Androgen Receptors Containing Expanded Polyglutamine Tracts Exhibit Progressive Toxicity when Stably Expressed in the Neuroblastoma Cell Line, SH-SY 5Y

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The pathogenesis of X-linked spinal and bulbar muscular atrophy (SBMA) has been traced to an expansion of repeated glutamine (Gln) residues within the amino terminus of the human androgen receptor (AR). To examine the mechanisms by which these expanded repeat ARs (Exp-ARs) are toxic to neurons, we have established and characterized a cell culture model by stably transfecting SH-SY 5Y neuroblastoma cells with cDNAs containing either normal AR (81 series; 23 Gins) or Exp-AR (902 series; 56 Gins). At a low passage number, no differences in cell morphology, growth properties, or susceptibility to toxic insults were observed between clones expressing normal AR or Exp-AR. Initially, both types of cultures were found to express similar levels of specific hormone binding in monolayer binding assays. Immunohistochemical studies demonstrated the vast majority of both the normal AR and Exp-AR were localized to the nucleus in the absence and presence of androgen. As the 902 series of clones were propagated, the Exp-AR content in the cells appeared to decline progressively. However, this decrease actually reflects a gradual disappearance of the Exp-AR cell population. No such selection occurred during the propagation of cells expressing the normal AR. This selection against cells expressing physiological levels of Exp-AR occurs in the absence of intracellular aggregates and suggests that mechanisms other than those involving the formation of aggregates underlie the observed toxicity of Exp-ARs. Exp Biol Med 228:982–990, 2003

Key words: androgen receptor; trinucleotide repeat; spinal and bulbar muscular atrophy

A number of progressive neurological diseases have now been traced to expansions of triplet repeats within the human genome (1, 2). In these diseases, expansions occur within both the noncoding and coding segments of the affected genes. In several [Huntington's disease (3) spinocellebellar ataxia-type 1 (4, 5), Machado-Joseph disease (6), dentatorubral pallidoluysian atrophy (7–9), and X-linked spinal and bulbar muscular atrophy [SBMA] (10)], the pathogenesis of the disease has been traced to an increased size of CAG repeats within the open reading frame of the disease gene. These expanded repeats encode polyglutamine tracts in the protein products.

In the case of X-linked SBMA (Kennedy's disease), the expanded polyglutamine stretch is located within the amino terminus of the human androgen receptor (AR) protein (10). In normal individuals, the number of repeated glutamine residues ranges from 12 to 30, with 20 through 23 representing the lengths most frequently observed (11–13). However, in patients with SBMA, the size range begins at 40 residues and extends to up to 72 (10). This region is important for transactivation by the receptor, and functional studies have suggested that this expanded glutamine repeat sequence reduces the transcriptional activity (14–16) as well as the ligand binding (17) of the AR. This may be further accentuated by decreased levels of AR expression (18, 19). Nonetheless, although these alterations may account for the subtle signs of androgen insensitivity that accompany this disorder, the gain of a toxic function is believed to underlie the pathogenesis of the motor neuron degeneration that is characteristic of this disease.

More recently, attention has focused on the fate in cultured cells of the mutant AR proteins that contain glutamine repeat expansions. Investigations by several groups have suggested that the pathogenesis of this disorder may be linked to the accumulation of smaller AR species (20–23). The results obtained are consistent with those from studies

This work was supported by the National Institutes of Health Grant DK 03892, by a grant from the Robert A. Welch foundation Grant I-1090, and by a grant from the Muscular Dystrophy Association.

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Received October 21, 2002.
Accepted April 25, 2003.

1535-3702/03/2288-0982$15.00

of other neurodegenerative diseases caused by expansion of glutamine repeats within the coding segment of other genes, such as Huntington's disease (24–26). It has been suggested that the formation of these fragments requires the actions of components of the cellular apoptotic machinery, and that the toxic effects involve impairment of the ubiquitin-proteasome system (20, 27). An additional key aspect of the toxicity of proteins containing expanded glutamine repeats—as yet unexplained—relates to the timing of the onset of the neurodegeneration characteristic of SBMA and other such disorders. Although each has been traced to inherited genetic changes, signs and symptoms of these neurodegenerative diseases do not appear until middle or later adulthood.

Many of the model systems that have been used to study the toxic effects of proteins containing glutamine repeat expansions have employed high-level expression of the respective proteins or expression of proteins with very long polyglutamine stretches. The experiments described here were performed to attempt to establish a model system that would permit a detailed study of the toxicity exhibited by ARs containing glutamine repeat expansions when expressed at levels approximating that observed in AR-expressing cells and tissues. Our results suggest that ARs containing expanded glutamine repeats exert toxic effects when expressed in the neuron-like cell line, SH-SY 5Y, and that this toxicity does not require the accumulation of aggregates containing proteolytic fragments derived from expanded repeat ARs.

