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Motoneuronal cell death is not correlated with aggregate formation of androgen receptors containing an elongated polyglutamine tract

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Spinal and bulbar muscular atrophy (SBMA) is associated with an abnormal expansion of the $(CAG)_n$ repeat in the androgen receptor (AR) gene. Similar mutations have been reported in other proteins that cause neurodegenerative disorders. The CAG-coded elongated polyglutamine (polyGln) tracts induce the formation of neuronal intracellular aggregates. We have produced a model to study the effects of potentially 'neurotoxic' aggregates in SBMA using immortalized motoneuronal cells (NSC34) transfected with AR containing polyGln repeats of different sizes [(AR.Q(n = 0, 23 or 46)]. Using chimeras of AR.Q(n) and the green fluorescent protein (GFP), we have shown that aggregate formation occurs when the polyGln tract is elongated and AR is activated by androgens. In NSC34 cells co-expressing the AR with the polyGln of pathological length (AR.Q46) and the GFP we have noted the presence of several dystrophic neurites. Cell viability analyses have shown a reduced growth/survival rate in NSC34 expressing the AR.Q46, whereas testosterone treatment partially counteracted both cell death and the formation of dystrophic neurites. These observations indicate the lack of correlation between aggregate formation and cell survival, and suggest that neuronal degeneration in SBMA might be secondary to axonal/dendritic insults.

INTRODUCTION

Spinal and bulbar muscular atrophy (SBMA) is an X-linked recessive disease characterized by the selective loss of anterior horn neuronal cells in the spinal cord, by the depletion of sensory neurons in the dorsal root ganglia and by the selective degeneration of motoneurons in the brainstem (motoneurons of the lower cranial nerves) (1). The loss of motoneurons in the spinal cord and in the bulbar region results in muscle weakness and atrophy, fasciculations, dysphagia and dysarthia. Signs of mild androgen insensitivity are often present, including partial loss of secondary male sexual characteristics, gynaecomastia and testicular atrophy. The molecular basis of the disease is an abnormal increase in the length of the $(CAG)_n$ repeat present in the first coding exon of the androgen receptor (AR) gene (2); the length of the repeat, which normally ranges from 15 to 35 CAG triplets, is increased to >38 in affected individuals (2,3). The mutation leads to an elongation of the polyglutamine (polyGln) tract present in the N-terminal transactivation domain of the encoded protein.

The biological functions and the mechanism of action of AR are well understood (4–8); however, the physiological function(s) of the polyGln domain in wild-type AR, as well as the mechanism by which its expansion leads to neurodegeneration are unknown. The AR is a ligand-activated transcription factor, normally confined in a multi-heteromeric inactive complex in the cell cytoplasm. After binding to the ligand [testosterone or 5α-dihydrotestosterone (DHT)], the receptor dissociates from the accessory proteins (heat shock proteins), translocates into the cell nucleus, dimerizes and, through its DNA-binding domain, interacts with specific androgen-responsive elements located in the promoter region of androgen-responsive genes (3,4). Activation of transcription is then mediated by specific transactivation domains present in the receptor and located in its N- and C-terminal regions (3,9,10); activation of transcription also requires the presence of coactivators (SRC-1, p300, p/CAF, CBP, etc.), that are stabilized in a heterocomplex by protein–protein interactions with the AR. The complex recruits general transcription factors to the TATA box, and also exerts histone acetyl transferase activity modifying the structure of histones and of chromatin (11); the two mechanisms act jointly to activate transcription of target genes (11). The elongation of the polyGln present in one of the transactivation domains of SBMA AR modifies the transcriptional behaviour of the mutated receptor (12–14), possibly by altering its interaction with the co-activators, and this may explain the endocrine changes observed in Kennedy's disease; however, motoneuronal cell death does not appear to be linked to a loss-of-function of the mutated AR, since neurodegeneration does not occur in patients with testicular feminization, who lack AR function (15–17). Moreover, many different types of neuron express the AR, in particular in the hypothalamus (18–21), but the predominant cell types degenerating in SBMA are represented by the spinal and bulbar motoneurons that may be

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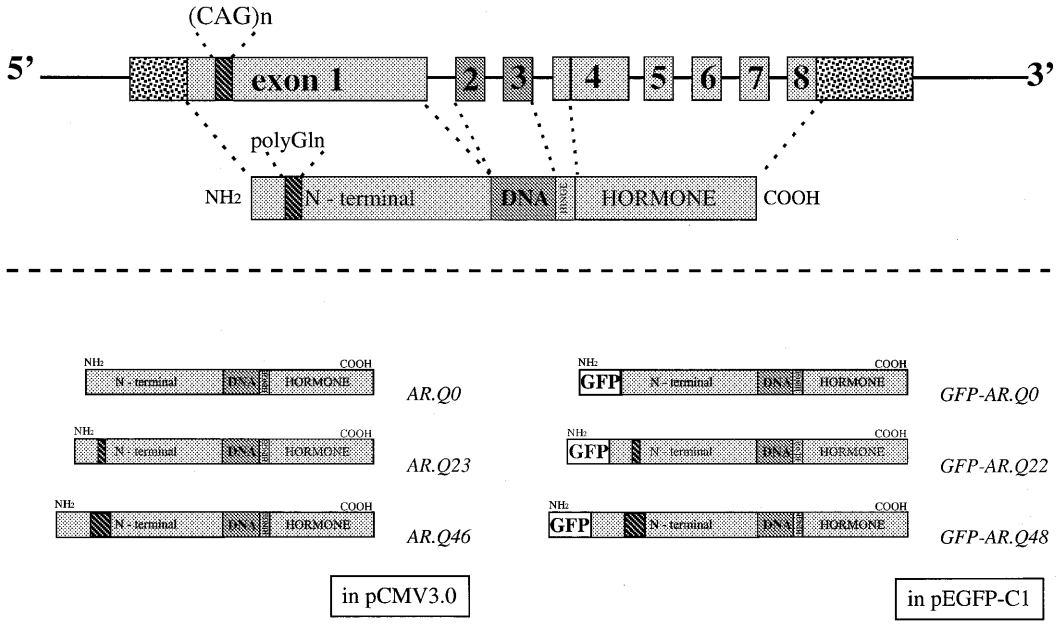

Figure 1. Schematic representation of the AR mutants transfected in NSC34.

particularly sensitive to the deleterious effects of the elongated polyGln tract (22). Interestingly, similar polyGln tract elongations have been found in a variety of proteins (huntingtin, atrophin, ataxins) which are involved in the pathogenesis of other neurodegenerative diseases (i.e. Huntington's disease, several types of spinocerebellar ataxia, dentatorubral and pallidoluysian atrophy, etc.) (23–32), indicating the existence of a common neurotoxic mechanism. Recent data indicate the presence of insoluble intracellular aggregates in all of these disorders including SBMA (33–52). The inclusions are analogous to those found in other neurodegenerative disorders (such as Alzheimer's, Parkinson's and prion diseases), suggesting that aggregates may be toxic for long-living postmitotic cells. Although aggregate formation may lead to cell death, this process has not been demonstrated and aggregate formation might not be the only cause of neurodegeneration. In this regard, Kennedy's disease offers a model to discriminate the well known biological functions of AR from the pathological functions acquired by the same receptor in SBMA. Despite this, very few studies have been done to ascertain whether in SBMA: (i) the spinal and bulbar motoneurons are a direct target of neurodegeneration; (ii) this process is secondary to other neuronal loss; and (iii) aggregate formation correlates with degeneration in these cells. We have developed an *in vitro* system to allow the analysis of possible direct toxic effects in motoneuronal cells of the elongated polyGln tract present in SBMA AR, and we have found that degeneration occurs in these cells, and that survival of motoneurons appears to be independent of aggregate formation.

