibility of using 17-AAG as a therapeutic agent for neurodegenerative diseases by examining its effects on mutant AR in cultured cells and in a mouse model of SBMA.

A model mouse of SBMA and a potent hormonal therapy

We had previously generated transgenic mice expressing the full-length human AR containing either 24 or 97 CAG repeats under the control of a cytomegalovirus enhancer and a chicken actin promoter [35]. The mice with 97 CAG repeats (AR-97Q) exhibited progressive motor impairment, while none of those with 24 CAG repeats (AR-24Q) showed abnormal phenotypes [35]. Other laboratories have

also generated various animal models of SBMA, almost all of which display phenotypic expressions of motor dysfunction [36]. For researching a truly effective molecular-targeted therapy, it is imperative to do so in a model that approximates the native state and metabolism of the disease-causing protein in vivo. We therefore consider that our SBMA mice carrying full-length AR are more beneficial for investigating therapeutic agents than those carrying the truncated one. Our transgenic mice indeed have a very severe phenotype, which is different to some extent from the human form of the disease, but they demonstrate polyQ-induced motor neuron degeneration and provide a beneficial tool to screen therapeutic agents to rescue this condition as we previously described [37]. For further details about the clinical features and our mouse model of SBMA, please refer to Katsuno et al. [38, 39].

We have already experimentally demonstrated several therapeutic approaches using this model [35, 37, 40, 41] and consequently confirmed that leuprorelin, a lutenizing hormone-releasing hormone agonist that reduces testosterone release from the testis, significantly rescued motor dysfunction and nuclear accumulation of mutant AR in our SBMA mice. Due to its minimal invasiveness established in human and its powerful efficacy demonstrated in the above model [37], this hormonal therapy has already been in human clinical trials with encouraging results [42]. However, it is an extremely specialized therapy for SBMA and cannot be applied to other polyQ diseases [35, 37, 38]. In contrast to this hormonal therapy, 17-AAG would be a potential therapeutic agent for SBMA as well as for other related diseases [15].

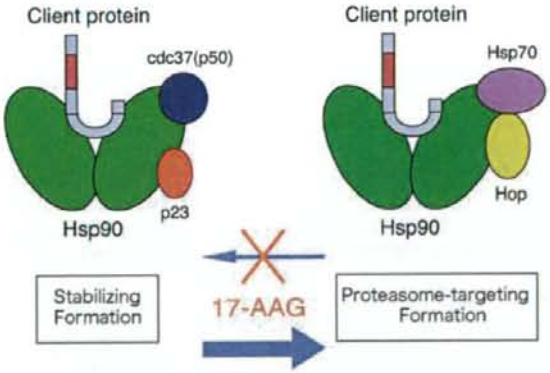

Fig. 1 Hsp90-inhibitor-induced change in Hsp90 complex. Hsp90 inhibitor (17-AAG) specifically binds ATP-binding site of Hsp90, resulting in a shift of the Hsp90 complex. To exert its effects on client proteins, Hsp90 functions in a multi-component complex of co-chaperone proteins including Hsp70, Hop, Cdc37, and p23. Two main forms of this complex exist. One complex is a proteasome-targeting form associated with Hsp70 and Hop, and the other is a stabilizing form with Cdc37 and p23. Hsp90 inhibitors block the progression of the Hsp90 complex toward the stabilizing form and shift it to the proteasomal-targeting form. This figure is modified from a model proposed by Neckers [14]

17-AAG alters the form of the Hsp90 complex, leading to proteasomal degradation of mutant AR

Hsp90 functions in a multi-component complex of chaperone proteins, including Hsp70, Hop, Cdc37, and p23, leading to the folding, activation, and assembly of Hsp90 client proteins [10]. In addition, the Hsp90 complexes are thought to exist in two main forms: one complex is a proteasome-targeting form associated with Hsp70 and Hop, and the other is a stabilizing form with Cdc37 and p23 [14, 43–45] (Fig. 1). Hop is known to independently bind to both Hsp90 and Hsp70, which promotes the Hsp90/Hsp70 linkage, and is thought to direct the triage decision for client proteins by bridging the Hsp90–Hsp70 interaction [45]. On the other hand, p23 is thought to modulate Hsp90 activity in the last stages of the chaperoning pathway, leading to the stabilization of Hsp90 client proteins in an ATP-dependent manner [46]. Hsp90 inhibitors, including 17-AAG, inhibit the ATP-dependent progression of the Hsp90 complex toward the stabilizing form and shift it to the proteasomal-targeting form, resulting in proteasomal degradation of the Hsp90 client protein [47, 48]. Steroid receptors, including the progesterone receptor and the glucocorticoid receptor, were the first Hsp90 client proteins to be identified [49, 50]. As for AR, Hsp90 is essential to maintain its high ligand-binding affinity and its stabilization [7, 12]. In practice, Hsp90 inhibitors reduce androgen ligand-binding affinity and induce the degradation of AR [12, 13].

To address the question of whether 17-AAG also promotes the degradation of the disease-causing protein of SBMA, polyQ-expanded mutant AR, we treated SH-SY5Y cells highly expressing the wild-type (AR-24Q) or mutant (AR-97Q) AR for 6 h with 36 μM 17-AAG or with dimethyl sulfoxide (DMSO) as control, in the absence or presence of the proteasome inhibitor, MG132. Immunoblot analysis demonstrated that the monomeric mutant AR decreased significantly more than the wild type did, suggesting that the mutant AR is more sensitive to 17-AAG than the wild type is. The degradation of wild-type and mutant AR by 17-AAG was completely blocked by the proteasome inhibitor, MG132 (Fig. 2a), suggesting that 17-AAG-facilitated degradation was dependent on the proteasome system as previously reported [47, 48].