Materials and Methods

Cell Lines and Reagents. The human neuroblastoma SH-SY 5Y cell line was obtained at passage 14 and was propagated as described in medium containing a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin ("growth medium") (28). Unless noted otherwise, cells were propagated in the absence of androgen. The plasmids encoding the normal human AR and the expanded repeat AR have been described previously (16, 29). G418 was obtained from GIBCO-BRL (Gaithersburg, MD). Medium and serum were obtained from Mediatech (Herndon, VA). Antibodies directed at the human AR have been described previously (30, 31). Antibodies to neuron specific enolase, the middle molecular weight neurofilament subunit, NF-M (BF10), and tyrosine hydroxylase were obtained from Chemicon International (Temecula, CA), Vector Laboratories (Burlingame, CA), and Calbiochem (La Jolla, CA), respectively.

Cell Transfection and Culture. Monolayers of SH-SY 5Y cells were transfected using calcium phosphate precipitation with plasmids encoding ARs containing normal (20 residues) or expanded (56 residues) glutamine repeat segments (32). Stable clones expressing these normal and mutant ARs were established by cotransfection with the plasmid pSV2neo, carrying the selectable marker, *neo*^{r}, followed by selection with the antibiotic G418 (400 μg/ml final concentration). Individual G418-resistant colonies were selected and propagated as individual clones. After the preservation of low-passage samples (Passage 1 [P1]), cultures representing the parental cell line transfected with the selectable marker and empty vector (pCMV) or transfected with the selectable marker in a combination with the normal human AR (the 81 series of cell clones) or the expanded-repeat AR (the 902 series of cell clones) were propagated continuously in growth medium containing G418. Subsequent passages are numbered in relation to these initial preserved clones. Passage 16 cells represent approximately 3.5 months in continuous culture. All stock cultures were passaged weekly in the "growth medium" (described above), and were fed again with fresh medium on Day 4 of each week. In the 7-day growth curve experiments, fresh medium was added to the cultures on Day 4. Unless otherwise noted, all cell cultures were propagated in the absence of androgen.

Immunoblot Assays. Cells were scraped, pelleted, resuspended in loading buffer (30), and stored at −80°C until use. Samples of the cell extracts were electrophoresed on 10% SDS PA gels and were transferred to nitrocellulose. Immunoreactive AR was detected by successive incubation with affinity-purified antibodies directed at selected epitopes within the AR protein (U402 U407, or R489 and ^{125}I-labeled goat anti-rabbit Fab2 [NEN Life Sciences Products, Boston, MA]). Antibodies U402, U407, and R489 recognize epitopes represented by amino acid residues 1 through 21, 200 through 220, and 893 through 917 of the AR, respectively (30, 31).

Immunofluorescence Microscopy. SH-SY 5Y cells were grown in plastic chamber-slides. Twenty hours before harvesting, samples were shifted to medium containing 5% charcoal-stripped serum containing no hormone or 2 n*M* mibolerone. At the time of harvesting, the chambers were washed twice with phosphate-buffered saline (PBS) and were fixed in 4% paraformaldehyde for 15 min and in acetone for 10 min. Unspecific binding was blocked by incubating with protein block-serum free in a humidified chamber at 4°C for 20 min and washed twice with PBS. Antibodies directed at the amino terminus of the human AR (U402) was added to the samples at 1:100 dilution in PBS and was incubated overnight at 4°C in a humidified chamber. After this incubation, the cells were washed twice with PBS and were incubated with fluorescein-conjugated goat anti-rabbit antibody at a dilution of 1:250 in 30% human serum for 2 hr at 4°C and were washed three times with PBS. The cells were incubated with rhodamine-conjugated avidin at a dilution of 1:600 for 1 hr at 4°C and were washed three times with PBS. The slides were mounted with Aqua-Mount and were observed using a Eclipse E1000M attachment cube (Nikon, Tokyo, Japan; FITC-HYQ and G-2/CTRITC) and a digital cooled SNAP camera. The images were processed using the MetaMorph imaging program.

Apoptosis Assay. Cultures of early passage cells (P2) were trypsinized and stained with Hoechst 33342 for 10 min. Apoptotic cells were identified on the basis of their typical morphological appearance, with chromatin condensation and nuclear fragmentation (33). The proportion of apoptotic cells was measured in approximately 300 cells in three separate determinations for each cell line.

Results

To examine the toxicity of ARs containing expanded glutamine repeats, we established a panel of transfected SH-SH5Y cell clones stably expressing normal or expanded glutamine repeat ARs, containing 20 and 56 glutamine residues, respectively. Colonies were isolated and propagated as individual clonal cell lines. Parallel cultures were transfected with the empty vector. SH-SY 5Y cells are a neuroblast subclone of the parent neuroblastoma cell line, SK-N-SH, however, the SH-SY 5Y population also contains a small proportion of epithelial-like and intermediate-type cells (34). As non-neuroblastic cells might have a higher propensity to take up DNA than neuroblasts, it was important to assess the neuronal properties of G418-resistant colonies. Characterization of the different clones revealed that they possess many similarities to the parental SH-SY 5Y cell line. Each of the clones exhibited a neuron-like morphology, and formed abundant neuritic processes in culture. In addition, all of the clones expressed neuron-specific markers, such as neuron-specific enolase, the middle molecular weight neurofilament subunit, NF-M, and tyrosine hydroxylase as assayed using immunofluorescence microscopy (data not shown). In all, 14 different clonal cell lines were established (11 different 902 clones and three different 81 clones). Five were examined in detail.