RESULTS

Preparation and characterization of the cellular models

Neuroblastoma–spinal cord (NSC) 34 is a mouse hybrid cell line displaying a multipolar neuron-like aspect, which has been fully characterized for its motoneuronal phenotype (53,54) and found not to express endogenous mouse AR (55), and is the parental line for the model.

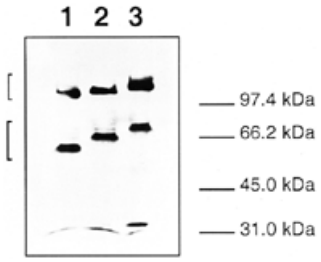

Figure 2. Western analysis of stably transfected NSC/AR.Q(n) performed using the AR(N-20) polyclonal antibody. The upper bands with molecular weight between 97 and 100 kDa are the different isoforms of AR; specific fragments from the N-terminal domain with molecular weights ranging from 50 and 69 kDa are detectable in all samples. Lane 1, cell extract from NSC/AR.Q0; lane 2, cell extract from NSC/AR.Q23; lane 3, cell extract from NSC/AR.Q46.

Figure 1 shows a schematic representation of the AR.Q(n) expression vectors utilized throughout the study. AR cDNA expression was driven by the potent Cytomegalovirus (CMV) promoter. pCMV/AR.Q23 expressed the wild-type AR with a polyGln tract of 23 Gln; in pCMV/AR.Q0 the CAG repeat was artificially removed (no polyGln tract); pCMV/AR.Q46 expressed AR with an elongated polyGln tract of 46 Gln; no additional amino acid changes were present. The plasmids were used to produce stably transfected cells that were named NSC/AR.Q0, NSC/AR.Q23 and NSC/AR.Q46, respectively. Expression of the desired cDNAs in NSC34 was tested by RT–PCR analysis followed by Southern analysis (data not shown). All clones expressed high levels of AR mRNA. In NSC/AR.Q46 the size of the CAG expansion remained stable, even in subsequent generations of cells, whereas the levels of mRNA coding for the AR gradually disappeared after

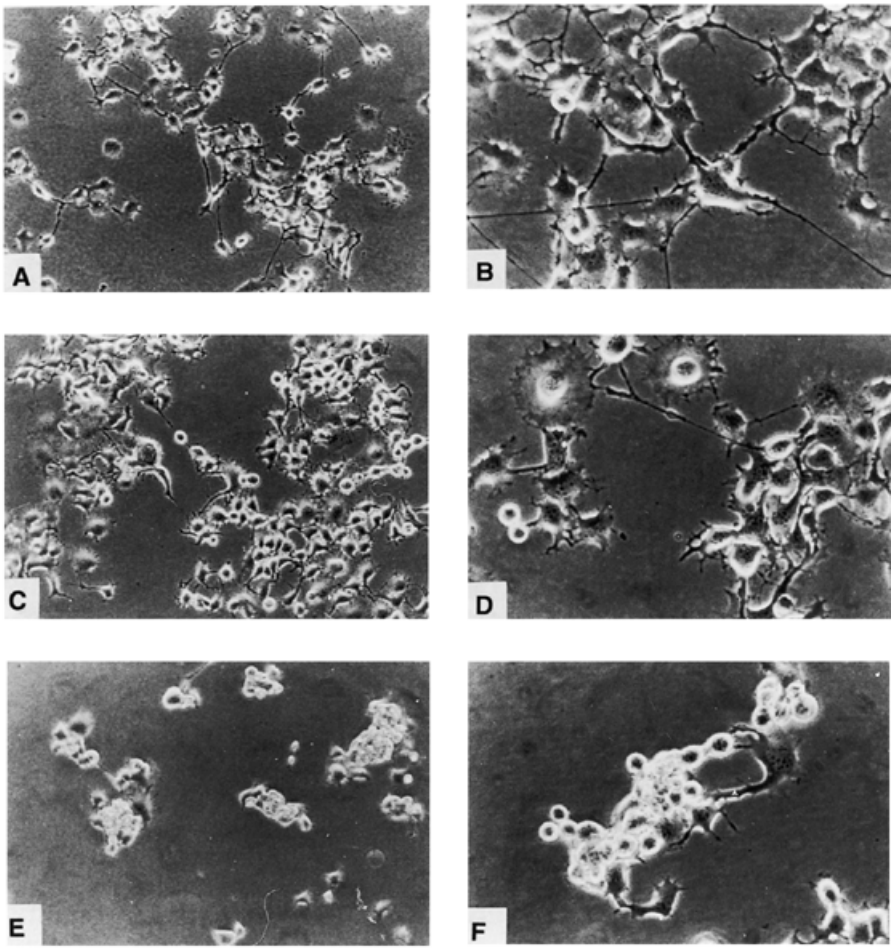

Figure 3. Cell morphology of stably transfected NSC/AR.Q(n). (**A** and **B**) NSC/AR.Q0; (**C** and **D**) NSC/AR.Q23; (**E** and **F**) NSC/AR.Q46. (A, C and E) Low magnification; (B, D and F) high magnification.

prolonged subculturing (data not shown), indicating the appearance of subclones expressing low levels of AR [confirmed by western analysis (data not shown)], possibly related to the deleterious effects of the elongated polyGln tract (see below), and this agrees with previous reports on SBMA AR (50).

Western analysis of cells transfected with recombinant ARs is shown in Figure 2. The levels of expression appeared comparable in all clones examined, and the different AR proteins were of the size expected for the presence of the corresponding polyGln tract. All samples showed the presence of a specific proteolytic fragment, apparently derived from the N-terminal region and from the DNA-binding domain of AR, which comprised the polyGln repeat and the epitope of the AR-N20 antibody, localized in the extreme N-terminal side of AR. Interestingly, C-terminally truncated fragments of AR may be toxic to motoneurons by initiating the transcription of specific genes in the absence of hormonal control (38), a possibility presently investigated in our laboratory.