Next, we examined changes in the Hsp90 chaperone complex in wild-type and mutant AR-expressing cultured cells after 17-AAG treatments. Immunoprecipitation with anti-AR antibody revealed that Hsp90-chaperone-complex-associated Hop was markedly increased, and p23 decreased in a 17-AAG dose-dependent manner, suggesting that 17-AAG resulted in the shifting of the AR-Hsp90 chaperone complex from a mature stabilizing form with p23 to a proteasome-targeting form with Hop. This chaperone complex shift appears to be very rapid as has been suggested previously [50, 51]. The loss of p23 from the mutant AR-Hsp90 complex was significantly greater than that from the wild-type one (Fig. 2b). Furthermore, these studies also strongly suggested that the mutant AR is more prone to be in the multi-chaperone complexes of

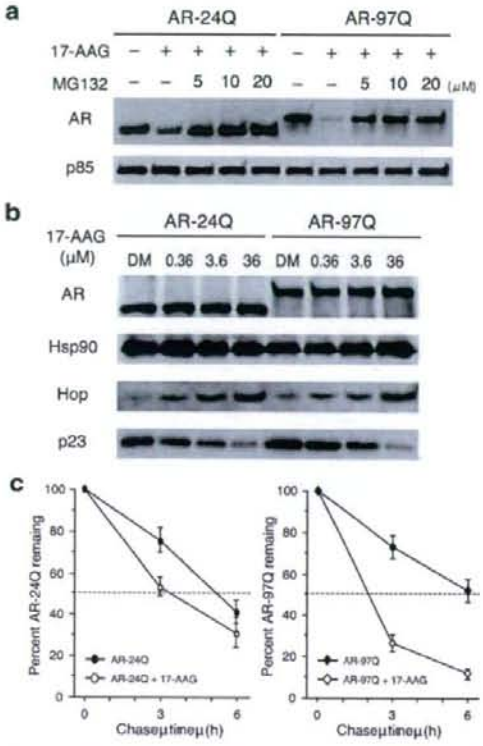

Fig. 2 a–c 17-AAG-induced changes in the AR–Hsp90 complex: correlation to proteasomal degradation. **a** 17-AAG treatment (36 μM, 6 h) of transfected SHSY5Y cells reduced the levels of mutant AR (AR-97Q) significantly more than the wild-type AR (AR-24Q); however, both decreases were completely blocked by the proteasomal inhibitor, MG132. **b** Immunoblots of lysates from transfected cells treated for 30 min with 17-AAG and immunoprecipitated with AR-specific antibody. The short time exposure to 17-AAG did not decrease the amount of mutant AR, but there were dose-dependent changes in both Hop and p23. There were no changes in the amounts of Hsp90 complexed with mutant AR. There were no changes in the expression of Hop, p23, and Hsp90 in whole lysates in the presence of 17-AAG (data not shown) **c** The effects of 17-AAG on the half-life of wild-type and mutant AR assessed from pulse-chase experiments. The amounts of AR-24Q remaining in the absence and presence of 17-AAG are indicated by *closed circles* (•) and *open circles* (○), respectively. The amounts of AR-97Q remaining in the absence and presence of 17-AAG are indicated by *closed* (♦) and *open* (◊) *diamonds*, respectively. Mutant AR was degraded more rapidly than the wild-type AR in the presence of 17-AAG. Values are expressed as means±SE (n=4)

Hsp90 with p23, which eventually enhances 17-AAG-dependent proteasomal degradation of mutant AR.

To determine whether the decrease in AR was due to protein degradation or to changes in RNA expression, the turnover of wild-type and mutant AR were then assessed with a pulse-chase labeling assay. In the presence of 17-AAG, the mutant AR and the wild-type AR had half-lives of 2 h and 3.5 h, respectively (Fig. 2c), while the mRNA levels for both the wild-type and mutant AR were quite similar [15]. These data indicate that 17-AAG preferentially degrades the mutant AR protein without altering mRNA levels. These in vitro studies indicated that the mutant AR was a good target protein of 17-AAG. To determine if it would also be preferentially degraded in vivo, we next examined the effects of 17-AAG in SBMA transgenic mice.

17-AAG ameliorates the phenotype in a mouse model of SBMA mouse without detectable toxicity

We administrated 17-AAG at doses of 2.5 or 25 mg/kg to males of the transgenic mouse model carrying full-length human AR with either 24Q or 97Q. The disease progression of AR-97Q mice treated with 25 mg/kg 17-AAG (Tg-25) was significantly ameliorated, and that of mice treated with the 2.5 mg/kg 17-AAG (Tg-2.5) was also mildly ameliorated (Fig. 3a). The AR-97Q mice treated with vehicle only (Tg-0) showed motor impairment assessed by the Rotarod task as early as 9 weeks after birth while the Tg-25 mice showed initial impairment only 18 weeks after birth and with less deterioration than the Tg-0 mice (P<0.005; Fig. 3a). Tg-2.5 mice showed intermediate levels of impairment in Rotarod performance (Fig. 3a). 17-AAG also significantly prolonged the survival rate of the Tg-2.5 and Tg-25 mice compared with the Tg-0 mice (P=0.004 and P<0.001, respectively; Fig. 3a). No lines were distinguishable in terms of body weight at birth; however, by 16 weeks, the Tg-0 mice showed obvious differences in body size, muscular atrophy, and kyphosis compared with the Tg-25 mice (Fig. 3b).

When mouse tissues were immunohistochemically stained for mutant AR using the 1C2 antibody, which specifically recognizes expanded polyQ, quantitative analysis revealed marked reductions in 1C2-positive nuclear accumulation in the spinal motor neurons and muscles of the Tg-25 mice compared with those of the Tg-0 mice (Fig. 3c).

Western blot analysis from lysates of the spinal cord and muscle of AR-97Q mice revealed high molecular weight mutant AR protein complex retained in the stacking gel as well as a band of monomeric mutant AR, whereas only the band of wild-type monomeric AR was visible in tissue from the AR-24Q mice (Fig. 3d). 17-AAG treatments significantly diminished both the high molecular weight complex and the monomer of mutant AR in the spinal cord and muscle of the AR-97Q mice but only slightly diminished the wild-type monomeric AR in AR-24Q mice (Fig. 3d). 17-AAG treatments decreased the amount of monomeric AR in AR-97Q mice by 64.4% in the spinal cord and 45.0% in the skeletal muscle, but only 25.9 and 12.5%, respectively, in AR-24Q mice (Fig. 3d). Thus, the reduction rate of the monomeric mutant AR was significantly higher than that of the wild-type AR in both spinal cord and skeletal muscle (P<0.001 and P<0.01 respectively). The levels of wild-type and mutant AR mRNA were similar in the respective mice treated with 17-AAG [15]. These observations indicate that 17-AAG markedly reduces not only the high molecular weight