Immunoblot analysis of the clones demonstrated the expression of immunoreactive AR that migrated as expected based on the length of the polymorphic glutamine repeats that they contained, i.e., ~112 kD for the normal AR and a slightly higher molecular weight for the polyglutamine-expanded species (Fig. 1). Control cell lines (transfected with the empty vector) and the SH-SY 5Y parental cell line did not express detectable levels of immunoreactive AR protein. Of note, analyses of these early passage stable cell lines expressing the expanded glutamine repeat AR using antibodies directed at a variety of internal epitopes did not detect abundant smaller molecular weight species, as has been reported in other assays systems using transient or high-level expression.

The localization of the normal and expanded glutamine repeat ARs in the two types of transfected cells was similar. In the absence of androgen, much of the AR was localized to the nucleus and only a small proportion was detected in the cytoplasm of the cells (Fig. 2). After the addition of 2 n*M* mibolerone, virtually all of the immunoreactive AR was found in the nucleus. Of note, the formation of intracellular aggregates has been reported as being a characteristic feature of proteins containing long polyglutamine tracts. In contrast to these reports, when the 81 and 902 cells strains were analyzed by immunofluorescence microscopy using antibodies directed at the human AR, we did not observe aggregates either in the cells expressing the normal or expanded glutamine repeat ARs.

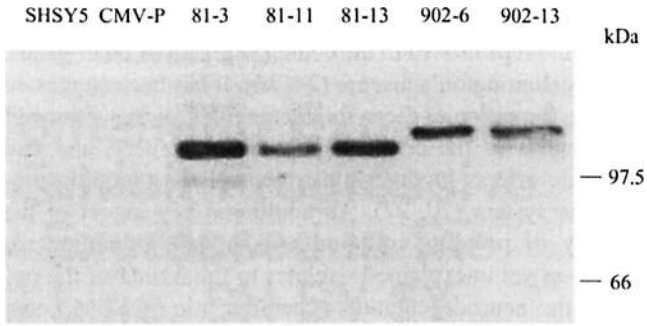

Figure 1. AR expression in the parental and derivative cell lines. To characterize the ARs expressed in the stably transfected cell lines, near confluent monolayer cultures of the individual pooled (-P) or clonal (numbered) cell strains were scraped and homogenized in loading buffer at passage 3. Aliquots of each sample were electrophoresed on a denaturing polyacrylamide gel and were transferred to a nitrocellulose membrane. Immunoreactive AR was detected using antibodies directed at the amino terminus of the protein (U402). As shown, immunoreactive AR of the predicted size of ~112 kD is detected in cells transfected with the expression vector encoding the normal human AR (20 glutamine residues; clones 81-3, -11, and -13). A slightly larger immunoreactive AR band is detected in cells transfected with the vector encoding the AR containing the expanded glutamine repeats (56 glutamine residues; clones 902-6 and 902-13). No immunoreactive AR was detected in the parental cell line (SH-SY 5Y) or in the cells transfected with the empty vector (CMV-P). Similar results were obtained when analyses were performed using antibodies directed at internal epitopes (anti-Internal-A antibodies) or at the carboxyl terminus of receptor protein (R 489; data not shown). Each lane contains 33 µg of cell extract protein. The positions of protein markers are shown to the right.

We sought to determine the effects of the expression of expanded glutamine repeat ARs on cell growth, cell morphology, and on recovery from toxic insults. At a low passage number, no obvious morphological differences were apparent between the different clones. Despite the application of a number of toxic insults (serum deprivation and shock with NaCl, glucose, or mannitol [35–37]), no substantial differences were evident between the parental SH-SY 5Y cell line, cells transfected with the empty pCMV vector, and cells expressing normal or expanded glutamine repeats AR (Fig. 3, A-C and data not shown).

After approximately 14 weeks in culture (greater than passage number 14), we observed that the cells from clones 902-6 and 902-13 began to show changes in morphology (Fig. 3F), with decreased substrate adherence and truncated neurite formation. These results are similar to those observed by Simeoni and colleagues (38). In addition, the 902-6 and -13 cell strains began to grow more slowly and exhibit increased serum dependence (Fig. 3C). Surprisingly, immunoblot analyses revealed that the AR was no longer expressed in the cells transfected with the expanded repeat AR at the time that these changes in morphology and growth began to appear (data not shown).