Cell morphology. An initial careful examination of the stably transfected clones, grown in standard conditions, did not reveal significant differences in cell morphology; however, when cells were plated at low density (<15 000 cells/cm^2), morphological changes were evident in NSC/AR.Q46. Figure 3A and B shows NSC/AR.Q0 observed at two different magnifications. Cells were adherent to the substrate showing a motoneuronal-like morphology with multipolar long neurites. The morphology of NSC/AR.Q23 (Fig. 3C and D) was very similar to that observed for NSC/AR.Q0. When NSC/AR.Q46 cells were plated at low density, cell morphology was markedly altered. Cells (Fig. 3E) tended to clump together forming anchorage-independent aggregates, with very few processes extending from the body. At higher magnification (Fig. 3F), the cells showed no signs of neurite formation in the first 2–3 days in culture. NSC/AR.Q46 cells were significantly less adherent to the substrate than NSC/AR.Q0 and NSC/AR.Q23. Interestingly, after reaching higher cell density, they began to differentiate acquiring a more pronounced neuronal-like morphology, with an appearance of extending processes, and becoming more similar to NSC/AR.Q0 or NSC/AR.Q23.

To directly demonstrate that AR.Q46 was responsible for altered morphology, NSC34 cells were co-transfected with AR.Q(n) and with the pEGFP-N1 plasmid to identify the AR.Q(n)-positive cells. Transfected cells were detected by fluorescence microscopy (transfection efficiency was esti-

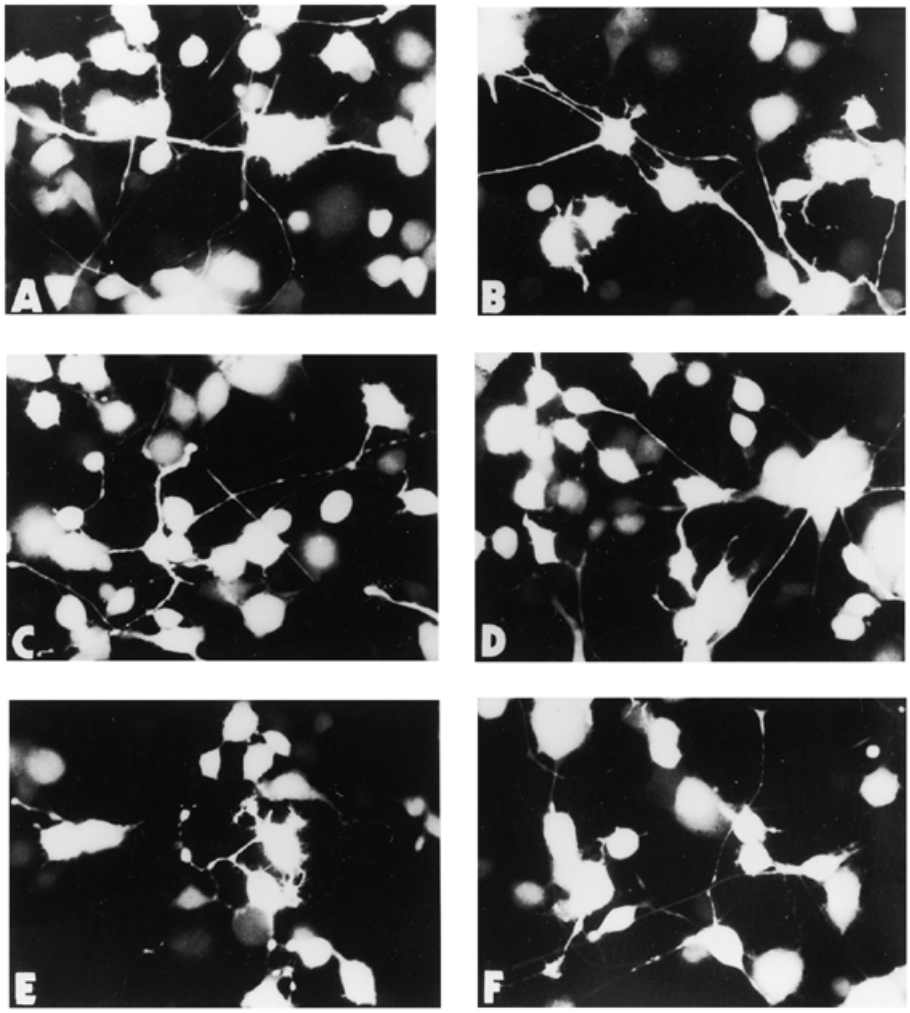

Figure 4. Cell morphology of NSC34 cells transiently transfected with pEGFP-N1 and the vectors expressing AR.Q(n). (**A**) Control NSC34 expressing AR.Q0; (**B**) NSC34 expressing AR.Q0 treated with 1 µM testosterone; (**C**) control NSC34 expressing AR.Q23; (**D**) NSC34 expressing AR.Q23 treated with 1 µM testosterone; (**E**) control NSC34 expressing AR.Q46; (**F**) NSC34 expressing AR.Q46 treated with 1 µM testosterone.

mated to range from 40 to 60%). Three days after transfection, many NSC34 cells expressing AR.Q46 (and pEGFP-N1) displayed short and dumpy neurites (Fig. 4E). The percentage of AR.Q46-containing cells showing aberrant phenotype was estimated to range between 25 and 30% of the total transfected fluorescent cells. In contrast, cells transfected with AR.Q0 (Fig. 4A) and AR.Q23 (Fig. 4C) showed the typical motoneuronal-like phenotype. Several cells were multipolar with long, well extended neurites. A 3 day treatment with testosterone did not modify neurite extension of control NSC34 (Fig. 4B and D, AR.Q0 and AR.Q23, respectively), but improved the ability of NSC34-expressing SBMA AR (Fig. 4F) to produce long, well extended neurites and a phenotype similar to that observed in control cells. NSC/AR.Q46 grown in the absence of testosterone showed a progressive increase in neurite damage. After 5 days, short neurites became dystrophic in a higher percentage of cells (30–40% of total transfected cells).

Mechanism of aggregate formation. To analyse whether altered cell morphology might be related to different intracellular localizations of the mutated AR protein, either in the presence or in the absence of testosterone, we analysed NSC34 cells transiently transfected with a chimeric protein composed of the green fluorescent protein (GFP) fused at the N-terminus of the AR. The constructs utilized are schematically represented in Figure 1, and have been already fully characterized in HeLa cells (52). In our motoneuronal model, similarly to HeLa cells, in the absence of testosterone, all types of receptor (wild-type and mutated) were found to be localized in the perinuclear region of the cytoplasm of motoneuronal cells (Fig. 5A, C and E, GFP–AR.Q0, GFP–AR.Q22 and GFP–AR.Q48, respectively), and no aggregates were present. After addition of testosterone, in the case of GFP–AR.Q0 and GFP–AR.Q22, the receptor moved into the nucleus (Fig. 5B and D, respectively). Conversely, after testosterone treatment GFP–AR.Q48-transfected cells showed intracellular aggregate formation, and in the aggregate-positive cells a small increase in the nuclear localization of the receptor protein was detectable (Fig. 5F). Identical results were obtained in electron microscopy (EM) analysis using NSC/AR.Q(n) (data not shown); the aggregates found by EM in NSC/AR.Q46 treated with testosterone (data