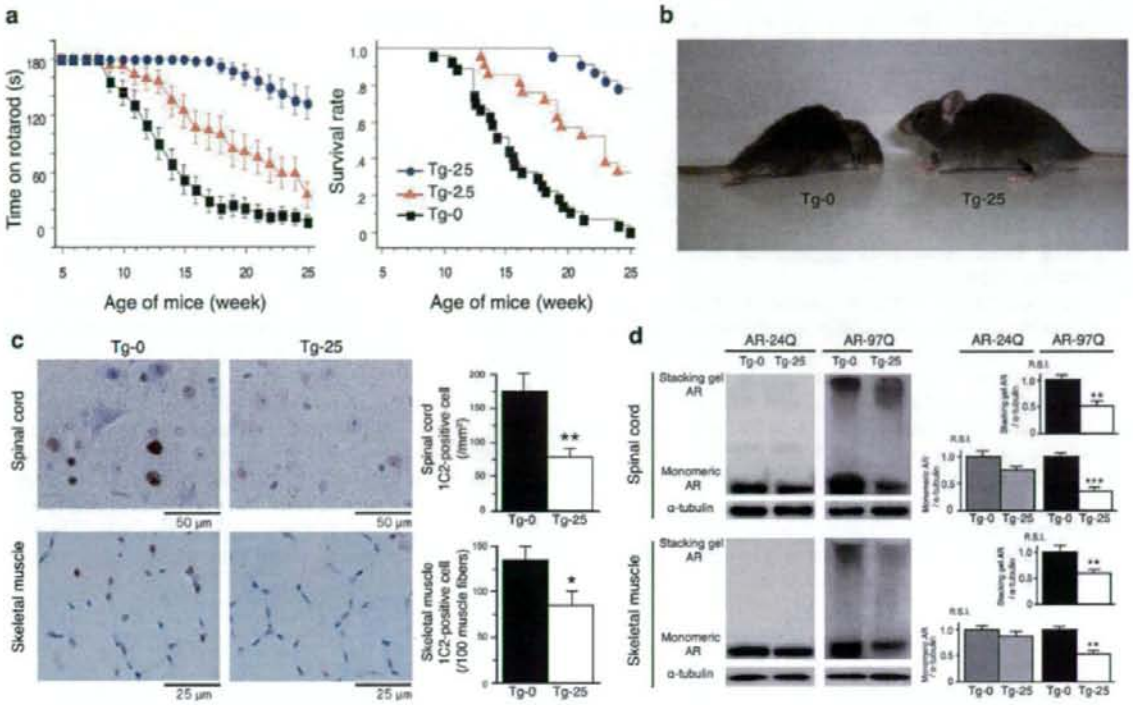

Fig. 3 a–d Effects of 17-AAG on transgenic SBMA mice. **a** Tg-0, Tg-2.5, and Tg-25 represent AR-97Q mice treated with vehicle alone and 2.5 and 25 mg/kg 17-AAG, respectively (each group, n= 27). The Tg-25 remained longer on the Rotarod than the Tg-0 mice did. A Kaplan–Meier plot shows the prolonged survival of Tg-2.5 and Tg-25 mice compared with the Tg-0 mice, which were all dead by 25 weeks of age (P=0.004, P<0.001, respectively). 17-AAG was less effective at the dose of 2.5 than 25 mg/kg in all parameters tested. **b** Representative photographs of a 16-week-old Tg-0 mouse (*left*) reveal an obvious difference in size and illustrate muscular atrophy and kyphosis compared with an age-matched Tg-25 mouse (*right*). **c**. Immunohistochemical staining with 1C2 antibody showed marked differences in diffuse nuclear staining and nuclear inclusions between DMSO-treated mice (Tg-0) and 17-AAG-treated (Tg-25) mice in the spinal anterior horn and skeletal muscle, respectively. There was a significant reduction in 1C2-positive cell staining in the spinal cord (**P<0.01) and skeletal muscle (*P<0.05) in the Tg-25 compared with the Tg-0. Values are expressed as means±SE (n=6). **d** Western blot analysis of tissue from AR-24Q and AR-97Q mice probed with an AR-specific antibody. In both spinal cord and muscle of mice treated with 17-AAG, there was a significant decrease in the amount of complexed, mutant AR in the stacking gel and monomeric mutant AR in AR-97Q mice, but only slightly less monomeric wild-type AR in AR-24Q mice compared with that from their respective, untreated control mice. Results of a densitometric analysis demonstrated that the 17-AAG-induced reduction of monomeric mutant AR was significantly greater than that of the wild-type monomeric AR. 17-AAG resulted in a 64.4% decline in monomeric mutant AR in the spinal cord and a 45.0% decline in the skeletal muscle, whereas, there was only a 25.9% decline in the spinal cord and a 12.5% decline in the skeletal muscle of AR-24Q mice. These results show significant differences in the reduction rate between wild-type and mutant AR in both spinal cord and skeletal muscle. Values are expressed as means±SE (n=5). Statistical differences are indicated by *asterisks* (*P<0.05; **P<0.01; ***P<0.001)

mutant AR complex but also the monomeric mutant AR protein by preferential degradation of mutant AR.

17-AAG facilitates the degradation of monomeric mutant AR, reducing its aggregation; a therapeutic approach that directly targets the disease-causing protein

In both cultured cells and transgenic SBMA mice, we have demonstrated both the efficacy and safety of 17-AAG [15]. Among the other proposed therapeutic approaches we have previously examined [35, 37, 40, 41], the efficacy of 17-AAG most closely approximated the very successful hormonal therapy using the LH-RH analog, leuprolein [15]. But, unlike leuprorelin, the Hsp90 inhibitor 17-AAG holds enormous potential for application to a wide-range of neurodegenerative diseases in addition to SBMA as previously reported [52–54]. For development of Hsp90 inhibitor treatment in neurological disorders, we regard this general versatility as very important.

In neurodegenerative diseases, recent studies have shown that disease-causing proteins in the process of aggregating have more toxic consequences than they do in either the nascent state or when in NI [55]. NIs have been thought to be a beneficial coping response to toxic mutant protein [56]. We have accumulated several pieces of data demonstrating that 17-AAG is capable of reducing aggregated protein in animal models of SBMA [15]. In both Western blot and filter trap analyses in AR-97Q

models, 17-AAG significantly diminished the insoluble high molecular weight complex of mutant AR as well as the soluble monomer. Moreover, in an immunostaining study of nervous tissue in AR-97Q mice, 17-AAG also significantly reduced diffuse nuclear staining. In SBMA patients, the extent of diffuse nuclear accumulation of mutant AR in motor and sensory neurons of the spinal cord was closely related to CAG repeat length [6]. We consider that 17-AAG had a curative effect on SBMA mice by reducing these toxic proteins as well as the soluble monomeric form.

It is difficult to determine whether 17-AGG facilitates the degradation of, specifically, these toxic intermediate proteins, as 17-AAG has the potent ability to also degrade their precursors (i.e., the monomers). One possible mode of 17-AAG action is that it may inhibit the aggregation of mutant AR via Hsp70 and Hsp40 induction. The pharmacological induction of Hsp70 and Hsp40 using Hsp90 inhibitors has already been shown to inhibit polyQ-induced abnormal aggregation of the huntingtin protein [57]. However, as 17-AAG displayed only a limited ability to induce Hsp70 and Hsp40 in mouse tissue [15], we think that the large decrease in AR seen in the insoluble fraction in vivo, rather than being a result of HSP induction, may be due to 17-AAG's potent ability to degrade the soluble monomeric form of mutant protein, thereby preventing aggregation in the first place. There is no doubt that a reduction of the main culprit protein must have curative properties against various neurodegenerative diseases. In fact, one therapeutic approach that directly reduced abnormal protein using RNA interference has already proved beneficial in various mouse models of polyQ diseases and amyotrophic lateral sclerosis [58–60].