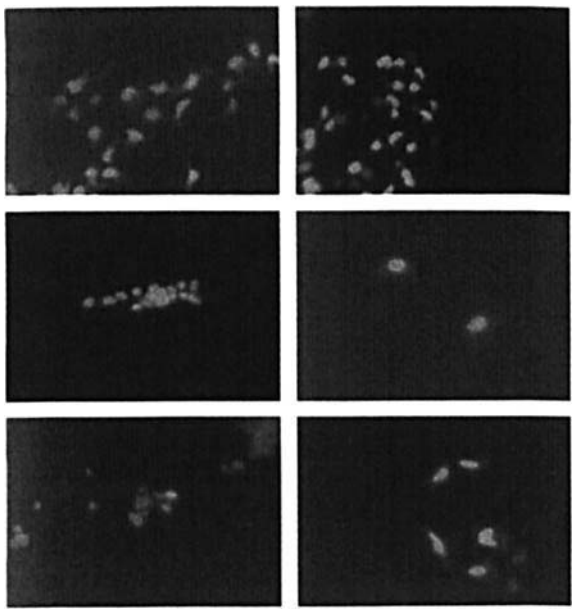

Figure 2. Immunohistochemistry of cell strains 81-3 (expressing ARs containing a normal-sized glutamine repeat) and strains 902-6 and 902-13 (expressing ARs containing an expanded glutamine repeat). In the absence of hormone, the AR is localized predominantly to the nucleus of expressing cells, with some accumulation in the cytoplasm. After the addition of saturating concentrations of agonist ligand (2 n*M* mibolerone), the AR immunoreactivity is localized exclusively to the nucleus. No intracellular aggregates of AR immunoreactivity are observed in any of the cell strains. High proportions of the cells of each strain are seen to express immunoreactive AR in such early passage cultures.

We then examined the changes of AR expression occurring as the different cell strains were passaged. In the cell strains expressing the normal AR, only small differences in AR expression levels were observed at higher passage numbers (Fig. 4, A and B). By contrast, levels of ARs containing an expanded glutamine repeat displayed progressive decreases as the cells were cultured. In the case of the 902-6 cell strain, in addition to the decrease in the expression of the intact AR, a smaller AR species fragment ~30 kDa in size increased with successive passage numbers. This fragment did not react with epitopes an antibody directed at an internal epitope (antibody U407 [which recognizes amino acids 200-220]; Fig. 4B), and thus is unlikely to represent a proteolytic fragment of the AR. In the case of the 902-13 cell strain, no immunoreactive AR fragments were observed; only a progressive decline in the level of AR expression was observed. These results parallel the results obtained using assays of ligand binding in intact cells (Table I).

To further examine the basis of these changes in the levels of AR expression, the proportion of cells expressing the AR was determined by immunofluorescence microscopy (Fig. 5). We observed that at early passage numbers, a high proportion of cells in all strains expressed the AR. As the cells were cultured, a gradual decrease in the proportion of cells that express the expanded repeat AR occurred. By contrast, the proportion of cells expressing the normal AR (strain 81-3) did not change appreciably with increasing passage number. These findings are tabulated in Table I. Of note, at no point were intracellular aggregates of AR evident in any of these analyses.

These experiments establish a paradigm in which the proportion of cells that stably express expanded glutamine repeat ARs decreases with increasing time of passage. An increased rate of apoptosis among the cells expressing the expanded glutamine repeats would represent one potential mechanism to explain these changes. To assess this possibility, the proportions of apoptotic cells were assessed by visualization of cells using immunofluorescence. The results of this analysis are presented in Table II. This experiment demonstrates that a slightly higher proportion of apoptotic cells are present in populations expressing either the normal or expanded repeat ARs. Despite this, the proportion of apoptotic cells in the lines expressing expanded glutamine repeats was not significantly higher in all of the cells strains compared with cells expressing the normal AR (81-3).

Discussion

SBMA is a progressive neurodegenerative disease caused by an expansion of a glutamine repeats within the amino terminus of the human AR (10). Although it was the first such disease to be described, a number of other neurodegenerative disorders have been identified whose pathogenesis can be traced to the expansion of CAG repeats in the open reading frame of the gene involved (3–9). A distinct group of disorders, usually not neurodegenerative, are caused the expansion of other types of triplet repeats that are located outside the open reading frame (1, 2). Those diseases in which the triplet expansion occurs in the untranslated segments of mRNA have been suggested to cause disease via abnormal interaction with RNA-binding proteins.

Several lines of evidence have suggested that the proteins containing expanded polyglutamine tracts, such as the AR, acquire a toxic property that is not possessed by the normal protein and that may involve accumulation of fragments derived from the protein containing the glutamine repeat expansion (20–23, 27, 39). Despite the insights that such experiments have provided, a number of important questions still remain pertaining to the mechanisms by which this toxicity emerges. This is particularly true as the onset of the diseases, like the majority of genetically-determined neurodegenerative diseases, most often occurs later in life (i.e., middle-age), in a time frame that is difficult to explain based on what is known regarding the expression of the gene products that have been implicated. In addition, it is remarkable that the toxicity observed in these diseases is restricted to cells of neural lineage, suggesting that some aspect of neuronal cell types might make them particularly susceptible to the effects of proteins containing the expanded glutamine repeats.