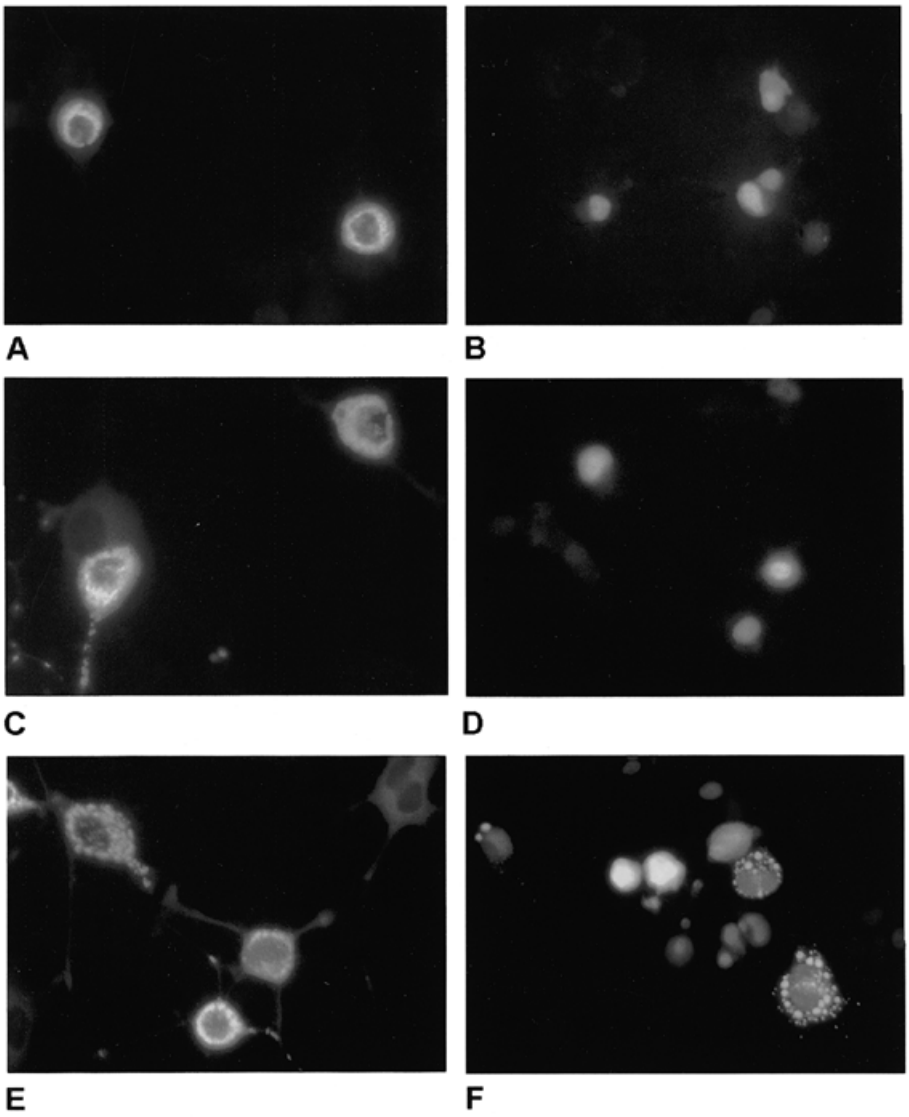

Figure 5. Aggregate formation in NSC34 transiently transfected with GFP–AR.Q(n). (**A**) Control NSC34 expressing GFP–AR.Q0; (**B**) NSC34 expressing GFP–AR.Q0 treated with 1 μM testosterone; (**C**) control NSC34 expressing GFP–AR.Q22; (**D**) NSC34 expressing GFP–AR.Q22 treated with 1 μM testosterone; (**E**) control NSC34 expressing GFP–AR.Q48; (**F**) NSC34 expressing GFP–AR.Q48 treated with 1 μM testosterone.

not shown) displayed a morphology similar to those found in HeLa cells (52) and to those found in related diseases (see below). The number of motoneuronal cells expressing GFP–AR.Q48 showing aggregates after testosterone treatment was estimated to range from 40 to 60% of the total transfected cells. Testosterone-treated/aggregate-negative NSC34(GFP–AR.Q48) cells showed a marked nuclear localization of the fluorescence, similar to that found in NSC34 cells expressing wild-type AR. The aggregates were predominantly localized in the cell cytoplasm and, in some cells, also in the neurite processes (Fig. 6A and B). Two types of aggregate were noted: 'small' aggregates diffusely localized throughout the cytoplasm and 'large' aggregates generally confined to the perinuclear region of the motoneurons. Inclusions in the neurite processes were generally similar in size to the 'small' aggregates (Fig. 6B). Occasionally, some aggregates were also detectable (5–10% of transfected cells) in GFP–AR.Q22-transfected motoneurons, in agreement with previous reports obtained in HeLa cells for the wild-type receptor (52). Figure 6C and D shows the results on immunolabelling studies utilizing antibodies to ubiquitin and the highly homologous NEDD8. Following extensive screening of multiple ubiquitin antisera, the GFP–AR.Q48 aggregates were shown to be weakly positive for ubiquitin (Fig. 6C) as are other polyGln expanded proteins such as huntingtin and ataxins (for a review see ref. 56). Aggregate-positive GFP–AR.Q48-expressing HeLa cells were readily NEDD8 positive (Fig. 6D) (52).

Studies on cell viability. Cell viability tests were also performed to determine the effects of the elongated polyGln tract on motoneuronal cell survival. The NSC/AR.Q46 cells showed a reduced growth/survival rate when compared with both NSC/AR.Q0 ($P < 0.01$) and NSC/AR.Q23 ($P < 0.05$) cells (Fig. 7A). After 6 days the cell viability of NSC/AR.Q46 was