Hsp70 is also known to accelerate proteasome-dependent degradation of polyQ abnormal protein [40, 61]. However, in our hands, mutant AR was markedly decreased after 17-AAG treatment even when Hsp70 and Hsp40 induction was completely blocked in the presence of a protein synthesis inhibitor [15], strongly suggesting that 17-AAG contributes to the preferential degradation of mutant AR mainly through Hsp90 chaperone complex formation and subsequent proteasome-dependent degradation rather than via Hsp70 and Hsp40 induction. Therefore, we think that, to reap the most therapeutic benefits, Hsp90 inhibitors should be applied against neurodegenerative diseases in which the causative protein is, like AR, an Hsp90 client protein.

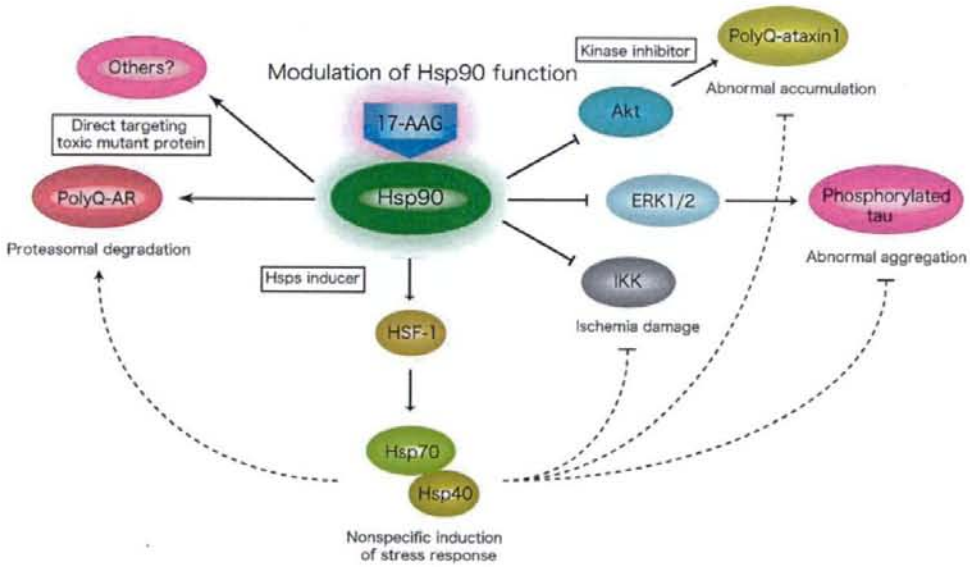

Fig. 4 Clinical application of Hsp90 inhibitors in neurological disorders. 17-AAG specifically binds to the Hsp90 ATP-binding site and disrupts its ATP-dependent function, resulting in inactivation and degradation of Hsp90 client proteins. Therapeutic approaches using Hsp90 inhibitors have now emerged that are unrelated to their role in inducing HSPs. In SBMA, where it may have its most effective potential, 17-AAG directly accelerates proteasomal degradation of the disease-causing protein, polyQ-expanded AR. However, it is still unclear whether other neurodegenerative disease-causing proteins are also Hsp90 client proteins. Many kinases involved in signal transduction do belong to the family of Hsp90 client proteins targeted by 17-AAG. ERK is associated with stabilizing phosphorylated tau. 17-AAG reduces the total amount of phosphorylated tau and its abnormal aggregation by inhibiting ERK activation. Following this same mechanism, 17-AAG might also reduce the abnormal accumulation of ataxin-1 by inhibiting Akt activation or alleviate cerebral infarction by inhibiting IKK activation, which is also an Hsp90 client protein, as well as by inducing HSPs. Hsp90 inhibitors are known to nonspecifically induce HSPs, although this effect was quite limited in our mouse model of SBMA. The induction of HSPs by Hsp90 inhibitors seems to play a supplementary role in neurodegenerative disorders

17-AAG functions with preserved UPS function

As we demonstrated in an in vitro study, 17-AAG-induced degradation requires a well-preserved proteasome function [15]. However, a question concerning the usefulness of this pharmacological approach to facilitate a self-clearing system has been raised [62]. It is generally accepted that the ubiquitin–proteasome system (UPS) is strongly involved in the pathology of polyQ diseases, as many components of the ubiquitin–proteasome and molecular chaperones are known to co-localize with polyQ-containing NIs [63, 64]. Previous reports of studies performed in cultured cell models suggested that an impairment of the UPS is probably a common pathology in polyQ diseases [65–67]. If this hypothesis were true, 17-AAG could not exert its pharmacological effect on polyQ diseases; its therapeutic effects are dependent on a preserved proteasome function [15, 47, 48, 62].

In this regard, recent studies using in vivo proteasome assays have raised serious questions concerning the impaired UPS hypothesis of polyQ diseases [68–70]. It has been reported that neuronal dysfunction developed without significant impairment of the UPS in a mouse model of SCA7 [69]. Consistent with this, it was also demonstrated that proteasome impairment did not contribute to pathogenesis in a mouse model of Huntington's disease (HD) [70]. Furthermore, in conditional mouse models of polyQ disease, genetic loss of the abnormal gene product led to a rapid clearance of pre-existing polyQ-mediated NIs and reversible improvement of the abnormal phenotypes [71, 72]. If the UPS were irreversibly damaged in polyQ diseases, then pre-existing NIs could not be diminished.

While it remains unclear what the difference is in proteasome function in in vitro and in vivo models, our research in a mouse model of SBMA indicates that impairment of the UPS is not a major etiology, at least in in vivo models of polyQ diseases. We therefore consider that treatment with 17-AAG, which takes advantage of a self-clearing system to target disease-causing proteins, is a reasonable therapeutic strategy against polyQ-related and other neurodegenerative diseases.

The expected beneficial effects of 17-AAG against other neurodegenerative diseases

Among neurodegenerative-disease-causing proteins, only AR in SBMA is established as an Hsp90 client protein at this time. It will be interesting to assess whether other neurodegenerative-disease-causing proteins also belong to the family of Hsp90 client proteins. Recent studies have already revealed that some Hsp90 client proteins exerted adverse influences on several neurological disorders [73–75], indicating that the clinical application of Hsp90 inhibitors could expand beyond the treatment of oncological diseases. With reference to previous reports, we now discuss the possibility that 17-AAG could be applicable to neurodegeneration other than SBMA (Fig. 4).

