neurodegenerative diseases: sometimes the chicken, sometimes the egg. Neuron 40, 427–446.

Cudkowicz, M.E., McKenna-Yasek, D., Sapp, P.E., Chin, W., Geller, B., Hayden, D.L., Schoenfeld, D.A., Hosler, B.A., Horvitz, H.R., Brown, R.H., 1997. Epidemiology of mutations in superoxide dismutase in amyotrophic lateral sclerosis. Ann. Neurol. 41, 210–221.

Glickman, M.H., Ciechanover, A., 2002. The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. 82, 373–428.

Hatakeyama, S., Matsumoto, M., Kamura, T., Murayama, M., Chui, D.H., Planel, E., Takahashi, R., Nakayama, K.I., Takashima, A., 2004. U-box protein carboxyl terminus of Hsc70-interacting protein (CHIP) mediates poly-ubiquitylation preferentially on four-repeat Tau and is involved in neurodegeneration of tauopathy. J. Neurochem. 91, 299–307.

Hatakeyama, S., Watanabe, M., Fujii, Y., Nakayama, K.I., 2005. Targeted destruction of c-Myc by an engineered ubiquitin ligase suppresses cell transformation and tumor formation. Cancer Res. 65, 7874–7879.

Hishikawa, N., Niwa, J., Doyu, M., Ito, T., Ishigaki, S., Hashizume, Y., Sobue, G., 2003. Dorfin localizes to the ubiquitylated inclusions in Parkinson's disease, dementia with Lewy bodies, multiple system atrophy, and amyotrophic lateral sclerosis. Am. J. Pathol. 163, 609–619.

Ishigaki, S., Liang, Y., Yamamoto, M., Niwa, J., Ando, Y., Yoshihara, T., Takeuchi, H., Doyu, M., Sobue, G., 2002a. X-Linked inhibitor of apoptosis protein is involved in mutant SOD1-mediated neuronal degeneration. J. Neurochem. 82, 576–584.

Ishigaki, S., Niwa, J., Ando, Y., Yoshihara, T., Sawada, K., Doyu, M., Yamamoto, M., Kato, K., Yotsumoto, Y., Sobue, G., 2002b. Differentially expressed genes in sporadic amyotrophic lateral sclerosis spinal cords—Screening by molecular indexing and subsequent cDNA microarray analysis. FEBS Lett. 531, 354–358.

Ishigaki, S., Hishikawa, N., Niwa, J., Iemura, S., Natsume, T., Hori, S., Kakizuka, A., Tanaka, K., Sobue, G., 2004. Physical and functional interaction between Dorfin and Valosin-containing protein that are colocalized in ubiquitylated inclusions in neurodegenerative disorders. J. Biol. Chem. 279, 51376–51385.

Ito, T., Niwa, J., Hishikawa, N., Ishigaki, S., Doyu, M., Sobue, G., 2003. Dorfin localizes to Lewy bodies and ubiquitylates synphilin-1. J. Biol. Chem. 278, 29106–29114.

Johnston, J.A., Ward, C.L., Kopito, R.R., 1998. Aggresomes: a cellular response to misfolded proteins. J. Cell Biol. 143, 1883–1898.

Julien, J.P., 2001. Amyotrophic lateral sclerosis. unfolding the toxicity of the misfolded. Cell 104, 581–591.

Jungmann, J., Reins, H.A., Schobert, C., Jentsch, S., 1993. Resistance to cadmium mediated by ubiquitin-dependent proteolysis. Nature 361, 369–371.

Lee, D.H., Sherman, M.Y., Goldberg, A.L., 1996. Involvement of the molecular chaperone Ydj1 in the ubiquitin-dependent degradation of short-lived and abnormal proteins in Saccharomyces cerevisiae. Mol. Cell. Biol. 16, 4773–4781.

Meacham, G.C., Patterson, C., Zhang, W., Younger, J.M., Cyr, D.M., 2001. The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. Nat. Cell Biol. 3, 100–105.

Miyazaki, K., Fujita, T., Ozaki, T., Kato, C., Kurose, Y., Sakamoto, M., Kato, S., Goto, T., Itoyama, Y., Aoki, M., Nakagawara, A., 2004. NEDL1, a novel ubiquitin–protein isopeptide ligase for dishevelled-1, targets mutant superoxide dismutase-1. J. Biol. Chem. 279, 11327–11335.

Murata, S., Minami, Y., Minami, M., Chiba, T., Tanaka, K., 2001. CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep. 2, 1133–1138.

Murata, S., Chiba, T., Tanaka, K., 2003. CHIP: a quality-control E3 ligase collaborating with molecular chaperones. Int. J. Biochem. Cell Biol. 35, 572–578.

Niwa, J., Ishigaki, S., Doyu, M., Suzuki, T., Tanaka, K., Sobue, G., 2001. A novel centrosomal ring-finger protein, dorfin, mediates ubiquitin ligase activity. Biochem. Biophys. Res. Commun. 281, 706–713.

Niwa, J., Ishigaki, S., Hishikawa, N., Yamamoto, M., Doyu, M., Murata, S., Tanaka, K., Taniguchi, N., Sobue, G., 2002. Dorfin ubiquitylates mutant SOD1 and prevents mutant SOD1-mediated neurotoxicity. J. Biol. Chem. 277, 36793–36798.

Oyake, D., Nishikawa, H., Koizuka, I., Fukuda, M., Ohta, T., 2002. Targeted substrate degradation by an engineered double RING ubiquitin ligase. Biochem. Biophys. Res. Commun. 295, 370–375.

Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X., et al., 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59–62.

Ross, C.A., Poirier, M.A., 2004. Protein aggregation and neurodegenerative disease. Nat. Med. 10, S10–S17 (Suppl.).

Rowland, L.P., Shneider, N.A., 2001. Amyotrophic lateral sclerosis. N. Engl. J. Med. 344, 1688–1700.

Sakamoto, K.M., Kim, K.B., Kumagai, A., Mercurio, F., Crews, C.M., Deshaies, R.J., 2001. Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. Proc. Natl. Acad. Sci. U. S. A. 98, 8554–8559.

Sakamoto, K.M., Kim, K.B., Verma, R., Ransick, A., Stein, B., Crews, C.M., Deshaies, R.J., 2003. Development of Protacs to target cancer-promoting proteins for ubiquitination and degradation. Mol. Cell Proteomics 2, 1350–1358.

Scheffner, M., Nuber, U., Huibregtse, J.M., 1995. Protein ubiquitination involving an E1–E2–E3 enzyme ubiquitin thioester cascade. Nature 373, 81–83.

Sherman, M.Y., Goldberg, A.L., 2001. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. Neuron 29, 15–32.

Shimura, H., Schwartz, D., Gygi, S.P., Kosik, K.S., 2004. CHIP–Hsc70 complex ubiquitinates phosphorylated tau and enhances cell survival. J. Biol. Chem. 279, 4869–4876.

Tanaka, K., Suzuki, T., Hattori, N., Mizuno, Y., 2004. Ubiquitin, proteasome and parkin. Biochim. Biophys. Acta 1695, 235–247.

Urushitani, M., Kurisu, J., Tateno, M., Hatakeyama, S., Nakayama, K., Kato, S., Takahashi, R., 2004. CHIP promotes proteasomal degradation of familial ALS-linked mutant SOD1 by ubiquitinating Hsp/Hsc70. J. Neurochem. 90, 231–244.