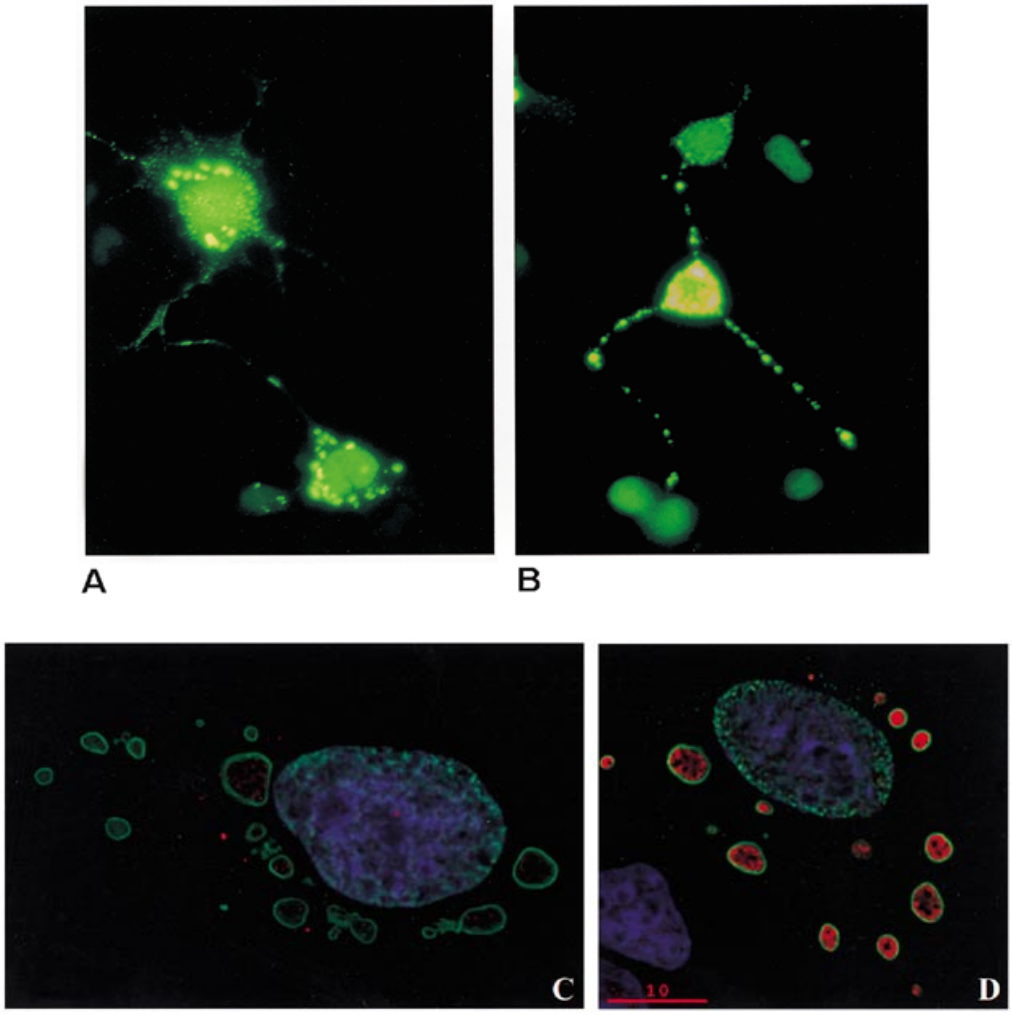

Figure 6. Aggregate formation in NSC34 transiently transfected with GFP–AR.Q48 treated with 1 µM testosterone. (**A**) 'Small' and 'large' aggregates in the cell cytoplasm of NSC34; (**B**) inclusions in the neurite elongations in NSC34; (**C**) ubiquitin profile of GFP–AR.Q48 aggregates; (**D**) ubiquitin-like (NEDD8) profile of GFP–AR.Q48 aggregates. Transiently transfected HeLa cells were used in conjunction with antisera to ubiquitin and NEDD8. GFP–AR.Q48 aggregates (green) in the cytoplasm were of various sizes and shown to be both ubiquitin [(C), red] and NEDD8 [(D), red] -positive. DAPI (blue) was used to counterstain DNA. Images were obtained with a wide-field fluorescence microscope and deconvolved.

reduced to 50% of that of NSC/AR.Q0 ($P < 0.01$), indicating that the elongated polyGln tract alters motoneuronal behaviour in culture. Testosterone treatment (Fig. 7B) generally increased the basal cell viability indicating that it may have a trophic effect on AR-transfected motoneurons. However, the effect of this hormone on NSC/AR.Q0 and NSC/AR.Q23 was not significant, whereas it significantly counteracted the decrease of survival in NSC/AR.Q46 ($P < 0.01$); interestingly, testosterone also induced the intracellular aggregation of the mutated protein (see above).

Cell viability tests were also performed on transiently transfected cells. The elongated polyGln tract of SBMA AR (Q46) exerted an adverse effect on motoneuronal cell viability (Fig. 8). The survival, measured in NSC34 expressing AR.Q46, was ~50% lower than that of cells expressing AR.Q0 ($P < 0.05$) in the absence of testosterone. Again, cells expressing AR.Q23 had an intermediate behaviour. Interestingly, testosterone significantly increased survival of NSC34 expressing AR.Q46 (an increase of 8.27 ± 0.09%, $P < 0.05$, over untreated cells; unpaired Student's *t*-test). The most potent testosterone metabolite, DHT, which is synthesized in motoneurons (7,55,57), was even more potent in this regard, inducing an increased survival of 10.01 ± 0.09% ($P < 0.01$; unpaired Student's *t*-test) (data not shown); however, the two treatments were not significantly different.

In order to induce a more pronounced motoneuronal phenotype, the proliferative capability of the hybrid NSC34 cells (which is due to the neuroblastoma component of the hybrid) was abolished by treating the cultures with hydroxyurea, which inhibits DNA replication, produces cell cycle arrest between the G_1 and S phases, and blocks the mitotic process. Under these conditions the survival rate of NSC/AR.Q46 was greatly affected, with a significant decrease in survival ratio after 3 days of treatment with hydroxyurea (NSC/AR.Q0, 79 ± 16% survival; NSC/AR.Q46, 56 ± 10% survival; NSC/AR.Q0 versus NSC/AR.Q46, $P < 0.05$); after 6 days in culture approximately two-thirds of NSC/AR.Q46 cells (35 ± 9% survival) had died, whereas only one-third (63 ± 11% survival) of NSC/

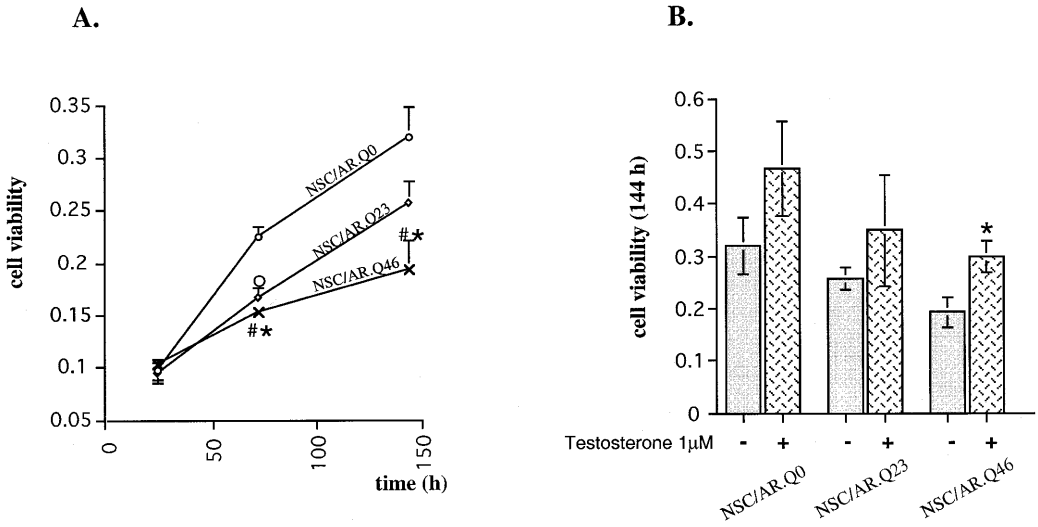

Figure 7. Cell viability of stably transfected NSC/AR.Q(n). (**A**) Growth/survival rate of NSC/AR.Q(n) at different times in culture (**P* < 0.01, NSC/AR.Q46 versus NSC/AR.QO; #*P* < 0.05, NSC/AR.Q46 versus NSC/AR.Q23; °*P* < 0.01, NSC/AR.Q23 versus NSC/AR.Q0); (B) comparison between control and testoterone-treated cells: growth/survival rate measured after 6 days in culture in the absence (grey bar) or presence (hatched bar) of 1 μM testosterone (**P* < 0.01, NSC/AR.Q46 plus testosterone versus NSC/AR.Q46 untreated; control versus testosterone-treated groups; unpaired Student's *t*-test).