Using 17-AAG as an inducer of HSPs

Hsp90 inhibitors are known to possess the unique pharmacological effect of inducing a stress response and, in addition to their use as anti-cancer agents, have also been developed as pharmacological HSP inducers [52, 76]. This pharmacological effect has already been confirmed in human clinical trials [22]. Murakami et al. were the first to show that the Hsp90 inhibitor herbimycin had the ability to induce Hsp70 in various cultured cell models [77]. Thereafter, Hsp90 proved to be a repressor of heat transcription factor (HSF-1) [78]. Hsp90 inhibitors cause a disassociation of HSF-1 from the Hsp90 complex and a trimerization of HSF-1, thereby resulting in HSP activation. Based on the ability to induce HSPs, Hsp90 inhibitors have also been applied to non-oncological diseases [52].

A great number of reports revealed that forced overexpression of Hsp70 resulted in acquisition of tolerance against various types of stresses and protection against apoptosis in various disease models [79]. In a wide range of polyQ disease models, both genetic and pharmacological overexpression of HSPs has been shown to suppress aggregate formation and cellular toxicity [63, 80, 81]. There is no doubt that HSP induction is beneficial for various neurodegenerative diseases [54]. We have also demonstrated that both genetic and pharmacological overexpression of Hsp70 significantly ameliorated expression of the abnormal phenotype in a transgenic mouse model of SBMA [40, 82].

Taking advantage of HSP induction, many studies have already showed that Hsp90 inhibitors exerted potential neuroprotective effects in: a model of HD [57, 83, 84], tauopathies [28, 85–87], Parkinson's disease [88–90], stroke [91, 92], and autoimmune encephalomyelitis [93]. In addition, Hsp90 inhibitors themselves have been shown to have some neuroprotective effects against various stresses, such as drug-induced toxicity, oxidative stress, and oxygen-glucose deprivation [94–97]. As for polyQ diseases, Sittler et al. [57] first showed that GA significantly suppressed aggregation of mutant huntingtin in a cultured cell model of HD via induction of the Hsp70 and Hsp40 heat shock response. Thus, the enhancement of cellular defenses using Hsp90 inhibitors is a very reasonable clinical application for neurodegenerative diseases.

In polyQ diseases, Bates and his colleagues [83] showed a progressive decrease in the expression of Hsp70 and Hsp40 in a mouse model of HD, which was also observed in our SBMA model [82]. The ability of Hsp90 inhibitors to significantly induce HSPs has been demonstrated only in cultured cell models and in the fly model, but not in mammals. Therefore, further investigation should be performed to address how much Hsp90 inhibitors can induce HSPs in mouse models of neurodegenerative disorders other than SBMA. Although it appears to be obvious that it would be advantageous for the treatment of neurodegeneration, in inducing HSPs by Hsp90 inhibitors, in view of our research finding in in vivo models, it would be unadvisable to rely only on the induction of nonspecific HSPs for human clinical trials.

Using 17-AAG as a kinase inhibitor in neurodegeneration

Phosphorylated tau is a representative disease-causing protein associated with tauopathies including fronto-temporal dementia, progressive supranuclear palsy, corticobasal degeneration, and multiple system atrophy. It is interesting to note that phosphorylated tau is a targeted protein of Hsp90 inhibitors [28, 85, 86]. Dou et al. recently showed that GA and 17-AAG indirectly blocked abnormal tau phosphorylation by inhibition of the Raf–MEK–extracellular signal-regulated kinase (ERK) pathway [98], of which upstream kinase Raf is also an Hsp90 client protein [10, 99]. ERK is known to mediate the activation and stabilization of phosphorylated tau [100, 101]. Along these same lines, LaFevre-Bernt and Ellerby [102] demonstrated that polyQ-expanded, mutant-AR-mediated neuronal cell death by ERK activation and that selective inhibition of the ERK pathway reduced polyQ-induced cell death. Based on this mechanism of inhibiting ERK activation, 17-AAG might also ameliorate abnormal phenotypic expression in the mouse model of SBMA. Furthermore, in other neurodegenerative disorders, evidence has accumulated suggesting that ERK activation is an important executor of neuronal damage [103–106]. Hsp90 inhibitors are well known to have the ability to inhibit various kinase activity [10]. This pharmacological effect of Hsp90 inhibitors, to reduce abnormal kinase activity, could be applied to neurodegenerative diseases as well as oncological diseases and could have far-reaching influence on the clinical application of Hsp90 inhibitors.

Zoghbi and colleagues demonstrated that Akt/PI3K was essential for stabilization and accumulation of mutant ataxin-1 in the polyQ-associated disease SCA1 [73, 107]. Akt is also an Hsp90 client protein, whose activity is significantly reduced by Hsp90 inhibition [108, 109]. Thus, reduction of Akt kinases activity by Hsp90 inhibition might be a therapeutic approach for SCA1. Although Akt/PI3K is believed to be a major pathway mediating survival signals in neuronal cells [110], their finding raises an issue about this hypothesis.

Hsp90 inhibitors have been found to have some neuroprotective effects such as on axonal regeneration in cultured cell models [94, 111]. Koprivica et al. demonstrated that epidermal growth factor receptor (EGFR) activation mediates inhibition of axon regeneration [74]. That EGFR is also an Hsp90 client protein [112, 113] might help explain how Hsp90 inhibition is related to axonal regeneration. In another example, GA markedly attenuates ischemic brain damage [91, 92] and IκB kinase (IKK), an Hsp90 client protein [114], plays an important role in ischemia-induced neuronal death [75, 115], suggesting that GA may ameliorate ischemia brain damage by reducing IKK activity as well as by inducing HSPs.

There is a caveat to this suggestion, however. If 17-AAG is to be applied clinically to treat neurodegenerative diseases with the expectation of reducing abnormal kinase activity, we should also keep in mind the possibility that 17-AAG might also inhibit some kinase activation that exerts cytoprotective effects against neuron degeneration. Taking HD as an example, the efficacy of GA has already been shown based on its ability to induce HSPs [57, 83]. However, a report recently released by Apostol et al. showed that ERK1/2 activation protects against mutant huntingtin-induced toxicity [116]. Furthermore, in a cultured cell model of HD carrying full-length huntingtin, various kinase activities were inhibited by mutant polyQ-expanded huntingtin, but not by normal huntingtin [117, 118]. If ERK activation plays a major role in protecting against HD phenotype expression, there is concern that 17-AAG might exert an adverse affect on HD by inhibiting ERK activation. Before applying 17-AAG to a neurological disorder, we should assess whether the kinase targeted by Hsp90 inhibitor is a true exacerbating factor for the pathology. While there may be some debate over whether 17-AAG should be used as a kinase inhibitor in neurodegeneration, if the application of 17-AAG is suitably performed, this agent would be expected to effectively inhibit abnormal kinase activity in several neurological disorders, possibly leading to cures for these diseases. Hence, this strategy is also of value to extend the versatility of Hsp90 inhibitors as therapeutic agents for neurological disorders.