Yoshida, Y., Tokunaga, F., Chiba, T., Iwai, K., Tanaka, K., Tai, T., 2003. Fbs2 is a new member of the E3 ubiquitin ligase family that recognizes sugar chains. J. Biol. Chem. 278, 43877–43884.

Alleviating Neurodegeneration by an Anticancer Agent

An Hsp90 Inhibitor (17-AAG)

MASAHIRO WAZA, HIROAKI ADACHI, MASAHISA KATSUNO, MAKOTO MINAMIYAMA, FUMIAKI TANAKA, AND GEN SOBUE

Department of Neurology, Nagoya University Graduate School of Medicine, Nagoya 466–8550, Japan

ABSTRACT: Heat shock proteins (HSPs) that function mainly as molecular chaperones play an important role in the folding and quality control of proteins. Compared with these chaperones, Hsp90 is unique in that it binds to substrate proteins, called Hsp90 client proteins. Hsp90 is involved in the folding, activation, and assembly of its client proteins in association with its co-chaperones. Because numerous oncoproteins belonging to the Hsp90 client protein family are selectively degraded 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. In spinal and bulbar muscular atrophy (SBMA), the pathogenic gene product is polyglutamine (polyQ)-expanded androgen receptor (AR), which belongs to the Hsp90 client protein family and is known to be degraded by 17-AAG. We have recently demonstrated that administration of an anticancer agent 17-AAG significantly ameliorated polyQ-mediated motor neuron degeneration by reducing the total amount of mutant AR. The ability of 17-AAG to 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 using a novel anticancer agent 17-AAG has emerged as a candidate for molecular-targeted therapies for neurodegenerative diseases.

KEYWORDS: neurodegeneration; polyglutamine; anticancer agent; Hsp90 inhibitor

INTRODUCTION

In chronic neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and polyglutamine (polyQ) diseases, abnormal

Address for correspondence: Gen Sobue, M.D., Ph.D., Department of Neurology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466–8550, Japan. Voice: +81-52-744-2385; fax: +81-52-744-2384.
e-mail: sobueg@med.nagoya-u.ac.jp

Ann. N.Y. Acad. Sci. 1086: 21–34 (2006).
doi: 10.1196/annals.1377.012

accumulation of disease-causing protein is a commonly observed characteristic.[1] A selective elimination of disease-causing protein would be a promising remedy for neurodegenerative disorders.

Among these neurodegenerative disorders, it is well confirmed that 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.[2] In polyQ diseases, spinal and bulbar muscular atrophy (SBMA) was first identified among these diseases[3] and is characterized by premature muscular exhaustion, slow progressive muscular weakness, atrophy, and fasciculation in bulbar and limb muscles.[4] 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 14–32; but ranges from 40 to 62 CAGs in SBMA patients.[5] A correlation exists between the number of CAG repeats and disease severity.[6,7] The pathologic features of SBMA are motor neuron loss in the spinal cord and brain stem,[4] and diffuse nuclear accumulations and nuclear inclusions (NIs) containing the mutant AR in the residual motor neurons and certain visceral organs.[8]

Heat shock protein (Hsp)90 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.[9–11] Hsp90 functions in a multicomponent 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.[12] 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 in clinical trials as a novel molecular-targeted agent for a wide range of malignancies.[13] AR also belongs to the Hsp90 client protein family and is degraded in the presence of Hsp90 inhibitors.[14–16]

We have recently demonstrated that 17-AAG markedly ameliorated polyQ-mediated motor neuron degeneration through degradation of mutant AR.[17] We consider that the ability to facilitate degradation of disease-causing protein would be of value when applied to SBMA and other related neurodegenerative diseases. This review discusses our research findings and other studies, and the clinical application of Hsp90 inhibitors to neurodegenerative diseases beyond neoplastic ones.

GENERATION OF LESS TOXIC HSP90 INHIBITOR, 17-AAG

The most classical Hsp90 inhibitor is geldanamycin (GA), a natural product that was developed as an antifungal agent.[18] Later, GA was also found to have

a potent and selective antitumor effect against a wide range of malignancies.[19] Although GA showed potential as a novel anticancer agent,[20] it was also found to have intolerable liver toxicity.[21] To overcome this GA-induced liver toxicity, scientists at the U.S. National Cancer Institute (NCI) succeeded in developing a new derivative of GA, 17-AAG, that shares its important biological activities[22] but shows less toxicity.[23] Owing to this promising derivative, Hsp90 inhibitors have taken a major developmental leap in their clinical applications, and 17-AAG is now in Phase II clinical trials with encouraging results as an anticancer agent.[24–28]

The antitumor effect of Hsp90 inhibitors is due to their ability to specifically bind to the Hsp90 adenosine 5'-triphosphate (ATP)-binding site, thereby modulating Hsp90 function and proteasomal degradation of Hsp90 client proteins.[29–31] Because numerous oncoproteins were shown to belong to the family of Hsp90 client proteins,[12] Hsp90 inhibitors are expected to become part of a new strategy in antitumor therapy.[20] Hsp90 inhibitors including GA and 17-AAG have been shown to have a higher selectivity for tumor cells compared with general antitumor agents.[32,33] This selectivity is due to the high affinity of 17-AAG for the Hsp90 client oncoproteins when they are incorporated in the Hsp90-dependent multichaperone complex, thereby increasing their binding affinity to 17-AAG more than 100-fold.[34] This high selectivity of 17-AAG to the incorporated Hsp90 client protein eventually minimizes its toxic side effects.[31] We think that this selectivity of Hsp90 inhibitors would also be advantageous for the treatment of neurodegenerative diseases.

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 (FIG. 1A).[16,35–37] As for AR, Hsp90 is essential to maintain its high ligand-binding affinity and its stabilization.[9,14] In practice, Hsp90 inhibitors reduce androgen ligand-binding affinity and induce the degradation of AR.[14,15] We also confirmed 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.[17]

17-AAG'S DEGRADATION OF MUTANT AR DEPENDS ON PROTEASOME

To determine whether or not 17-AAG promotes the degradation of the 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 dimethylsulfoxide (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 did the wild type, suggesting that the mutant AR is more sensitive to 17-AAG than is the wild type. The degradation of wild-type and mutant AR by 17-AAG was completely

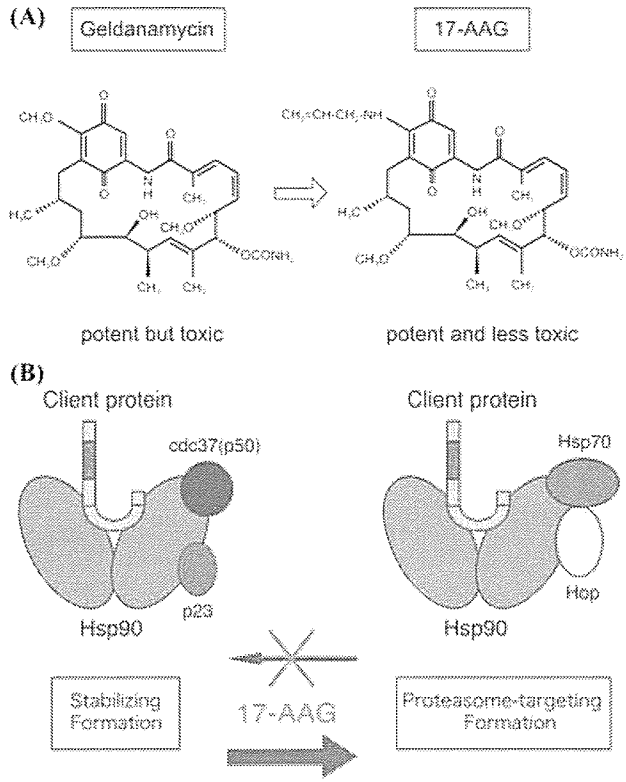

FIGURE 1. Hsp90 inhibitors and the pharmacological effect. (A) Geldanamycin (GA) is the most classical Hsp90 inhibitor. Although GA has a potent and selective antitumor effect against a wide range of malignancies, this agent has intolerable liver toxicity. The 17-AAG, a new derivative of GA, shares its important biological activities but shows less toxicity. (B) The 17-AAG specifically binds ATP-binding site of Hsp90, resulting in a shift of the Hsp90 complex. 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.[16]