AR.Q0 cells were lost (NSC/AR.Q0 versus NSC/AR.Q46, $P < 0.001$).

DISCUSSION

SBMA is an inherited form of lower motoneuron degeneration caused by the expansion of a CAG repeat in the AR gene, which results in an elongation of the polyGln tract normally present in the AR protein.

In order to clarify the mechanism(s) of motoneuronal death occurring in SBMA, we have designed new cellular models by transfecting a mouse spinal cord motoneuron × neuroblastoma hybrid cell line (NSC34) with human AR cDNAs with CAG repeats of different sizes (AR.Q0, AR.Q23 and AR.Q46). NSC34 are known to be very valuable in testing neurotoxicity (53,54). Using our model we have shown, in essence, that neurodegeneration may occur directly in isolated motoneuronal cells, and that the adverse effect(s) exerted by the mutated SBMA AR is apparently distinct from the appearance of intracellular aggregates brought about by the mutated protein. Indeed, the presence of aggregates may have a protective effect (56,58).

The transfected motoneuronal cells produced an SBMA AR protein and retained the polyGln repeat of the expected size, even after repeated subcultures; this result differs from those found in other artificial *in vivo* systems (59,60), in which instability (either elongation or contraction) of the polyGln tract has been described (60). In stable clones, the levels of SBMA AR.Q46 expression gradually decreased after several platings (20–30 passages), indicating that the potential toxicity of the mutated receptor may induce selection of low expressing AR.Q46 clones; this finding may explain the decreased intracellular levels of mutated AR (with respect to wild-type AR) observed in other motoneuronal cell lines (50). When the proliferative capability of the hybrid NSC34 cells was abolished with hydroxyurea, NSC/AR.Q46 showed a dramatic decrease in their survival rate; the appearance of the intrinsic toxicity of the elongated polyGln tract in these postmitotic motoneurons suggests that the process of motoneuronal degeneration occurring in Kennedy's disease is not secondary to other degenerative phenomena.

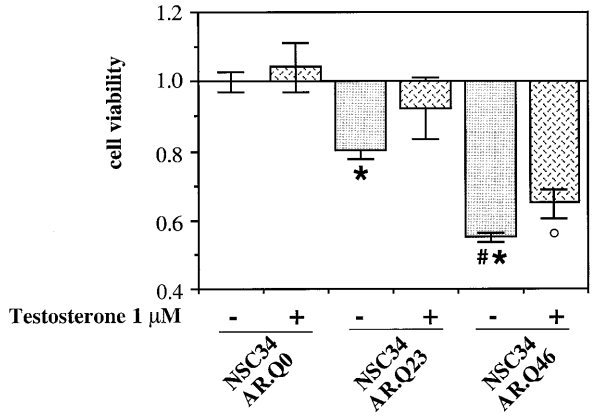

Figure 8. Cell viability of NSC34 transiently transfected with AR.Q(n) after 4 days in culture in the absence (grey bar) or presence (hatched bar) of 1 μM testosterone (°*P* < 0.05, NSC34 expressing AR.Q46 testosterone-treated versus NSC34 expressing AR.Q46 untreated; **P* < 0.05 NSC34 expressing either AR.Q46 or AR.Q23 untreated versus NSC34 expressing AR.Q0 untreated; #*P* < 0.05 NSC34 expressing AR.Q46 untreated versus NSC34 expressing AR.Q23 untreated; comparison between untreated groups, Scheffé test; control versus testosterone-treated groups, unpaired Student's *t*-test).

Cell morphology in NSC34 cells expressing AR.Q46 was markedly altered. Neurite dimension, number and extension was abnormal in cells growing at low density. NSC/AR.Q46 cells were poorly adherent, but were able to replicate and to grow; by increasing density levels they acquired a neuronal-like morphology. This agrees with the survival data, suggesting that the cells might produce putative adhesion

molecules and/or growth factors acting through autocrine/paracrine mechanisms. The factor(s) may partially overcome the toxicity of the elongated polyGln tract in NSC/AR.Q46 cells.

In transient co-transfection experiments, the neurites in cells containing AR.Q46 also appeared affected, being short and dystrophic, and producing abnormal branching after prolonged time in culture. Dystrophic neurites, as a consequence of the expression of elongated polyGln tracts, have also been reported *in vivo*, since axonopathies have also been detected in SBMA patients (22). Altered neurites have also been described in transgenic animal models for Huntington's disease (45,61). These observations indicate that an initial insult in motoneurons may occur in the neurites; the loss of connection with other cells in the spinal cord and/or with the target muscle finally results in the loss of motoneuronal cell body.

Testosterone treatment in this model markedly reversed the adverse effect of the polyGln expansion on neurite extension, and motoneuronal cells acquired a fairly normal phenotype. Testosterone also significantly increased survival of motoneuronal cells expressing SBMA AR. Whether both of these effects can be ascribed to the trophic effect that this hormone exerts on motoneurons (62,63) remains to be determined. Motoneurons in the spinal cord are androgen-sensitive; in adult male rats, androgen deprivation decreases the somatic size and the dendritic length of the motoneurons, as well as the number of chemical and electrical synapses (gap junctions). This phenomenon is reversed by androgen replacement therapy (62). In motoneurons, androgens are also involved in the regulation of their own receptors, and of structural proteins like actin, β-tubulin and those of the gap junction channels (62). Preliminary observations performed in our laboratory, using differential display-PCR analysis, indicate that testosterone induces the gene expression of a protein responsible for neurite extension (i.e. neuritin) in NSC34 transfected with the mouse AR. All these data support the hypothesis of a trophic effect of androgens on NSC34 cells.

Despite the numerous data appearing in recent years on the various types of CAG-related disorder, the mechanism of neurodegeneration remains obscure. A large body of evidence has very recently emerged indicating the formation of neuronal intracellular aggregates that may be toxic for long-living post-mitotic cells like neurons (33–46,61,64). This hypothesis has been put forward mainly because of the close similarity of these insoluble intracellular inclusions to those found in other neurodegenerative disorders (such as Alzheimer's disease, prion disorders and Parkinson's disease). Proteolytic fragments of mutated proteins may be important in initiating the process of aggregate formation (45), which apparently takes place in the cell nucleus (47). However, aggregation also seems to involve the neuronal processes. Why aggregates are formed only when the CAG repeat reaches a critical size (>40) is still a matter of discussion. It is known that *in vitro* synthetic peptides containing poly-L-Gln are insoluble and tend to acquire a β-pleated sheet structure, stabilized by hydrogen bonds between their main- and side-chain amides; because of this, they form polar zippers (65–67); the structure is generally unstable with <40 Gln, but becomes more stable with >40 Gln, because of an entropy gain. The notion that aggregates are formed as an abnormal cell response to the presence of polyGln of unusual length is supported by the observation that aggregates are ubiquitin or NEDD8 positive, possibly blocking the proteolytic pathway (49,52,68).