Conclusion

When considering a role for molecular chaperones in neurological disorders, it should be said that Hsp70 and Hsp40 have received most of the attention, especially in neurodegenerative diseases, as these chaperones have the desirable ability to refold abnormal proteins or to carry them to degradation as a part of the system of protein quality control [54, 119]. Compared with this, Hsp90 is not known to directly fold non-native proteins but rather to bind to substrate proteins only at a late stage of folding [120]. Considering our research findings and those of the other above-mentioned reports, in addition to its role in malignancies, Hsp90 exerts an adverse influence on the nervous system in some situations. In this case, it is reasonable to consider modulating Hsp90 function appropriately.

The ability of 17-AAG to facilitate the degradation of abnormal toxic protein through the modulation of Hsp90 function would be directly applicable to SBMA and probably other neurodegenerative disorders as well. But we should keep in mind that 17-AAG is not a panacea for neurological disorders because it has only limited ability to induce Hsp70 and Hsp40 in vivo. 17-AAG is expected to exert the most effective therapeutic potential against diseases in which the main etiological factor is mediated by Hsp90 client proteins, like AR in SBMA. We believe that more research should be invested in examining the effects of Hsp90 inhibitors on neurodegeneration and that suitably modulating Hsp90 function has great potential to become a new molecular-targeted therapy against a wide range of neurodegenerative diseases.

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Review

Pathogenesis, animal models and therapeutics in Spinal and bulbar muscular atrophy (SBMA)

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Abstract

Spinal and bulbar muscular atrophy (SBMA) is a hereditary neurodegenerative disease characterized by slowly progressive muscle weakness and atrophy of bulbar, facial, and limb muscles. The cause of SBMA is expansion of a trinucleotide CAG repeat, which encodes the polyglutamine tract, in the first exon of the androgen receptor (AR) gene. SBMA chiefly occurs in adult males, whereas neurological symptoms are rarely detected in females having mutant AR gene. The cardinal histopathological finding of SBMA is loss of lower motor neurons in the anterior horn of spinal cord as well as in brainstem motor nuclei. Animal models carrying human mutant AR gene recapitulate polyglutamine-mediated motor neuron degeneration, providing clues to the pathogenesis of SBMA. There is increasing evidence that testosterone, the ligand of AR, plays a pivotal role in the pathogenesis of neurodegeneration in SBMA. The striking success of androgen deprivation therapy in SBMA mouse models has been translated into clinical trials. In addition, elucidation of pathophysiology using animal models leads to emergence of candidate drugs to treat this devastating disease: HSP inducer, Hsp90 inhibitor, and histone deacetylase inhibitor. Utilizing biomarkers such as scrotal skin biopsy would improve efficacy of clinical trials to verify the results from animal studies. Advances in basic and clinical researches on SBMA are now paving the way for clinical application of potential therapeutics.

Keywords: Spinal and bulbar muscular atrophy; Polyglutamine; Androgen receptor; Testosterone; Luteinizing hormone-releasing hormone analog; Heat shock protein; Geranylgeranylacetone; 17-Allylamino geldanamycin; Histone deacetylase inhibitor; Axonal transport

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History and nomenclature

More than a hundred years have elapsed since the first description of spinal and bulbar muscular atrophy (SBMA) from Hiroshi Kawahara, who described the clinical and hereditary characteristics of two Japanese brothers with progressive bulbar palsy (Kawahara, 1897). This work was followed by several reports on similar cases with or without X-linked pattern of inheritance (Katsuno et al., 2004). SBMA is also known as Kennedy disease (KD), named after William R. Kennedy, whose study on 11 patients from 2 families depicted the clinical, genetical, and pathological features of this disorder (Kennedy et al., 1968). Other names for this disease are bulbospinal neuronopathy and bulbospinal muscular atrophy.

In 1991, the cause of SBMA was identified as the expansion of a trinucleotide CAG repeat in the androgen receptor (AR) gene (La Spada et al., 1991). This was the first discovery of polyglutamine-mediated neurodegenerative diseases, and subsequent studies using transgenic animal models opened the door to development of pathogenesis-based therapies for this devastating disease.

Clinical features

SBMA exclusively affects adult males. The prevalence of this disease is estimated to be 1–2 per 100,000, whereas a considerable number of patients may have been misdiagnosed as other neuromuscular diseases including amyotrophic lateral sclerosis (Fischbeck, 1997). Patients of various ethnic backgrounds have been reported around the world.

Major symptoms of SBMA are weakness, atrophy, and fasciculations of bulbar, facial and limb muscles (Sperfeld et al., 2002; Katsuno et al., 2004). In extremities, involvement is usually predominant in proximal musculature. The onset of weakness is usually between 30 and 60 years but is often preceded by nonspecific symptoms such as postural tremor and muscle cramps. Although fasciculations in the extremities are rarely present at rest, they are easily induced when patients hold their arms horizontally or bend their legs while lying on their backs. These contraction fasciculations are especially noticeable in the face, neck, and tongue and are usually present in the early stage. Fatigability after exercise might also be accompanied. Bilateral facial and masseter muscle weakness, poor uvula and soft palatal movements, and atrophy of the tongue with fasciculations are often encountered. Speech has a nasal quality in most cases due to reduced velopharyngeal closure. Advanced cases often develop dysphagia, eventually resulting in aspiration or choking. Muscle tone is usually hypotonic, and no pyramidal signs are detected. Deep tendon reflex is diminished or absent with no pathological reflex. Sensory involvement is largely restricted to vibration sense which is affected distally in the legs. Cerebellar symptoms, dysautonomia, and cognitive impairment are absent. Patients occasionally demonstrate signs of androgen insensitivity such as gynecomastia, testicular atrophy, dyserection, and decreased fertility, some of which are detected before the onset of motor symptoms. Abdominal obesity is common, whereas male pattern baldness is rare in patients with SBMA.