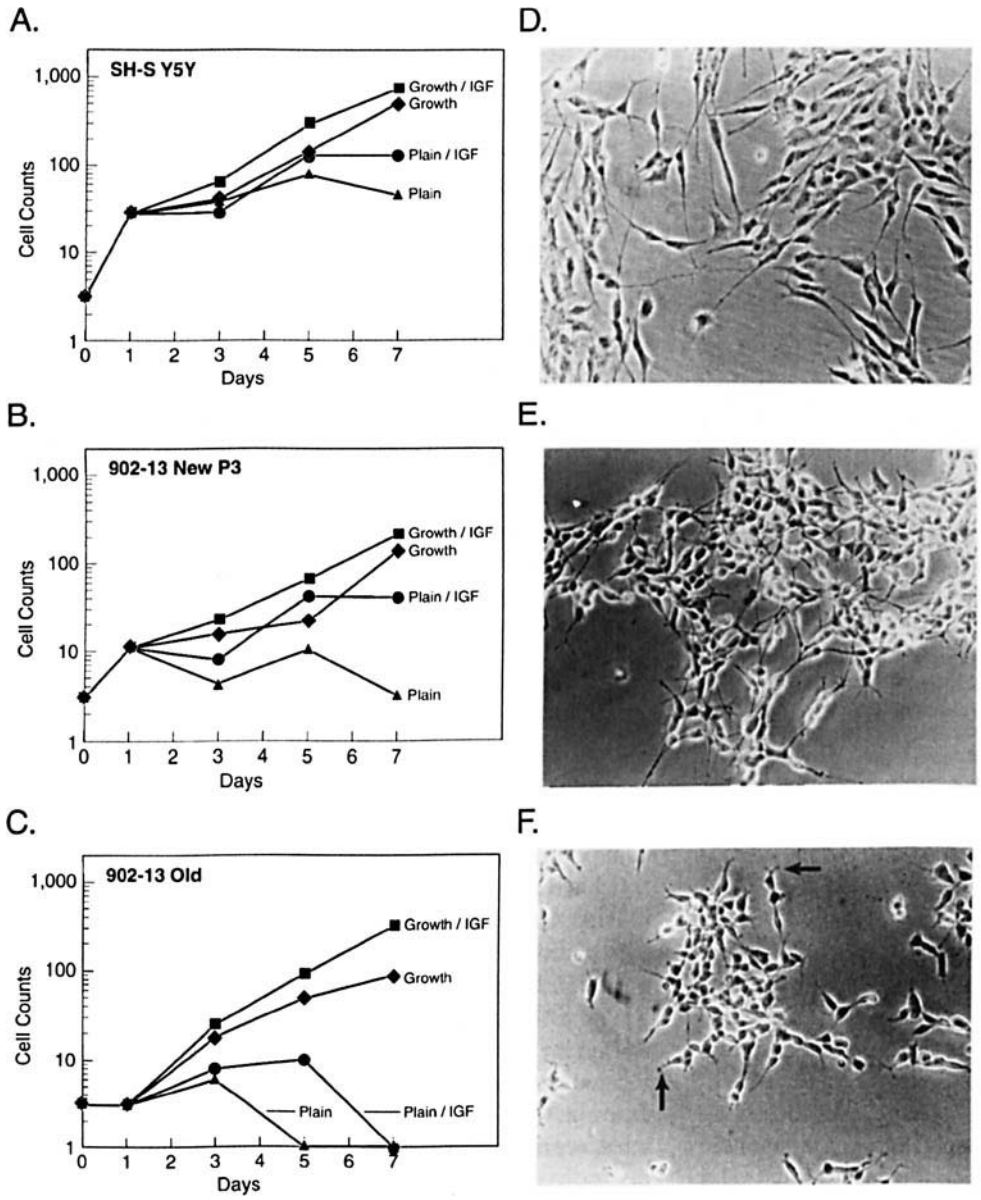

Figure 3. Growth and morphological changes appear in the cell lines that express the expanded glutamine repeat AR. In initial experiments, growth rates were similar between the SH-SY 5Y parental cell line, cells transfected with the empty expression vector, and cells transfected with ARs containing normal or expanded glutamine repeats. Growth curves from early passage parental SH-SY 5Y cells (A) and the clonal cell line 902-13 (expressing ARs with expanded glutamine repeats; B) are presented as representative. With continued passage, alterations in growth appeared only in cells expressing expanded glutamine repeats. These changes were reflected principally as diminished adherence to the culture dishes (data not shown) and an increased dependence on serum (displayed for the 902-13 cell line in C). These changes in growth rate are paralleled by changes in morphology. Early passage cells (SH-SY 5Y parental cell line, cells transfected with the empty expression vector, and cells transfected with ARs containing normal or expanded glutamine repeats) were indistinguishable morphologically and were similar to the passage 2 SH-SY 5Y parental cell strain shown in D. With increased passage number, although the cells expressing the AR containing normal-sized glutamine repeats (81-3) continued to form abundant long neurites (E), cells expressing ARs containing expanded glutamine repeats (strain 902-13) began to form short, stubby neurites (F).

In our studies, we established stable cell lines to establish a culture model in which to examine potential toxicities of ARs containing expanded glutamine repeats. Transfection and selection of the neuroblastoma cell line SH-SY 5Y with plasmids encoding the normal or expanded repeat ARs led to the isolation of clonal cell strains expressing the normal or expanded repeat ARs at comparable levels. Characterization of these cell populations demonstrated that the cell strains initially grew in a fashion indistinguishable from the parental strain and continued to express markers characteristic of neuron-like cells. Of note, with continued passage, the cells expressing the expanded glutamine repeats gradually began to display alterations of cell morphology and growth not observed in the parental cell line or in cells transfected with the normal AR. Furthermore, we found that these changes occurred despite the absence of detectable AR or AR fragments.