blocked by MG132 (FIG. 2A), suggesting that 17-AAG-faciiltated degradation was dependent on the proteasome system as previously reported.[38,39]

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 was 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. 2B), while mRNA levels for both the wild-type and mutant AR were quite similar.[17] These data indicate that 17-AAG preferentially degrades the mutant AR protein without altering mRNA levels.

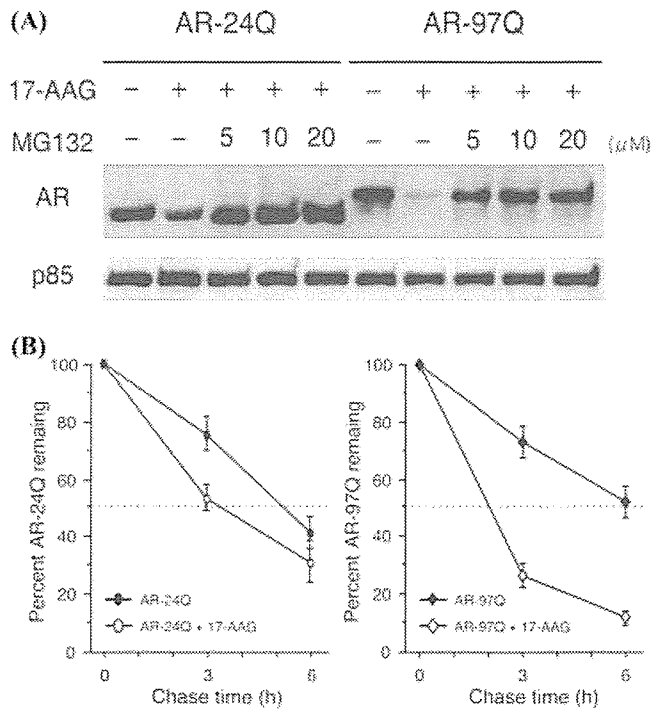

FIGURE 2. The 17-AAG's degradation of mutant AR depends on proteasome function. (A) 17-AAG treatment (36 μM, 6 h) of transfected SH-SY5Y cells reduced the levels of mutant AR (AR-97Q) significantly more than wild-type AR (AR-24Q), however, both decreases were completely blocked by the proteasomal inhibitor, MG132. (B) Effects of 17-AAG on the half-life of wild-type and mutant AR assessed from pulse-chase experiments. Amounts of AR-24Q remaining in the absence and presence of 17-AAG are indicated by closed circles (●) and open circles (○), respectively. 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).

Thus, these in vitro studies indicated that the mutant AR was a good target protein of 17-AAG. To determine whether 17-AAG has the ability to degrade the toxic disease-causing protein 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 WITHOUT DETECTABLE TOXICITY

Referring to previous reports,[15,36,40] we administered 17-AAG thrice a week on alternate days 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). AR-24Q mice treated with 17-AAG displayed no altered phenotypes (data not shown). To evaluate toxic effects of 17-AAG, we examined blood samples from 25-week-old mice treated with 25 mg/kg 17-AAG for 20 weeks. Hematological examination demonstrated that 17-AAG resulted in neither infertility nor liver or renal dysfunction in the AR-97Q male mice at the dose of 25 mg/kg.[17]

When mouse tissues were immunohistochemically stained for mutant AR using the 1C2 antibody, which specifically recognizes expanded polyQ, quantitative analysis revealed a marked reduction in 1C2-positve nuclear accumulation in the spinal motor neurons of the Tg-25 mice compared with those of the Tg-0 mice (FIG. 3B).

Western blot analysis from lysates of the spinal cord 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. 3C). 17-AAG treatments significantly diminished both the high-molecular-weight complex and the monomer of mutant AR in the spinal cord of the AR-97Q mice, whereas they only slightly diminished the wild-type monomeric AR in AR-24Q mice (FIG. 3C). The levels of wild-type and mutant AR mRNA were similar in the respective mice treated with 17-AAG.[17] These observations indicate that 17-AAG markedly reduces not only the high-molecular-weight mutant AR complex but also the monomeric mutant AR protein by preferential degradation of mutant AR.

MOLECULAR-TARGETED THERAPY FOR DISEASE-CAUSING PROTEIN BY AN HSP90 INHIBITOR—BEYOND HSP INDUCER

Hsp90 inhibitors are known to possess the unique pharmacological effect of inducing a stress response, and, in addition to their use as anticancer agents, have also been developed as pharmacological HSP inducers.[41,42] In neurological disorders, many studies have already shown that, taking advantage of HSP-induction, Hsp90 inhibitors exerted potential neuroprotective effects in a model of HD,[43–45] tauopathies,[46–48] PD,[49–51] stroke,[52,53] and autoimmune encephalomyelitis.[54] In considering the role for molecular chaperones in neurological disorders, Hsp70 and Hsp40 have received most of the attention, especially in neurodegenerative diseases.[55–56] because 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.[55–57] However, in our cultured cell models, mutant AR was markedly decreased following 17-AAG treatment even when Hsp70 and Hsp40 induction was completely blocked in the presence of a protein synthesis inhibitor.[17] In SBMA mice, the Western blot analysis