Electromyogram shows neurogenic abnormalities, and distal motor latencies are often prolonged in nerve conduction study. Both sensory nerve action potential and sensory evoked potential are reduced or absent. Endocrinological examinations frequently reveal partial androgen resistance with elevated serum testosterone level. Serum creatine kinase level is elevated in the majority of patients. Hyperlipidemia, liver dysfunction, and glucose intolerance are also detected in some patients. Profound facial fasciculations, bulbar signs, gynecomastia, and sensory disturbance are the main clinical features distinguishing SBMA from other motor neuron diseases, although gene analysis is indispensable for diagnosis. Female patients are usually asymptomatic, but some express subclinical phenotypes including high amplitude motor unit potentials on electromyography (Sobue et al., 1993).

The progression of SBMA is usually slow, but a considerable number of patients need assistance to walk in their fifties or sixties. Life-threatening respiratory tract infection often occurs in the advanced stage of the disease, resulting in early death in some patients. No specific therapy for SBMA has been established. Testosterone has been used in some patients, although it has no effects on the progression of SBMA.

Etiology

The cause of SBMA is expansion of a trinucleotide CAG repeat, which encodes the polyglutamine tract, in the first exon of the androgen receptor (AR) gene (La Spada et al., 1991). The CAG repeat within AR ranges in size from 9 to 36 in normal subjects but from 38 to 62 in SBMA patients. Expanded polyglutamine tracts have been found to cause several neurodegenerative diseases including SBMA, Huntington's disease, several forms of spinocerebellar ataxia, and dentatorubral-pallidoluysian atrophy (Gatchel and Zoghbi, 2005). These disorders, known as polyglutamine diseases, share salient clinical features including anticipation and somatic mosaicism, as well as selective neuronal and nonneuronal involvement despite widespread expression of the mutant gene. There is an inverse correlation between the CAG repeat size and the age at onset or the disease severity adjusted by the age at examination in SBMA as documented in other polyglutamine diseases (Doyu et al., 1992). These observations explicitly suggest that common mechanisms underlie the pathogenesis of polyglutamine diseases.

AR, the causative protein of SBMA, is an 110-kDa nuclear receptor which belongs to the steroid/thyroid hormone receptor family (Poletti, 2004). AR mediates the effects of androgens, testosterone, and dihydrotestosterone, through binding to an androgen response element in the target gene to regulate its expression. AR is essential for major androgen effects including normal male sexual differentiation and pubertal sexual development, although AR-independent nongenomic function of androgen has been reported. AR is expressed not only in primary and secondary sexual organs but also in nonreproductive organs including the kidney, skeletal muscle, adrenal gland, skin, and nervous system, suggesting its far-reaching influence on a variety of mammalian tissues. In the central nervous system, the expression level of AR is relatively high in spinal and brainstem

motor neurons, the same cells which are vulnerable in SBMA. The AR gene is located on chromosome Xq11–12. This 90-kb DNA contains eight exons coding for the functional domains specific to the nuclear receptor family. The first exon codes for the N-terminal transactivating domain. Exons 2 and 3 code for the DNA-binding domain, whereas exons 4 through 8 code for the ligand-binding domain. The N-terminal transactivating domain, in which a CAG trinucleotide repeat locates, possesses a major transactivation function maintained by interaction with general transcriptional coactivators such as c-AMP response element binding protein-binding protein (CBP), TAFII130, and steroid receptor coactivator-1 (SRC-1). The CAG repeat beginning at codon 58 in the first exon of AR encodes polyglutamine tract. The length of this repeat is highly variable because of the slippage of DNA polymerase upon DNA replication. Whereas its abnormal elongation causes SBMA, the shorter CAG repeat is likely to increase the risk of prostate cancer (Clark et al., 2003). Transcriptional coactivators also possess glutamine-rich regions modulating protein–protein interaction with the N-terminal transactivating domain of AR.

The expansion of a polyglutamine tract in AR has been implicated in the pathogenesis of SBMA in two different, but not mutually exclusive, ways: loss of normal AR function induces neuronal degeneration; and the pathogenic AR acquires toxic property damaging motor neurons. Since AR possesses trophic effects on neuronal cells, one can assume that loss of AR function may play a role in the pathogenesis of SBMA. Expansion of the polyglutamine tract mildly suppresses the transcriptional activities of AR, probably because it disrupts interaction between the N-terminal transactivating domain of AR and transcriptional coactivators (Poletti, 2004). Although this loss of function of AR may contribute to the androgen insensitivity in SBMA, the pivotal cause of neurodegeneration in SBMA has been believed to be a gain of toxic function of the pathogenic AR due to expansion of the polyglutamine tract. This hypothesis is supported by the observation that motor impairment has never been observed in severe testicular feminization (Tfm) patients lacking AR function or in AR knockout mice. Moreover, a transgenic mouse model carrying an elongated CAG repeat driven by human AR promoter demonstrated motor impairment, suggesting that the expanded polyglutamine tract is sufficient to induce the pathogenic process of SBMA (Adachi et al., 2001).

Aggregation of abnormal protein has been considered to be central to the pathogenesis of neurodegenerative diseases such as Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, and prion disease. An expanded polyglutamine stretch alters conformation of causative proteins, resulting in aggregation of the proteins. It is now widely accepted that aggregation of these abnormal proteins in neurons is the primary event in the pathogenesis of polyglutamine diseases. The rate-limiting step of aggregation has been proposed to be the formation of oligomeric nucleus, which may occur form after a repeat length-dependent conformational change of polyglutamine monomer from a random coil to a parallel, helical β-sheet (Wyttenbach, 2004). Several experimental observations indicate that formation of toxic oligomers, or intermediates, of abnormal polyglutamine-containing protein instigates a series of cellular events which lead to neurodegeneration (Muchowski and Wacker, 2005). This hypothesis is likely to be the case in SBMA.

Pathology

Histopathological studies provide important information on the pathogenesis of polyglutamine-mediated neurodegeneration. The fundamental histopathological finding of SBMA is loss of lower motor neurons in the anterior horn of spinal cord

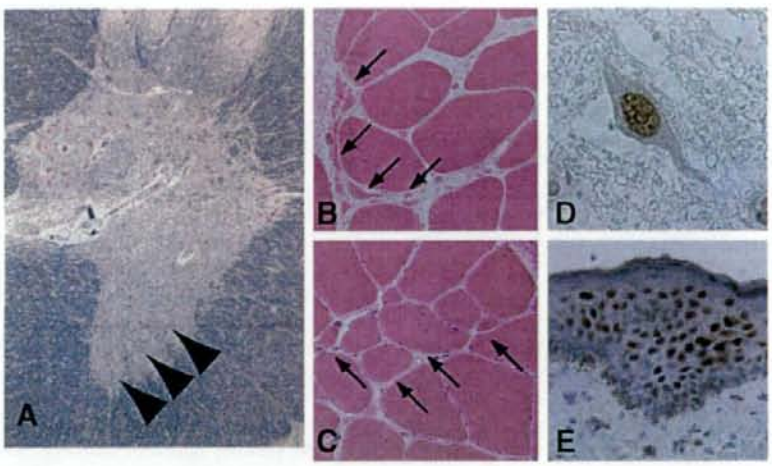

Fig. 1. Histopathology of SBMA. (A) A transverse section of spinal cord demonstrates marked depletion of motor neurons in the anterior horn. (B and C) HE staining of skeletal muscle shows both neurogenic (B, arrows) and myogenic changes (C, arrows). (D) A residual motor neuron in the lumbar anterior horn shows a diffuse nuclear accumulation of pathogenic androgen receptor detected by anti-polyglutamine antibody. (E) Nuclear accumulation of pathogenic AR is also detected in nonneuronal tissues such as scrotal skin (E).