Further characterization demonstrated that differences in the levels of AR expression were evident between the cell strains expressing the normal AR (81 series) and those expressing the expanded glutamine repeat ARs (902 series) well before any changes in growth rate or morphology were evident. Specifically, we found that continued passage of the cell strains led to progressive decreases in the level of expression of the expanded repeat AR in strains 902-6 and 902-13. This decrease does not occur in the strains expressing the normal AR, despite the expression of similar levels of AR in the initial clonal cell lines. Immunofluorescence microscopy experiments revealed that these changes represent changes in the proportion of cells that express the expanded repeat AR. Consistent with the stable levels of expression of the normal AR (strain 81-3), the proportion of cells expressing the normal AR in this strain did not decrease as passage number increases.

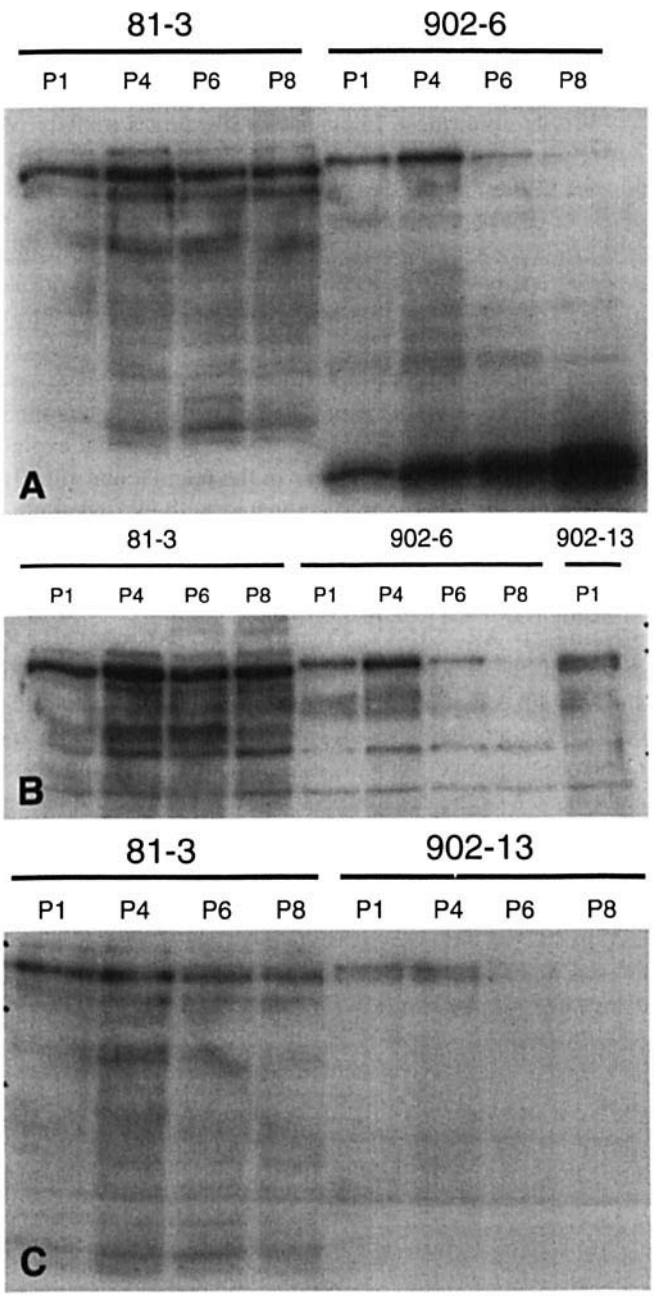

Figure 4. AR is lost as the SH-SY 5Y clones expressing expanded glutamine repeats ARs containing are propagated. (A) Immunoblot analysis of AR expression in successive passages of the 81-3 and 902-6 cell strains using an antibody directed at residues 1 through 20 of the amino-terminal of the AR (antibody U402). In samples prepared from early passage cells, similar levels of AR are detected, whereas in samples prepared from later passage cultures, levels of intact AR are observed to decrease progressively with successive culture only in the cells expressing the expanded glutamine repeat (902-6). Of note, with increasing passage, a shorter immunoreactive AR species is seen to accumulate in the 902-6 strain. (B) Immunoblot analysis of AR expression in successive passages of the 81-3 and 902-6, and 902-13 cell strains using an antibody directed at an epitope internal to the amino-terminal of the AR (U407; residues 200-220). The result of this analysis are similar to those depicted in Figure 3A. The principal finding of this immunoblot is to demonstrate that the small immunoreactive AR species that is increasing apparent in the later passages of the 902-6 cell strain is not identified with the U407 antibody. This finding suggests that the amino acid residues recognized by the U407 antibody (amino acids 200-220) are lacking from this immunoreactive AR species. Based on this observation and the apparent size of the immunoreactive fragment, simple proteolysis appears unlikely. This suggests the possibility that additional processing, either at the RNA or protein level, may lead this fragment to be inefficiently recognized by the U407 antibody. (C) Immunoblot analysis of AR expression in successive passages of the 81-3 and 902-13 cell strains using an antibody directed at the amino-terminal of the AR. In samples prepared from early passage cells, similar levels of AR are detected. In samples prepared from later passage cultures, intact AR levels decrease progressively only in the cells expressing the expanded glutamine repeat (902-13). Smaller immunoreactive AR fragments do dot accumulate in the 902-13 cell strain, as is observed in the 902-6 cell strains (Fig. 3A).