In our study, aggregation did not occur in NSC34 cells expressing SBMA GFP–AR.Q48 under basal conditions; inclusions were formed after activation of the receptor by testosterone. Our data agree with those already obtained in several types of cell (52). In fact, it has been shown that SBMA AR forms predominantly cytoplasmic aggregates in transfected mammalian cells (COS cells, mouse neuroblastoma NB2a/d1 cells, MN-1 neuronal cells, glioma–neuroblastoma hybrid cells, NG108-15 cells) (48,49,69,70), with few intranuclear inclusions (38,49,52,70,71). The structure of the AR aggregates has been extensively characterized, and found to be comparable with that observed in other CAG-related disorders; AR.Q46 or GFP–AR.Q48 aggregates formed in HeLa cells, after testosterone treatment, included NEDD8 (52) and ubiquitin. The difficulty in obtaining positive ubiquitin labeling (52) is likely more than just a technical issue (e.g. antibody affinity/specificity), since studies with polyGln-expanded ataxin-1 done in parallel experiments using various ubiquitin antibodies all were positive (unpublished data). Whether nuclear translocation of AR, which normally occurs with hormone binding, is important for the formation of aggregates is unknown (49), but their cytoplasmic localization in cell culture appears to contradict this hypothesis. Some inclusions are also clearly detectable in the cell processes, where they may be responsible for axonal or dendritic transport alterations. In our model, neuronal processes of cells expressing elongated polyGln tracts appeared to be dystrophic (see above). The kinetics of aggregate formation in NSC34 cells (and in HeLa cells) clearly differed from the rate of survival. In fact, cell death occurred in motoneuronal cells expressing SBMA AR in the absence of testosterone when inclusions were not detectable. On the other hand, cell survival was increased by hormone addition, a treatment which induces formation of large intracellular aggregates. Cell survival and aggregate formation were inversely correlated, suggesting that these inclusions were not involved in cell death observed in our motoneurons. Recently, it has been postulated that nuclear (or cytoplasmic) inclusions are not necessarily toxic to the cells; Sisodia (72) has proposed that those structures can even be beneficial for cell survival, mainly by removing toxic proteins from the soluble cell compartments. On the basis on these data, and of the most recent experimental observations showing a lack of correlation between polyGln-induced apoptosis and the formation of nuclear aggregates *in vivo* (56,58,73), and in particular the results presented in this paper, it appears that the whole topic should be re-evaluated. In particular, attention should be devoted to the apoptotic process, since it has been shown recently that the AR of SBMA may serve as a substrate for the cysteine protease cell death executioners, the caspases. Caspase cleavage is a critical step in cytotoxicity; in fact, the AR is cleaved by a caspase-3 subfamily protease at Asp146, and this cleavage is increased during apoptosis indicating an involvement in neural cell death in CAG-related disorders (51). The existence, in basal conditions, of microaggregates that may alter some important cell function (e.g. mitochondrial functions, neurite transports, etc.) remains to be determined. These might be derived from the AR proteolytic fragments, observed in our motoneuronal cells by western analysis; since

these lack the hormone-binding domain they might be active in a hormone-independent fashion. This hypothesis is now under study in our laboratory. The microaggregates might also be responsible for the formation of the big inclusions seen in the presence of testosterone in NSC34 cells expressing SBMA AR since, on hormone addition, they may clump together and be removed from the site where their adverse effect takes place, thus decreasing their potential toxicity.

In conclusion, transfected NSC34 cells are a good model in which to study neurodegeneration occurring in Kennedy's disease. The lack of correlation between cell death and aggregate formation suggests that other mechanisms may be responsible for neurodegeneration. Finally, motoneuronal cells transfected with the mutated AR identified in Kennedy's patients, may be useful to seek new drugs able to counteract neurodegeneration occurring in all CAG-related diseases.

MATERIALS AND METHODS

Reagents

AR(N-20), an affinity-purified rabbit polyclonal antibody raised against a synthetic peptide reproducing an amino acid sequence located in the N-terminal region of the human AR, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Defined fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). All the tissue culture reagents were from Seromed, Biochrom (Berlin, Germany). Charcoal stripped fetal bovine serum (CS-FBS) was prepared as previously described (74) and used in all experiments in which steroid hormone treatments were utilized. Chemicals were obtained from Sigma (St Louis, MO). NSC34 cells were kindly provided by Dr Neil R. Cashman (McGill University, Montreal, Canada).

Plasmids

The plasmid expressing the wild-type AR (here named pCMV.AR/Q23) was obtained by subcloning the full-length AR cDNA in pCMV3, and has been described previously (17); The pCMV.AR/Q46 vector, expressing the SBMA AR, was prepared by subcloning the CAG repeat of a patient with proven Kennedy's disease into the backbone of the AR cDNA in the restriction sites *Nar*I and *Afl*II, which surround the CAG repeat. For the construction of the pCMV.AR/Q0 vector, an *Nar*I–*Afl*II fragment containing zero CAG repeats was generated by PCR utilizing the following primers: Gln0, 5′-**CGC AGC ACC TCC C**<u>**GG CGC C**</u>**AG TTT GCT GCT GCT G***GG TGA GGA TGG TTC TCC CCA AGC C*, and Gln0, 3′-CTG CTT AAG CCG GGG AAA GTG GGG CCC. The 5′-oligonucleotide contained nucleotides located immediately upstream (nt 300–333, bold) and downstream (nt 394–420, italic) of the CAG repeat coding for the polyGln tract, and incorporated the unique *Nar*I site of the AR sequence (underlined). The 3′-oligonucleotide was derived from nt 680–654 of the AR cDNA, and incorporated the unique *Afl*II site of the sequence. A Gln0 fragment was amplified by PCR using the wild-type AR as a template and these two primers (annealing-extension at 68°C for 90 s, and denaturing at 95°C for 60 s for 33 cycles using vent polymerase). The resulting band was subcloned as an *Nar*I–*Afl*II fragment in the AR cDNA. The absence of additional mutations and the of Gln-coding sequence was verified by sequence analysis. A schematic representation of the coded proteins is provided in Figure 1. The GFP–AR fusion protein expression vectors were generated by insertion of the AR cDNA in the multiple cloning site of pEGFP-C1, as has been described previously (52). In the chimeric proteins, the GFP protein was linked to the N-terminal region of the wild-type and mutated AR proteins. The length of the CAG in these constructs was modified by repeated amplification in bacteria, and was determined by sequence to be 22 CAGs in the wild-type and 48 CAGs in the SBMA AR.