as well as in brainstem motor nuclei except for the third, fourth and sixth cranial nerves (Fig. 1A) (Sobue et al., 1989). The number of nerve fibers is reduced in the ventral spinal nerve root, reflecting motor neuronopathy. Sensory neurons in the dorsal root ganglia were less severely affected, and large myelinated fibers demonstrate a distally accentuated sensory axonopathy in the peripheral nervous system. Neurons in the Onufrowicz nuclei, intermediolateral columns, and Clarke's columns of the spinal cord are generally well preserved. Muscle histopathology includes both neurogenic and myogenic findings: there are groups of atrophic fibers with a number of small angular fibers, fiber type grouping and clamps of pyknotic nuclei as well as variability in fiber size, hypertrophic fibers, scattered basophilic regenerating fibers, and central nuclei (Figs. 1B and C).

A pathologic hallmark of polyglutamine diseases is the presence of nuclear inclusions (NIs). In SBMA, NIs containing the pathogenic AR are found in the residual motor neurons in the brainstem and spinal cord as well as in nonneuronal tissues including prostate, testis, and skin (Li et al., 1998). These inclusions are detectable using antibodies recognizing a small portion of the N-terminus of the AR protein, but not by those against the C-terminus of the protein. This observation implies that the C-terminus of AR is truncated or masked upon formation of NI. A full-length AR protein with expanded polyglutamine tract is cleaved by caspase-3, liberating a polyglutamine-containing toxic fragment, and the susceptibility to cleavage is polyglutamine repeat length-dependent (Kobayashi et al., 1998). Thus, proteolytic cleavage is likely to enhance the toxicity of the pathogenic AR protein. Electron microscopic immunohistochemistry shows dense aggregates of AR-positive granular material without limiting membrane, both in the neural and nonneural inclusions, in contrast to the other polyglutamine diseases in which NIs take the form of filamentous structures. Although NI is a disease-specific histopathological finding, its role in pathogenesis has been heavily debated. Several studies have suggested that NI may indicate cellular response coping with the toxicity of abnormal polyglutamine protein (Arrasate et al., 2004). Instead, the diffuse nuclear accumulation of the mutant protein has been considered essential for inducing neurodegeneration in polyglutamine diseases including SBMA.

An immunohistochemical study on autopsied SBMA patients using an anti-polyglutamine antibody demonstrates that diffuse nuclear accumulation of the pathogenic AR is more frequently observed than NIs in the anterior horn of spinal cord (Adachi et al., 2005). Intriguingly, the frequency of diffuse nuclear accumulation of the pathogenic AR in spinal motor neurons strongly correlates with the length of the CAG repeat in the AR gene. No such correlation has been found between NI occurrence and the CAG repeat length. Similar findings have also been reported on other polyglutamine diseases. Taken together, it appears that the pathogenic AR containing an elongated polyglutamine tract principally accumulates within the nuclei of motor neurons in a diffusible form, leading to neuronal dysfunction and eventual cell death. In support of this hypothesis, neuronal dysfunction is halted by genetic modulation preventing nuclear import of the pathogenic polyglutamine-containing protein in cellular and animal models of polyglutamine diseases (Gatchel and Zoghbi, 2005).

Since human AR is widely expressed in various organs, nuclear accumulation of the pathogenic AR protein is detected not only in the central nervous system but also in nonneuronal tissues such as scrotal skin (Figs. 1D and E). The degree of pathogenic AR accumulation in scrotal skin epithelial cells tends to be correlated with that in the spinal motor neurons in autopsy specimens, and it is well correlated with CAG repeat length and inversely correlated with the motor functional scale (Banno et al., in press). These findings indicate that scrotal skin biopsy with anti-polyglutamine immunostaining is a potent biomarker with which to monitor SBMA pathogenic processes. Since SBMA is a slowly progressive disorder, appropriate biomarkers would help improve the power and cost effectiveness of longitudinal clinical treatment trials.

Molecular pathogenesis and therapeutic strategies

Ligand-dependent pathogenesis in animal models of SBMA

SBMA is unique among polyglutamine diseases in that the pathogenic protein, AR, has a specific ligand, testosterone, which alters the subcellular localization of the protein by favoring its nuclear uptake. AR is normally confined to a multiheteromeric inactive complex in the cell cytoplasm and translocates into the nucleus in a ligand-dependent manner. This ligand-dependent intracellular trafficking of AR appears to play important roles in the pathogenesis of SBMA.

In order to investigate ligand effect in SBMA, we generated transgenic mice expressing the full-length human AR containing 24 or 97 CAGs under the control of a cytomegalovirus enhancer and a chicken β-actin promoter (Katsuno et al., 2002). This model recapitulated not only the neurologic disorder but also the phenotypic difference with gender which is a specific feature of SBMA. The mice with 97CAGs (AR-97Q) exhibited progressive motor impairment, although those with 24 CAGs did not show any manifested phenotypes. Affected AR-97Q mice demonstrated small body size, short life span, progressive muscle atrophy, and weakness as well as reduced cage activity, all of which were markedly pronounced and accelerated in the male AR-97Q mice, but either not observed or far less severe in the female AR-97Q mice. The onset of motor impairment detected by the rotarod task was at 8 to 9 weeks of age in the male AR-97Q mice while 16 weeks or more in the females. The 50% mortality ranged from 66 to 132 days of age in the male AR-97Q mice, whereas mortality of the female AR-97Q mice remained only 10 to 30% at more than 210 days. Western blot analysis revealed the transgenic AR protein smearing from the top of the gel in the spinal cord, cerebrum, heart, muscle, and pancreas. Although the male AR-97Q mice had more smearing protein than their female counterparts, the female AR-97Q mice had more monomeric AR protein. The nuclear fraction contained the most of smearing pathogenic AR protein. Diffuse nuclear staining and less frequent NIs detected by 1C2, an antibody specifically recognizing the expanded polyglutamine tract, were demonstrated in the neurons of spinal cord,