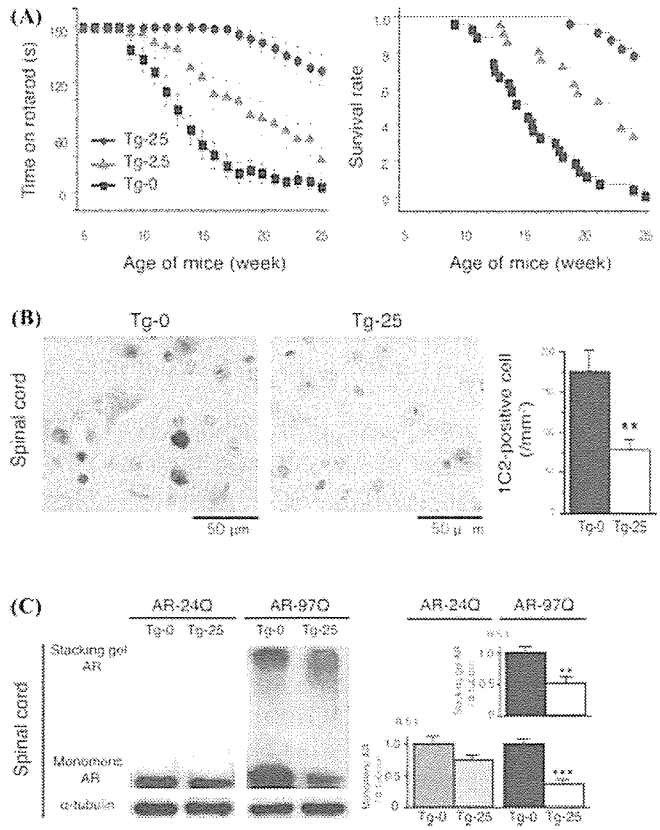

FIGURE 3. Effects of 17-AAG on transgenic SBMA mice. (A) Tg-0, Tg-2.5, and Tg-25 represent AR-97Q mice treated with vehicle alone, 2.5, and 25 mg/kg 17-AAG, respectively (each group: n = 27). The Tg-25 remained longer on the Rotarod than did the Tg-0 mice. A Kaplan–Meier plot shows the prolonged survival 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). The 17-AAG was less effective at the dose of 2.5 mg/kg than 25 mg/kg in all parameters tested. (B) Immunohistochemical staining with 1C2 antibody showed marked differences in diffuse nuclear staining and NIs between DMSO-treated mice (Tg-0) and 17-AAG-treated (Tg-25) mice in the spinal anterior horn. There was a significant reduction in 1C2-positive cell staining in the spinal cord (**$P < 0.01$) in the Tg-25 compared with the Tg-0. Values are expressed as means ± SE (n = 6). (C) Western blot analysis of tissue from AR-24Q and AR-97Q mice probed with an AR-specific antibody. In both spinal cords of mice treated with 17-AAG, there was a significant decrease in the amount of 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. 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, whereas there was only a 25.9% decline in the spinal cord of AR-24Q mice. Values are expressed as means ± SE (n = 5). Statistical differences are indicated by asterisks; *, $P < 0.05$; **, $P < 0.01$.

revealed that the inductions of Hsp70 and Hsp40 were statistically significant, but were also not as pronounced as those arising from genetic manipulation in our previous study.[17,57] 17-AAG thus displayed the limited ability to induce Hsp70 and Hsp40 in mouse tissue, while mutant AR was significantly decreased in Western blot analysis and histopathological study (FIG. 3).

As for SBMA, Thomas et al. have published an interesting report that Hsp90 inhibitor inhibited the aggregation of polyQ-expanded mutant AR in HSF-1 (-/-) mouse embryonic fibroblasts where HSPs were not induced, suggesting that the induction of stress proteins was not necessary for the reduction of mutant AR aggregation by Hsp90 inhibitors.[58] Furthermore, they first demonstrated that Hsp90 inhibitors prevented the aggregation of the mutant AR by the inhibition of Hsp90-dependent trafficking machinery. It seems that Hsp90 would also play a major role in the acceleration of mutant AR aggregation.

Although it would be advantageous for the treatment of neurodegeneration to induce HSPs by Hsp90 inhibitors, considering our research finding in in vivo models, it would be unadvisable to rely only on the induction of nonspecific HSPs for human clinical trials. In SBMA, where it may have its most effective potential, 17-AAG directly accelerates proteasomal degradation of the disease-causing protein, polyQ-expanded AR. 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. We think the induction of HSPs by Hsp90 inhibitors seems to play a supplementary role in neurodegenerative disorders.

CLINICAL APPLICATION OF 17-AAG TO NEUROLOGICAL DISORDERS

In the cultured cells and mouse models of SBMA, we have shown both the efficacy and safety of 17-AAG.[17] Based on our data, 17-AAG would be a candidate for therapeutic agents for SBMA via the ability to facilitate the degradation of mutant protein.

We also confirmed that leuprorelin, a lutenizing hormone-releasing hormone agonist that reduces testosterone release from the testis, significantly rescued motor dysfunction in our SBMA mice.[59] Due to its minimal invasiveness, established in human, and its powerful action, this hormonal therapy has already been in human clinical trials with encouraging results.[60] However, it is an extremely specialized therapy for SBMA and cannot be applied to other polyQ diseases.[59,61,62] In contrast to this hormonal therapy, 17-AAG would be a potential therapeutic agent for SBMA as well as other related diseases.[17] We think that this anticancer agent 17-AAG holds enormous potential for application to a wide range of neurodegenerative diseases in addition to SBMA as previously reported.[41,55,63] For development of Hsp90 inhibitor treatment in neurological disorders, we regard this general versatility as very important. Among neurodegenerative disease-causing proteins, only AR in SBMA

is established as an Hsp90 client protein at this time. However, evidence has accumulated that some Hsp90 client proteins would exert adverse influences on several neurodegenerative disorders.[64–66]

It is well known that many kinases involved in signal transduction belong to the family of Hsp90 client proteins targeted by 17-AAG. Phosphorylated tau is a representative disease-causing protein associated with tauopathies including fronto-temporal dementia, progressive supranuclear palsy, corticobasal degeneration, and multiple system atrophy. Interestingly, phosphorylated tau is a targeted protein of Hsp90 inhibitors.[46] 17-AAG reduces the total amount of phosphorylated tau and its abnormal aggregation. Dou et al. showed that GA and 17-AAG indirectly blocked abnormal tau phosphorylation by inhibition of the Raf-MEK-ERK pathway,[67] of which upstream kinase Raf is also an Hsp90 client protein.[12,68] Extracellular signal-regulated kinase (ERK) is known to mediate the activation and stabilization of phosphorelated tau.[69,70] Along these same lines, LaFevre-Bernt & Ellerby 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.[71] 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 that ERK activation is an important executor of neuronal damage.[72–75] 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. For further development of Hsp90 inhibitors beyond malignancies, it is of considerable importance to assess whether or not other Hsp90 client proteins would exert an adverse effect on neurological disorders.

INTEGRATION OF NEURONAL AND NEOPLASTIC DISORDERS

Most antitumor agents that have been therapeutically applied to neurodegenerative diseases[76,77] have some cytotoxic effects on normal cells, which must be overcome in any clinical application against neurodegeneration. Because neurodegenerative diseases generally follow a chronic progression and the medical treatment is long-standing compared with that for malignancy, the toxic side effects should be extensively suppressed. In contrast to general antitumor agents, the effects of 17-AAG have been known to have a high selectivity to tumor cells that would yield desirable results for neurodegenerative diseases.[32,33]

Considering our research findings and those of the other above-mentioned reports, in addition to its role in malignancies, Hsp90 client protein exerts an adverse influence on the nervous system in some situations. In this case,

it is reasonable to consider modulating Hsp90 function appropriately, where 17-AAG would exert the maximum pharmacological effect.

Until now, when considering the role of molecular chaperones in neurological disorders, Hsp70 and Hsp40 have received most of the attention, especially in neurodegenerative diseases.[55,56] However, evidence has accumulated that Hsp90 would be an important therapeutic target in neurodegenerative disorders.[17,58,67] We believe that 17-AAG, an Hsp90 inhibitor, has great potential to become a new molecular-targeted therapy against a wide range of neurodegenerative diseases as well as malignancies. It is not too much to say that neuronal and neoplastic disorders have something in common, so crossover researches between them should be performed for their further development.

ACKNOWLEDGMENTS

We thank the National Cancer Institute and Kosan Biosciences for kindly providing 17-AAG. This work was supported by a Center-of-Excellence (COE) grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan, grants from the Ministry of Health, Labour and Welfare, Japan, by a grant from the Naito Foundation, and by a grant from the Kanae Foundation.