Cell cultures

NSC34 are hybrid cell lines obtained by fusion of mouse motoneuron-enriched embryonic day 12–14 spinal cord cells (from GD 14–16) with mouse motoneuronal cells (53); the hybrid displays a multipolar neuron-like phenotype and possesses most of the motoneuronal properties. The cells were initially tested by RT–PCR (data not shown) and were found not to express endogenous mouse AR. The motoneurons were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% defined FBS, 1 mM glutamine and antibiotics (penicillin G K-salt, Squibb, 100 UI/ml and streptomycin sulphate, Squibb, 100 μg/ml), and grown at 37°C in a humidified atmosphere (5% CO_2– 95% air) in 25 cm^2 flasks (Corning, Cambridge, MA) changing the medium every 2–3 days. Every week the cells were detached from the plate by mechanical dissociation in culture medium and replated at a density of 5×10^4 cells/flask. When cells were treated with steroid hormones, FBS was always replaced with CS-FBS (5%) to eliminate endogenous steroids. Cells stably transfected with the AR.Q(n) cDNAs were routinely maintained in the same medium containing 6 μl/ml of geneticin (G148, 25 mg/ml). In the series of experiments performed on transfected cells differentiated towards the motoneuronal phenotype, the cell cycle was blocked with 500 μM hydroxyurea (for up to 6–9 days).

Transfection of NSC34 cells

The cells were either transiently or stably transfected. In the first case, Lipofectamine Plus (Gibco BRL, Gaithersburg, MD) was used; in the second case Lipofectin (Gibco BRL) was selected as transfectant. In both procedures, the day before transfection, the cells were plated in 25 cm^2 flasks at 40–50% of confluence. On the day of transfection, the medium was replaced with serum-free, antibiotic-free DMEM and then the cells were transfected according to the manufacturer's protocol. In transient transfections, cells were transfected for 3 h, then the medium was replaced with standard growth medium containing CS-FBS. Cells were allowed to grow in these conditions for 2 or 3 days, and were then used for single point 3-[4,5-dimethyl(thiazol-2-yl)-3,5-diphenyl] tetrazolium bromide tetrazolium salt (MTT, Fluka Biochemika, Switzerland) assays [AR.Q(n) expression vector experiments], fluorescent microscope evaluation of cell morphology [co-transfection of AR.Q(n)-expressing plasmids with pEGFP-N1 plasmid; plasmid DNA used at 5:1 ratio] or aggregate formation [with GFP–AR.Q(n)-expressing plasmids]. For each stable cell line (NSC/AR.Q0, NSC/AR.Q23 and NSC/

AR.Q46) utilized in this study, the following amounts of plasmids were used in the transfection procedure: 2 μg of Ar cDNA expressing plasmid DNA in combination with 0.2 μg of pSV2neo DNA (10:1 ratio). The cells were transfected overnight in serum-free, antibiotic-free DMEM; the medium was then replaced with standard growth medium. Cells were allowed to grow in these condition for 3 days and then 12 μl/ml of G148 (25 mg/ml) was added to select stably transfected clones; after 1 week the cells were mechanically detached, replated in new flasks and maintained in a high concentration of geneticin for another 7 days, then utilized for RT–PCR screening of AR (wild-type or mutated) mRNA expression using RT–PCR according to the procedure described previously (75)

Western blot and immunocytochemistry analyses

Transfected cells were harvested and homogenized in TE buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA) using a sonicator. The homogenate was centrifuged at 10 000 *g* for 30 min and the supernatants constituted the cell cytosols. Protein concentration was determined using the Bradford assays (76). Western immunoblot analysis was done by resolving the samples obtained from NSC/AR.Q0, NSC/AR.Q23 and NSC/AR.Q46 cells on 7.5% SDS–PAGE [0.1% SDS, 7.5% polyacrylamide gel containing 0.075% *N*,*N*′-methylenebisacrylamide (BioRad, Hercules, CA)] (77). Approximately 50 μg of protein was loaded in each lane, separated, blotted using a liquid transfer apparatus (BioRad) and analysed using AR(N-20) (1:500). Immunoreactive bands were visualized using the enhanced chemiluminescence detection kit reagents (Amersham, Milan, Italy).

HeLa cells were transiently transfected with GFP–AR.Q(n) vectors and subsequently processed for immunolabelling as described (52). Anti-NEDD8 (a kind gift from E. Yeh, Department of the University of Texas at Houston Health Science Center, Houston, TX) routinely showed positive results as described previously (52). A monoclonal ubiquitin antibody (Accurate Chemical, Westbury, NY) was found that marked GFP–AR.Q48 aggregates in transiently transfected HeLa cells. DNA was counterstained with DAPI. Routine FITC filters were used to detect GFP and a Texas Red-conjugated secondary antibody was used for ubiquitin and NEDD8.

Microscopy

To analyse cell morphology on living stably transfected clones, a Leitz microscope was used. Images were obtained at either 10× or 20× magnification. In the studies performed using the EGFP protein/AR co-transfection or the GFP–AR chimera studies in living NSC34 cells, a Zeiss Axiovert microscope has been used. The instrument was equipped for fluorescence analysis with the sets of filters necessary to detect GFP emissions. Images were obtained at 20× or 32× magnification. Anti-NEDD8 and ubiquitin fluorescence images were obtained with a deconvolution-based Zeiss Axiovert system (Applied Precision, Issaqua, WA).

Cell proliferation and survival

MTT analysis. The MTT assay was used either on transiently or stably transfected cells. The assay was performed in sextuplicate after 72 h of transfection, in the presence or absence of 1 μM testosterone or DHT. In stably transfected cells, a more complex experimental design was utilized. Briefly, stably transfected NSC/AR.Q0, NSC/AR.Q23 and NSC/AR.Q46 cells were plated in 24-well culture dishes (Corning, Corning, NY) at densities ranging from 8×10^3 (low density) to $>15 \times 10^3$ (high density) cells/cm^2, and maintained in complete DMEM containing N2 supplement (Gibco BRL). Samples were analysed at various time intervals (see Results) and each point was obtained using six different replicates. On the day of the assay, the medium was replaced with MTT stock solution (1.5 mg MTT/ml DMEM without phenol red).

A fresh MTT stock solution was prepared for all the assays required to obtain a single growth/survival curve in order to avoid experiment variability. Prefiltered MTT solution (300 μl) was added to each well and incubated at 37°C for 1 h. The formazan derivative formed from conversion of MTT was solubilized using 500 μl of isopropanol by mechanical resuspension, followed by incubation at room temperature for 10 min on a rocking platform. The amount of purple formazan derivative formed was determined by measuring the absorbance at 550 nm. In the case of the assays performed in the presence of hydroxyurea to determine cell survival, the values at different time intervals were compared with the initial absorbance values taken as a reference. Hormone treatments were performed using a final concentration of 1 μM testosterone or DHT in complete DMEM containing 5% CS-FBS. Statistical analysis has been performed using an unpaired Student's *t*-test or the Scheffé test for comparisons (see figure legends).

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