Two groups have reported studies of cells stably transfected with cDNAs encoding ARs containing different-sized glutamine repeats. In the experiments of Brooks *et al.* (19), a mouse motor neuron hybrid cell was stably transfected with cDNAs expressing normal and expanded repeat ARs. In these experiments, no differences were identified in the growth behavior or responses to stress between cells expressing the two types of AR, and only subtle functional differences between the ARs, were detected. Simeoni *et al.* (38) reported studies that examined clones of mouse neuroblastoma-spinal cord hybrid cells that expressed ARs containing normal, contracted, or expanded repeat ARs. These investigators observed alterations of cell growth and morphology that appeared as cells expressing the expanded repeat ARs were propagated. These same authors noted that the expression of AR mRNA in these cells declined at higher passage numbers. In this latter work, the relationship between the appearance of alterations in growth and morphology and the changes in AR expression was not presented.

Our experiments bear some similarity to the results of both studies. In keeping with the results of both groups, clonal cell strains that expressed similar levels of AR were isolated. In addition, our experiments demonstrated that at early passages, the cell strains expressing either type of AR (normal or expanded glutamine repeats) displayed growth and morphology properties similar to those of the parental cell line. In agreement with the studies of Simeoni *et al.* (38), we found that cells expressing the expanded glutamine repeat ARs began to display alteration of morphology and increased serum dependence.

Table I. Proportion of Cells Expressing AR Protein at Different Passages

Passage	Cell line	FITC-green	TRITC-red	(%)	Specific binding
P-2	81–3	27 ± 4.0	37 ± 7.0	73	346.5
P-2	902–6	10 ± 1.5	13 ± 1.0	77	430.8
P-9	81–3	25 ± 7.5	30 ± 4.5	83	561.8
P-9	902–6	2 ± 1.0	43 ± 1.5	5	35.8

Note. AR protein was visualized using a rabbit polyclonal antibody (U402) directed at the amino terminus of the AR. FITC-conjugated goat anti-rabbit immunoglobulin (whole molecule) was used to detect the specifically bound anti-AR antibody molecules. Cell nuclei were visualized using a TRITC-rhodamine conjugated egg white avidin. The number of stained nuclei and AR-expressing cells represent the average numbers of stained cells (±SD) in five fields each containing from 15 to 50 cells per field. Cell lines were treated with 2 n*M* Mb for 72 h before nuclear staining or immunohistochemistry. The levels of specific binding of [^{3}H]-dihydrotestosterone in the different cell strains as determined in monolayer binding assays are shown to the right (picomoles per milligram of protein).

We anticipated that such changes might reflect the accumulation of AR fragments that exerted deleterious effects on the cells expressing the expanded repeats. To our surprise, we discovered that the full-length AR expression could not be detected in the cell lines transfected with the expanded repeat AR cDNAs at the time that these alterations became evident. Careful studies examining the time course of AR expression revealed that although growth and morphology changes did not emerge until passage 14, populations of cells that had lost expression of the full-length AR containing the expanded glutamine repeat accounted for the bulk of the 902-6 and 902-13 cultures by passage 9.

We interpret these findings as reflecting a toxicity of ARs containing expanded glutamine repeats. This toxicity appears to exert a selective pressure that results in the emergence of cell populations with reduced expression of intact ARs containing the expanded glutamine repeats. The nature of this toxicity remains to be identified, but appears to be distinct from pathways that result in the formation of intranuclear inclusions.

An increase in the proportion of cells undergoing apoptosis as a result of the expression of expanded glutamine repeat ARs would represent one of several potential explanations for the observed changes in the populations of cells that express the AR (normal compared with expanded glutamine repeat). To examine this possibility, we performed experiments to examine the proportion of apoptotic cells in early passage cultures of each of the cell types. Cells expressing no AR (SH-SY 5Y parental cells), normal AR (81-3 cell line), and expanded glutamine repeat ARs (902-6 and 902-13 cell strains) were examined using immunofluorescence after staining with Hoechst 33342. These experiments demonstrate an increased proportion of apoptotic cells in each of the cell strains that express any form of the AR, whether normal or containing glutamine repeat expansions. Although this increased rate of apoptosis may well contribute to the changing proportion of cells the express