REFERENCES

1. Ross, C.A. & M.A. Poirier. 2004. Protein aggregation and neurodegenerative disease. Nat. Med. 10(Suppl.): S10–S17.
2. Di Prospero, N.A. & K.H. Fischbeck. 2005. Therapeutics development for triplet repeat expansion diseases. Nat. Rev. Genet. 6: 756–765.
3. La Spada, A.R., E.M. Wilson, D.B. Lubahn, et al. 1991. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352: 77–79.
4. Sobue, G., Y. Hashizume, E. Mukai, et al. 1989. X-linked recessive bulbospinal neuronopathy. A clinicopathological study. Brain 112(Pt 1): 209–232.
5. Tanaka, F., M. Doyu, Y. Ito, et al. 1996. Founder effect in spinal and bulbar muscular atrophy (SBMA). Hum. Mol. Genet. 5: 1253–1257.
6. Doyu, M., G. Sobue, E. Mukai, et al. 1992. Severity of X-linked recessive bulbospinal neuronopathy correlates with size of the tandem CAG repeat in androgen receptor gene. Ann. Neurol. 32: 707–710.
7. Atsuta, N., H. Watanabe, M. Ito, et al. 2006. Natural history of spinal and bulbar muscular atrophy (SBMA): a study of 223 Japanese patients. Brain 129: 1446–1455.
8. Adachi, H., M. Katsuno, M. Minamiyama, et al. 2005. Widespread nuclear and cytoplasmic accumulation of mutant androgen receptor in SBMA patients. Brain 128: 659–670.
9. Fang, Y., A.E. Fliss, D.M. Robins, et al. 1996. Hsp90 regulates androgen receptor hormone binding affinity in vivo. J. Biol. Chem. 271: 28697–28702.
10. Georget, V., B. Terouanne, J.C. Nicolas, et al. 2002. Mechanism of antiandrogen action: key role of hsp90 in conformational change and transcriptional activity of the androgen receptor. Biochemistry 41: 11824–11831.

11. POLETTI, A. 2004. The polyglutamine tract of androgen receptor: from functions to dysfunctions in motor neurons. Front. Neuroendocrinol. 25: 1–26.
12. PRATT, W.B. & D.O. TOFT. 2003. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp. Biol. Med. (Maywood) 228: 111–133.
13. NECKERS, L. 2002. Hsp90 inhibitors as novel cancer chemotherapeutic agents. Trends Mol. Med. 8: S55–S61.
14. VANAJA, D.K., S.H. MITCHELL, D.O. TOFT, et al. 2002. Effect of geldanamycin on androgen receptor function and stability. Cell Stress Chaperones 7: 55–64.
15. SOLIT, D.B., F.F. ZHENG, M. DROBNJAK, et al. 2002. 17-Allylamino-17-demethoxygeldanamycin induces the degradation of androgen receptor and HER-2/neu and inhibits the growth of prostate cancer xenografts. Clin. Cancer Res. 8: 986–993.
16. NECKERS, L. 2002. Heat shock protein 90 inhibition by 17-allylamino-17-demethoxygeldanamycin: a novel therapeutic approach for treating hormone-refractory prostate cancer. Clin. Cancer Res. 8: 962–966.
17. WAZA, M., H. ADACHI, M. KATSUNO, et al. 2005. 17-AAG, an Hsp90 inhibitor, ameliorates polyglutamine-mediated motor neuron degeneration. Nat. Med. 11: 1088–1095.
18. DEBOER, C.M.P., R.J. WNUK , D.H. PETERSON. 1970. Geldanamycin, a new antibiotic. J. Antibiot. (Tokyo) 23: 442–447.
19. WHITESELL, L., S.D. SHIFRIN, G. SCHWAB, et al. 1992. Benzoquinonoid ansamycins possess selective tumoricidal activity unrelated to src kinase inhibition. Cancer Res. 52: 1721–1728.
20. NECKERS, L., T.W. SCHULTE & E. MIMNAUGH. 1999. Geldanamycin as a potential anti-cancer agent: its molecular target and biochemical activity. Invest. New Drugs 17: 361–373.
21. SUPKO, J.G., R.L. HICKMAN, M.R. GREVER, et al. 1995. Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. Cancer Chemother. Pharmacol. 36: 305–315.
22. SCHULTE, T.W. & L.M. NECKERS. 1998. The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. Cancer Chemother. Pharmacol. 42: 273–279.
23. PAGE, J., J. HEATH, R. FULTON, et al. 1997. Comparison of geldanamycin (NSC-122750) and 17-allylaminogeldanamycin (NSC-330507D) toxicity in rats. Proc. Am. Assoc. Cancer Res. 38: 308.
24. GOETZ, M.P., D. TOFT, J. REID, et al. 2005. Phase I trial of 17-allylamino-17-demethoxygeldanamycin in patients with advanced cancer. J. Clin. Oncol. 23: 1078–1087.
25. BANERJI, U., A. O'DONNELL, M. SCURR, et al. 2005. Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies. J. Clin. Oncol. 23: 4152–4161.
26. GREM, J.L., G. MORRISON, X.D. GUO, et al. 2005. Phase I and pharmacologic study of 17-(allylamino)-17-demethoxygeldanamycin in adult patients with solid tumors. J. Clin. Oncol. 23: 1885–1893.
27. RAMANATHAN, R.K., D.L. TRUMP, J.L. EISEMAN, et al. 2005. Phase I pharmacokinetic-pharmacodynamic study of 17-(allylamino)-17-demethoxygeldanamycin (17AAG, NSC 330507), a novel inhibitor of heat shock protein 90, in patients with refractory advanced cancers. Clin. Cancer Res. 11: 3385–3391.

28. HEATH, E.I., M. GASKINS, H.C. PITOT, et al. 2005. A Phase II trial of 17-Allylamino-17- Demethoxygeldanamycin in patients with hormone-refractory metastatic prostate cancer. Clin. Prostate Cancer 4: 138–141.
29. WHITESELL, L., E.G. MIMNAUGH, B. DE COSTA, et al. 1994. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc. Natl. Acad. Sci. USA 91: 8324–8328.
30. PRODROMOU, C., S.M. ROE, R. O'BRIEN, et al. 1997. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. Cell 90: 65–75.
31. WORKMAN, P. 2004. Altered states: selectively drugging the Hsp90 cancer chaperone. Trends Mol. Med. 10: 47–51.
32. KAMAL, A., L. THAO, J. SENSINTAFFAR, et al. 2003. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature 425: 407–410.
33. NECKERS, L. & Y.S. LEE. 2003. Cancer: the rules of attraction. Nature 425: 357–359.
34. KAMAL, A., L. THAO, J. SENSINTAFFAR, et al. 2003. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature 425: 407–410.
35. SULLIVAN, W., B. STENSGARD, G. CAUCUTT, et al. 1997. Nucleotides and two functional states of hsp90. J. Biol. Chem. 272: 8007–8012.
36. EGORIN, M.J., E.G. ZUHOWSKI, D.M. ROSEN, et al. 2001. Plasma pharmacokinetics and tissue distribution of 17-(allylamino)-17-demethoxygeldanamycin (NSC 330507) in CD2F1 mice1. Cancer Chemother. Pharmacol. 47: 291–302.
37. MCCLELLAN, A.J., M.D. SCOTT & J. FRYDMAN. 2005. Folding and quality control of the VHL tumor suppressor proceed through distinct chaperone pathways. Cell 121: 739–748.
38. MIMNAUGH, E.G., C. CHAVANY & L. NECKERS. 1996. Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. J. Biol. Chem. 271: 22796–22801.
39. BONVINI, P., H. DALLA ROSA, N. VIGNES, et al. 2004. Ubiquitination and proteasomal degradation of nucleophosmin-anaplastic lymphoma kinase induced by 17-allylamino-demethoxygeldanamycin: role of the co-chaperone carboxyl heat shock protein 70-interacting protein. Cancer Res. 64: 3256–3264.
40. BELIAKOFF, J., R. BAGATELL, G. PAINE-MURRIETA, et al. 2003. Hormone-refractory breast cancer remains sensitive to the antitumor activity of heat shock protein 90 inhibitors. Clin. Cancer Res. 9: 4961–4971.
41. WHITESELL, L., R. BAGATELL & R. FALSEY. 2003. The stress response: implications for the clinical development of hsp90 inhibitors. Curr. Cancer Drug Targets 3: 349–358.
42. BAGATELL, R., G.D. PAINE-MURRIETA, C.W. TAYLOR, et al. 2000. Induction of a heat shock factor 1-dependent stress response alters the cytotoxic activity of hsp90-binding agents. Clin. Cancer Res. 6: 3312–3318.
43. SITTLER, A., R. LURZ, G. LUEDER, et al. 2001. Geldanamycin activates a heat shock response and inhibits huntingtin aggregation in a cell culture model of Huntington's disease. Hum. Mol. Genet. 10: 1307–1315.
44. HAY, D.G., K. SATHASIVAM, S. TOBABEN, et al. 2004. Progressive decrease in chaperone protein levels in a mouse model of Huntington's disease and induction of stress proteins as a therapeutic approach. Hum. Mol. Genet. 13: 1389–1405.

45. AGRAWAL, N., J. PALLOS, N. SLEPKO, et al. 2005. Identification of combinatorial drug regimens for treatment of Huntington's disease using Drosophila. Proc. Natl. Acad. Sci. USA 102: 3777–3781.
46. DOU, F., W.J. NETZER, K. TANEMURA, et al. 2003. Chaperones increase association of tau protein with microtubules. Proc. Natl. Acad. Sci. USA 100: 721–726.
47. PETRUCELLI, L., D. DICKSON, K. KEHOE, et al. 2004. CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation. Hum. Mol. Genet. 13: 703–714.
48. DICKEY, C.A., J. DUNMORE, B. LU, et al. 2006. HSP induction mediates selective clearance of tau phosphorylated at proline-directed Ser/Thr sites but not KXGS (MARK) sites. FASEB J. 20: 753–755.
49. AULUCK, P.K. & N.M. BONINI. 2002. Pharmacological prevention of Parkinson disease in Drosophila. Nat. Med. 8: 1185–1186.
50. AULUCK, P.K., M.C. MEULENER & N.M. BONINI. 2005. Mechanisms of suppression of {alpha}-synuclein neurotoxicity by geldanamycin in drosophila. J. Biol. Chem. 280: 2873–2878.
51. FLOWER, T.R., L.S. CHESNOKOVA, C.A. FROELICH, et al. 2005. Heat shock prevents alpha-synuclein-induced apoptosis in a yeast model of Parkinson's disease. J. Mol. Biol. 351: 1081–1100.
52. LU, A., R. RAN, S. PARMENTIER-BATTEUR, et al. 2002. Geldanamycin induces heat shock proteins in brain and protects against focal cerebral ischemia. J. Neurochem. 81: 355–364.
53. GIFFARD, R.G., L. XU, H. ZHAO, et al. 2004. Chaperones, protein aggregation, and brain protection from hypoxic/ischemic injury. J. Exp. Biol. 207: 3213–3220.
54. MURPHY, P., A. SHARP, J. SHIN, et al. 2002. Suppressive effects of ansamycins on inducible nitric oxide synthase expression and the development of experimental autoimmune encephalomyelitis. J. Neurosci. Res. 67: 461–470.
55. MUCHOWSKI, P.J. & J.L. WACKER. 2005. Modulation of neurodegeneration by molecular chaperones. Nat. Rev. Neurosci. 6: 11–22.
56. LEVY, Y. & A. GORSHTEIN. 2005. Chaperones and disease. N. Engl. J. Med. 353: 2821–2822; author reply 2821-2822.
57. ADACHI, H., M. KATSUNO, M. MINAMIYAMA, et al. 2003. Heat shock protein 70 chaperone overexpression ameliorates phenotypes of the spinal and bulbar muscular atrophy transgenic mouse model by reducing nuclear-localized mutant androgen receptor protein. J. Neurosci. 23: 2203–2211.
58. THOMAS, M., J.M. HARRELL, Y. MORISHIMA, et al. 2006. Pharmacologic and genetic inhibition of hsp90-dependent trafficking reduces aggregation and promotes degradation of the expanded glutamine androgen receptor without stress protein induction. Hum. Mol. Genet. 15: 1876–1883.
59. KATSUNO, M., H. ADACHI, M. DOYU, et al. 2003. Leuprorelin rescues polyglutamine-dependent phenotypes in a transgenic mouse model of spinal and bulbar muscular atrophy. Nat. Med. 9: 768–773.
60. BANNO, H., H. ADACHI, M. KATSUNO, et al. 2006. Mutant androgen receptor accumulation in spinal and bulbar muscular atrophy scrotal skin: a pathogenic marker. Ann. Neurol. 59: 520–526.
61. KATSUNO, M., H. ADACHI, A. KUME, et al. 2002. Testosterone reduction prevents phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. Neuron 35: 843–854.
62. KATSUNO, M., H. ADACHI, F. TANAKA, et al. 2004. Spinal and bulbar muscular atrophy: ligand-dependent pathogenesis and therapeutic perspectives. J. Mol. Med. 82: 298–307.