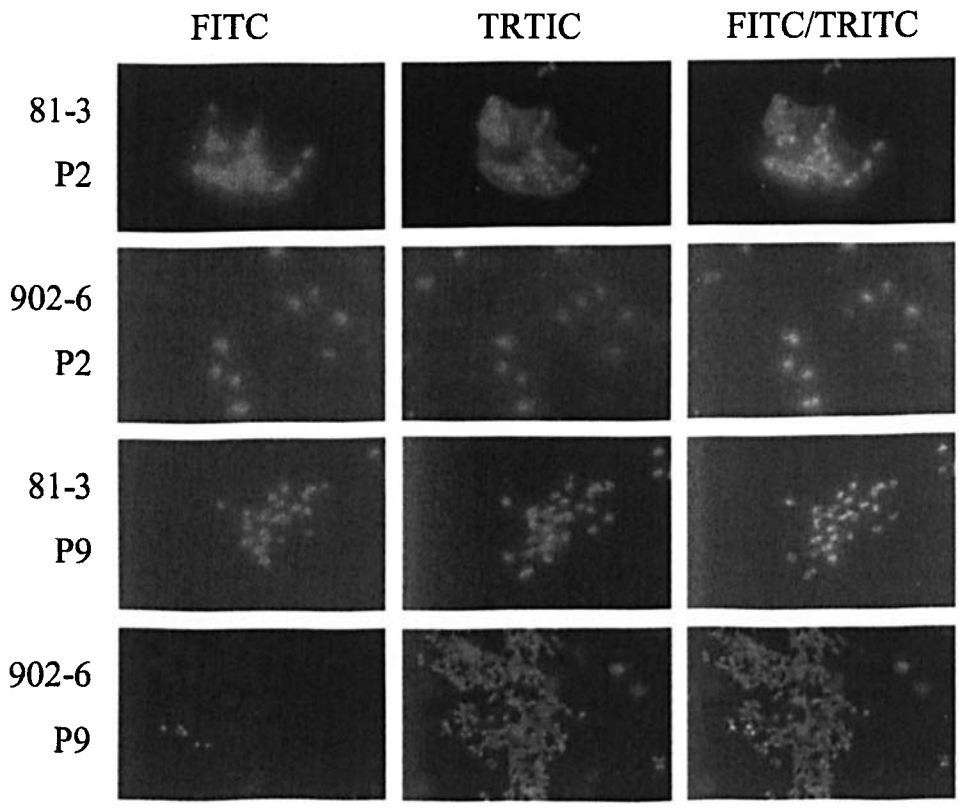

Figure 5. The decreased expression observed in cells expressing expanded glutamine repeat ARs reflects the emergence of cells lacking AR expression. Immunofluorescence microscopy was performed of early and late passage cell strains 81-3 (expressing ARs containing normal glutamine repeat) and strains 902-6 (expressing ARs containing expanded glutamine repeat) using an antibody directed at the amino terminus of the AR (U402). At early passages, a high proportion of the cells (expressing the normal or expanded repeat ARs) can be seen to express the AR. At higher passage numbers, similar proportions of the cells expressing the normal AR (strain 81-3) are observed to express detectable levels of immunoreactive AR. By contrast, at higher passages, a decreased proportion of cells expressing the expanded repeat AR (strain 902-6) is detected. All immunohistocytochemistry was performed after culture of the cells with saturating concentrations of mibolerone (2 n*M*) for 24 hr. Results similar to those depicted for the 902-6 strain are obtained when cultures of the 902-13 strain are performed. It is assumed that the small immunoreactive species observed in the 902-6 strain in immunoblot assays is lost from the samples during the fixation or incubation processes.

Table II. Proportion of Apoptotic Cells in Cell Lines Expressing the Normal or Expanded Glutamine Repeat AR

Cell strain	Number of cells examined	Proportion of apoptotic cells	Statistical significance compared with:		
			SH-SY 5Y	81-3	902-13
SH-SY 5Y	1104	4.08 ± 0.19	—		
81-3	957	5.72 ± 1.20	$P < 0.05$	—	
902-13	1153	7.87 ± 0.36	$P < 0.0005$	$P < 0.01$	—
902-6	1096	6.24 ± 1.05	$P < 0.005$	N.S.	$P < 0.025$

Note. Cultures of the SH-SY 5Y parental cell line the early passage cultures of each of the cell strains were grown as described in "Methods". Aliquots of the cell lines were trypsinized and stained with Hoeschst 33342 dye and were examined to determine the proportion of apoptotic cells, identified on the basis of their typical morphological appearance, with chromatin condensation and nuclear fragmentation (33). The numbers above represent the total number of cells counted in three separate determinations. The means ± SDs derived from these measurements are presented. The statistical significance of differences were obtained using Student's *t*-test. N.S., not significant; —, not applicable.

the AR, this cannot be the sole explanation. This is most clearly demonstrated by the observation that the proportion of apoptotic cells measured in one of the two cell lines expressing the expanded glutamine repeats AR (902-6) does not differ from the proportion in the 81-3 cell line that expresses the normal glutamine repeat AR. This finding suggests the additional mechanisms may serve alter the growth or viability of cells expressing expanded glutamine repeats.

In summary, the studies in the present manuscript suggest novel properties by which proteins containing glutamine repeat expansion can exert toxicity in a human neuroblastoma cell line. These effects are reflected in progressive changes in the proportion of cells that express ARs containing expanded glutamine repeats, but not the normal AR, during continued propagation. These changes are not reflections of supraphysiological levels of AR expression nor do they reflect differences in the levels of expression between the strains. Furthermore, these changes do not require the formation of intracellular aggregates and cannot be explained on the basis of differences in the proportion of apoptotic cells in these populations. Finally, our studies also demonstrate that the cell populations that we have studied are dynamic during a period of weeks. As this evolving heterogeneity is likely to complicate the interpretation of mechanistic studies, such experiments will optimally use cell strains in which these proteins can be expressed at physiological levels in a tightly regulated and inducible fashion.

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