63. KAMAL, A., M.F. BOEHM & F.J. BURROWS. 2004. Therapeutic and diagnostic implications of Hsp90 activation. Trends Mol. Med. 10: 283–290.
64. CHEN, H.K., P. FERNANDEZ-FUNEZ, S.F. ACEVEDO, et al. 2003. Interaction of Akt-phosphorylated ataxin-1 with 14-3-3 mediates neurodegeneration in spinocerebellar ataxia type 1. Cell 113: 457–468.
65. KOPRIVICA, V., K.S. CHO, J.B. PARK, et al. 2005. EGFR activation mediates inhibition of axon regeneration by myelin and chondroitin sulfate proteoglycans. Science 310: 106–110.
66. HERRMANN, O., B. BAUMANN, R. DE LORENZI, et al. 2005. IKK mediates ischemia-induced neuronal death. Nat. Med. 11: 1322–1329.
67. DOU, F., L.D. YUAN & J.J. ZHU. 2005. Heat shock protein 90 indirectly regulates ERK activity by affecting Raf protein metabolism. Acta Biochim. Biophys. Sin. (Shanghai) 37: 501–505.
68. STANCATO, L.F., Y.H. CHOW, K.A. HUTCHISON, et al. 1993. Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system. J. Biol. Chem. 268: 21711–21716.
69. FERRER, I., R. BLANCO, M. CARMONA, et al. 2001. Phosphorylated map kinase (ERK1, ERK2) expression is associated with early tau deposition in neurones and glial cells, but not with increased nuclear DNA vulnerability and cell death, in Alzheimer disease, Pick's disease, progressive supranuclear palsy and corticobasal degeneration. Brain Pathol. 11: 144–158.
70. PEI, J.J., H. BRAAK, W.L. AN, et al. 2002. Up-regulation of mitogen-activated protein kinases ERK1/2 and MEK1/2 is associated with the progression of neurofibrillary degeneration in Alzheimer's disease. Brain Res. Mol. Brain Res. 109: 45–55.
71. LAFEVRE-BERNT, M.A. & L.M. ELLERBY. 2003. Kennedy's disease. Phosphorylation of the polyglutamine-expanded form of androgen receptor regulates its cleavage by caspase-3 and enhances cell death. J. Biol. Chem. 278: 34918–34924.
72. MURRAY, B., A. ALESSANDRINI, A.J. COLE, et al. 1998. Inhibition of the p44/42 MAP kinase pathway protects hippocampal neurons in a cell-culture model of seizure activity. Proc. Natl. Acad. Sci. USA 95: 11975–11980.
73. CHEUNG, E.C. & R.S. SLACK. 2004. Emerging role for ERK as a key regulator of neuronal apoptosis. Sci. STKE 2004: PE45.
74. SUBRAMANIAM, S., U. ZIRRGIEBEL, O. VON BOHLEN UND HALBACH, et al. 2004. ERK activation promotes neuronal degeneration predominantly through plasma membrane damage and independently of caspase-3. J. Cell Biol. 165: 357–369.
75. ZHU, X., H.G. LEE, A.K. RAINA, et al. 2002. The role of mitogen-activated protein kinase pathways in Alzheimer's disease. Neurosignals 11: 270–281.
76. FERRANTE, R.J., H. RYU, J.K. KUBILUS, et al. 2004. Chemotherapy for the brain: the antitumor antibiotic mithramycin prolongs survival in a mouse model of Huntington's disease. J. Neurosci. 24: 10335–10342.
77. RAVIKUMAR, B., C. VACHER, Z. BERGER, et al. 2004. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat. Genet. 36: 585–595.

Gene Expression Profiling toward Understanding of ALS Pathogenesis

FUMIAKI TANAKA, JUN-ICHI NIWA, SHINSUKE ISHIGAKI, MASAHISA KATSUNO, MASAHIRO WAZA, MASAHIKO YAMAMOTO, MANABU DOYU, AND GEN SOBUE

Department of Neurology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

ABSTRACT: Although more than 130 years have gone by since the first description in 1869 by Jean-Martin Charcot, the mechanism underlying the characteristic selective motor neuron degeneration in amyotrophic lateral sclerosis (ALS) has remained elusive. Modest advances in this research field have been achieved by the identification of copper/zinc superoxide dismutase 1 (SOD1) as one of the causative genes for rare familial ALS (FALS) and by the development and analysis of mutant SOD1 transgenic mouse models. However, in sporadic ALS (SALS) with many more patients, causative or critical genes situated upstream of the disease pathway have not yet been elucidated and no available disease models have been established. To approach genes causative or critical for ALS, gene expression profiling in tissues primarily affected by the disease has represented an attractive research strategy. We have been working on screening these genes employing and combining several new technologies such as cDNA microarray, molecular indexing, and laser capture microdissection. Many of the resultant genes are of intense interest and may provide a powerful tool for determining the molecular mechanisms of ALS. However, we have barely arrived at the starting point and are confronting an enormous number of genes whose roles remain undetermined. Challenging tasks lie ahead of us such as identifying which genes are really causative for ALS and developing a disease model of SALS with due consideration for the expression changes in those genes.

KEYWORDS: ALS; SOD1; gene expression analysis; cDNA microarray; molecular indexing; laser capture microdissection

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative and fatal human disorder characterized by loss of motor neurons in the spinal cord, brain stem,

Address for correspondence: Dr. Gen Sobue, Department of Neurology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Voice: +81-52-744-2385; fax: +81-52-744-2384.
e-mail: sobueg@med.nagoya-u.ac.jp

Ann. N.Y. Acad. Sci. 1086: 1–10 (2006).
doi: 10.1196/annals.1377.011

and motor cortex, presenting as weakness of the limbs, speech abnormalities, and difficulties in swallowing.[1] The terminal phases of the disease involve respiratory insufficiency and half of the patients die within 3 years after the onset of symptoms. ALS can be inherited as an autosomal dominant trait in a subset of individuals who make up 5% to 10% of the total population of those affected. In addition, 20% to 30% of familial ALS (FALS) cases are associated with a mutation in the copper/zinc superoxide dismutase 1 gene (SOD1).[2] However, more than 90% of ALS patients are sporadic, not showing any familial trait. Since there have been no available disease models for sporadic ALS (SALS) as of now, transgenic mouse models or cell culture models[3] of ALS associated with SOD1 mutations have proven very useful in studying the initial mechanisms underlying this neurodegenerative disease of unknown etiology. The use of an animal model makes it possible and easy to investigate the different stages of disease progression including the early preclinical phase.

One of the experimental approaches toward a more comprehensive understanding of the molecular changes occurring in ALS is gene expression study[4] employing array-based methods or a differential display and its related techniques. Using transgenic mouse models expressing the SOD1 gene with a G93A mutation, we performed cDNA microarray analysis[5] to reveal the transcriptional profiles of affected tissues, namely, spinal anterior horn tissues. This analysis revealed an upregulation of genes related to an inflammatory process together with a change in apoptosis-related gene expression at the presymptomatic stage prior to motor neuron death.

Next, we extended our gene expression study from mouse to human postmortem spinal anterior horn tissues obtained from SALS patients. In this analysis, we employed a molecular indexing technique, a modified version of the differential display developed by Kato in 1995.[6] These PCR-based screening procedures have the advantage of being able to cover an unrestricted range of expressed genes including even hitherto unknown ones. As a result, we have successfully cloned a novel gene designated "dorfin,"[7] the expression of which was upregulated in SALS spinal cords.

Using spinal anterior horn tissues of SOD1 mutant mice or SALS patients as starting materials, these gene expression studies[5,7] have shed considerable light on the pathogenesis of FALS and SALS. However, in the spinal anterior horn tissues of ALS spinal cords, there are reduced numbers of motor neurons with glial cell proliferation. The alteration of the gene expression in the spinal anterior horn tissues could reflect the number of motor neurons and glial cells during disease progression. Such a disadvantage in using anterior horn tissues as starting materials prompted us to try to extract a pure motor neuron-specific gene expression profile. To this end, we employed the technology of laser capture microdissection[8] combined with T7-based RNA amplification and cDNA microarrays, which culminated in the successful detection of a total of 196 genes considered important for the SALS molecular mechanism.[9]

GENE EXPRESSION ANALYSIS FOR MUTANT SOD1 MOUSE MODEL OF ALS

We analyzed both temporal and differential gene expressions in the lumbar spinal anterior horn tissues of the transgenic mouse models expressing the SOD1 gene with a G93A mutation and the controls.[5] In this analysis, we detected a significant upregulation of 30 specific transcripts and downregulation of 7 transcripts in the spinal cords of mutant SOD1 mice[5] (TABLE 1). Before 11 weeks of age, mutant SOD1 mice are free of a disease phenotype, but they begin to decline rapidly in motor function after 14 weeks. The employment of mice for gene expression analysis provides a great advantage in obtaining data in the preclinical stage.

Interestingly, we found an upregulation of genes related to an inflammatory process together with a change in apoptosis-related gene expression at 11 weeks of age in the preclinical stage prior to motor neuron death.[5] The representative inflammatory-related genes elevated in their expression at this stage were the tumor necrosis factor (TNF)-α gene, which is a proinflammatory cytokine, and the Janus tyrosine-protein kinase 3 (JAK3), a necessary component of cytokine receptor signaling (TABLE 1). At a subsequent disease stage of 14 or 17 weeks of age, many more genes associated with an inflammatory process such as cathepsin D, serine protease inhibitor (SPI) 2–4, and cystatin C precursor, CD68, CD147, and clusterin increased their expression (TABLE 1). A histopathological evaluation showed glial cell activation and proliferation as early as 11 weeks of age and continuing to advance until 17 weeks.[10] A temporal increase in the expression level observed in these genes might reflect an inflammatory response with activated microglia and reactive astrocytes.

On the other hand, caspase-1, an initiator of the neuronal apoptotic cascade, was also upregulated at a presymptomatic 11 weeks of age (TABLE 1). An interrelationship between the inflammatory reaction and apoptotic pathway has been demonstrated. In addition to its role as an initiator of neuronal apoptotis, extracellular caspase-1 converts interleukin-1β (IL-1β) into a mature form. Thus, caspase-1 activation in motor neurons contributes to an inflammatory pathway with early astrocytosis and microglial activation in mutant SOD1 mice. In contrast, there is strong evidence for an inflammatory response involving microglial activation that leads to neuronal apoptosis.[11] Activated microglia express neurotoxic cytokines and substances such as TNF-α, proteases, oxyradicals, and small reactive molecules.[12] A nearly simultaneous upregulation of genes related to an inflammatory process and apoptotic initiation at the preclinical stage might contribute to the relentless neurodegenerative process making for a detrimental cycle. At 14 weeks of age, an early phase of the symptomatic stage, a key executioner of apoptosis, caspase-3, resulting from caspase-1 activation, began to be upregulated.[13] This finding agrees with

TABLE 1. Differentially expressed predominant genes detected in spinal anterior horn or spinal motor neurons from SOD1 mutant mice and sporadic ALS patients

Analysis object	SOD1 G93A mutant mice[5,13] spinal anterior horn	SALS patients[7,18] spinal anterior horn	SALS patients[9] spinal anterior horn	SALS patients[9] spinal motor neuron
Analysis method	cDNA microarray	Molecular indexing	cDNA microarray	cDNA microarray
	30/1176	46/entire mRNA	37/4845	52/4845
Upregulated genes in ALS	TNF-α*†	dorfin*	KIAA0231	death receptor 5 (DR5)*
	JAK3†	TAFII30*	fibrinogen A a polypeptide	cyclin A1, cyclin C*, ephrin A1*
	cathepsin D	neugrin*	presenilin 1	caspase 1, caspase 3, caspase 9
	serine protease inhibitor (SPI) 2–4		ephrin A1	acetyl-coenzyme A transporter*, NF-κB
	cystatin C precursor		transcription factor NF-Atc	ciliary neurotrophic factor (CNTF)
	CD68*, CD147		SH3-binding protein 2	hepatocyte growth factor (HGF)
	clusterin		integrin alpha E precursor (ITGAE)	glial cell line-derived neurotrophic factor (GDNF)
	caspase-1*†, caspase-3		cysteine dioxygenase, type 1	KIAA0231*
	GFAP*, vimentin			glutamate receptor subunit 2 (GLUR-2)
	Bcl-xL			interleukin-1 receptor antagonist
	c-fos, junD			TNF receptor-associated factor 6 (TRAF6)
Downregulated genes in ALS	7/1176	38/entire mRNA	8/4845	144/4845
	XIAP	metallothionein-3*	glutamate receptor, metabotropic 6	dynactin 1 (p150)*, TRK-C*, midkine, musashi 1
	$GABA_A$-receptor-α1	MRP8*	cholecystokinin A receptor	microtubule-associated protein 1A, 4
		ubiquitin-like protein 5*	signal recognition particle 14kD	microtubule-associated protein tau
			syntaxin 1B	early growth response 3 (EGR3)*
			sex-determining region Y (SRY)-box 11	BCL2-antagonist/killer 1 (Bak)*
				cellular retinoic acid-binding protein 1 (CRABP1)*
				retinoic acid receptor-α

Principal genes showing expression changes of 3.0-fold increase and 0.3-fold decrease are listed. Fold-change is calculated by dividing the fluorescence signals of each ALS sample by those of control samples.*Gene expression changes were confirmed by other methods such as reverse transcription-polymerase chain reaction (RT-PCR) or *in situ* hybridization.†Genes upregulated in 11-week-old mice.

the result that at 14 weeks of age XIAP mRNA downregulation occurred in the spinal cords of mutant SOD1 mice (TABLE 1) since XIAP is a direct inhibitor of caspase-3, -7, and -9.[14]

DISCOVERY OF NOVEL GENES ASSOCIATED WITH ALS PATHOGENESIS

To identify genes differentially expressed in the anterior horn tissues of the human SALS spinal cord, we adopted molecular indexing, a modified version of the differential display.[6] The entire mRNA population is identified and displayed by 3′ end cDNA fragments generated by class IIS restriction enzyme digestion and PCR.[6] Accordingly, molecular indexing provides a significant advantage in expression analysis for unknown genes. Among 84 fragments differentially expressed in SALS cloned in the first screening procedure, we noticed a fragment with an unknown sequence overexpressed in SALS spinal cords. We cloned it using RACE methods and named it dorfin (double ring-finger protein)[7] (TABLE 1).

Dorfin contains a RING-IBR (in between ring finger) domain at its N terminus and mediated ubiquitin ligase (E3) activity.[7] Interestingly, dorfin is predominantly localized and overexpressed in the ubiquitinated neuronal hyaline inclusion bodies found in the motor neurons of SALS patients as well as FALS patients with a SOD1 mutation and of mutant SOD1-transgenic mice.[15,16] An in vitro assay revealed that dorfin physically bound and ubiquitylated various SOD1 mutants and enhanced their degradation, and that its overexpression protected neural cells against the toxic effects of mutant SOD1 and reduced SOD1 inclusions.[15,17] These findings suggest that dorfin, an E3 ligase, may play some protective role in the pathogenesis of FALS and SALS via the ubiquitylation and degradation of its substrates, mutant SOD1, and others yet to be identified.

Besides dorfin, we have detected 30-kDa TATA binding protein-associated factor (TAFII30) and neugrin as upregulated genes in the SALS spinal cord[18] (TABLE 1). On the other hand, metallothionein-3, macrophage-inhibiting factor-related protein-8 (MRP-8) and ubiquitin-like protein 5 were downregulated in their expression[18] (TABLE 1).

MOTOR NEURON-SPECIFIC GENE EXPRESSION PROFILE IN SALS

As noted above, even using spinal anterior horn tissues consisting of heterogeneous cell types including motor neurons and glial cells as starting materials, gene expression studies have successfully shed light on the genes related to the pathogenesis of FALS and SALS.[5,7,18] However, the constitution