□원저 : 실험연구□

Expression of Expanded Polyglutamine Disease Proteins in *Drosophila* (*Drosophila* Polyglutamine Disease Models)

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Purpose : Polyglutamine diseases are a group of diseases caused by the expansion of a polyglutamine tract in the protein. The present study was performed to verify if polyglutamine disease transgenic *Drosophila* models show similar dysfunctions as are seen in human patients.

Methods : Polyglutamine disease transgenic *Drosophila* were tested for their climbing ability. And using genetic methods, the effects of anti-apoptotic gene *bcl-2* and chemical chaperones on neurodegeneration were observed. Also, spinocerebellar ataxia 2 (SCA2) transgenic *Drosophila* lines were generated for future studies.

Results : Expanded forms of spinocerebellar ataxia 3 (SCA3) transgenic protein causes characteristic locomotor dysfunction when expressed in the nervous system of *Drosophila* but the anti-apoptotic gene *bcl-2* shows no evidence of ameliorating the deleterious effect of the expanded protein. However, Glycerol, a chemical chaperone, seemed to reduce the toxicity, at least in the eyes of the transgenic flies. The level SCA2 expression is too weak in the transgenic SCA2 *Drosophila* for evaluation.

Conclusion : SCA3 transgenic *Drosophila* show ataxic behavior as observed in human patients. Chemical chaperones such as glycerol may prove beneficial in this class of genetic disease, which has no current method of cure. **(Korean J Pediatr 2005;48:425-432)**

Key Words : Polyglutamine, Machdo-Joseph disease (MJD), Spinocerebellar ataxia 3 (SCA3), *Drosophila*, Behavioral dysfunction, *bcl-2*, Spinocerebellar ataxia 2 (SCA2), Chaperones

Introduction

Several autosomal dominant genetic neurological diseases are caused by an expanded CAG repeat encoding a segment of polyglutamines within an expressed open-reading frame.

An expanded polyglutamine tract confers a dominantly toxic property upon these otherwise unrelated proteins[1, 2]. Whereas the normal polyglutamine repeat length is of no pathological consequence, expansion of the polyglutamine tract beyond a critical threshold leads to neuronal loss and a degenerative phenotype[3].

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Studies with Huntington's disease (HD), Dentatorubral pallidoluysian atrophy (DRPLA) and spinocerebellar ataxia (SCA) types show that apoptosis appears to be operative in patients and model systems[3].

Machado-Joseph disease (MJD), also known as spinocerebellarataxia type 3 (SCA3), is the most common dominantly inherited progressive ataxia caused by polyglutamine expansion[4]. The disease gene *MJD1* encodes an intracellular protein of unknown function, ataxin-3. The CAG/glutamine repeat region lies near the carboxyl terminus, and is normally composed of 12-37 repeats. However, affected individuals show a regional expansion to greater than 40 repeats. Ataxin-3 is a cytoplasmic protein expressed ubiquitously throughout the body. In affected individuals, mutant ataxin-3 is predominantly found in nuclear inclusions (NIs) in neurons of the ventral pons, whereas normally it is cytoplasmically localized[5, 6].

Spinocerebellar ataxia 2 (SCA2) is another progressive

ataxia caused by polyglutamine expansion. The gene causing SCA2 has been named ataxin-2. Expansion of the polyglutamine tract, located at the N-terminus of ataxin-2, is linked to the accumulation of intracellular inclusions and neuronal cell death[7]. The normal length of the polyglutamine tract threshold is 14-30 repeats. Disease occurs when the tract expands beyond 35 repeats. In our previous study [8], we reported that In the Korean population occurrence of SCA2 is higher than that of SCA3.

Present study was performed to investigat the behavioral manifestations of neurodegeneration induced in *Drosophila* by expanded polyglutamine. Flies previously described[9] were used to produce adult-onset neurodegeneration and various behavioral tests were performed to characterize the behavioral dysfunction associated with such neurodegeneration. In addition, as polyglutamine degeneration is reported to be partly apoptotic[10], the anti-apoptotic gene *bcl-2* was co-experessed in *MJD*tr-Q78s to determine whether *bcl-2* could rescue or reduce polyglutamine induced toxicity. Also, as the cytotoxicity of polyglutamine is thought to occur by protein misfolding caused by the expanded polyglutamine tracts, chemical chaperones were used to mimic the effect of molecular chaperones.

Materials And Methods

1. Immunohistochemistry

Fly heads were prepared for frontal sections (10 μm) in paraffin blocks. After deparaffinization, the sections were fixed in 0.5% formaldehyde/phosphate buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH7.2) for 1 hr, and then washed in PBS for 10 min. The samples were blocked in PBSG (0.2% BSA/1% Goat serum/0.01% saponin/PBS) for 30 min, and washed in several changes of 0.01% saponin/PBS. Anti-HA primary antibody was then applied at 1 : 200 in PBSG for 1 hr at room temperature in a humid chamber. After washing in several changes of 0.01% saponin/PBS, HRP-conjugated secondary antibody was added at 1 : 500 in PBSG and the samples incubated for 1 hr. They were then washed in several changes of 0.01% saponin/PBS and Sigma DAB tablets in PBS were used for staining, which was stopped by washing in PBS. They were then dehydrated through a graded ethanol series and mounted with Permount, and observed under a Nikon E600 (Nikon; Kawasaki, Japan) phase-contrast microscope.

2. Climbing assay

The climbing assay was performed as described[11] with some modifications. Individual flies of the genotype *elav-GAL4UAS : MJD*tr-Q78w and *elav-GAL4UAS : MJD*tr-Q78m were placed in a glass vial and vortexed for 10 sec. The time for each fly to reach a height of 7 cm was recorded. More than 100 hundred flies of each genotype and sex were tested.

3. Chemical chaperones

Gelycerol and dimethylsulfoxide (DMSO) were used as chemical chaperones to mimic the effects of molecular chaperones[12]. These chemicals were added to the fly-media, concentration ranging from 0.01 M to 1 M. More than 30 larvae of each trangenic lines were reared in each food vial. To roughly estimate the degeneration of eye structure pseudopupil analysis was performed[13].

4. Generation of SCA2 trangenic *Drosophila*

SCA2 gene with 22 (SCA2-CAG22) or 58 (SCA2-CAG58) polyglutamine repeats were cloned into pUAST *Drosophila* vectors and injected into W1118 line embryos to generate SCA2 transgenic flies.

Results

1. Expression of *MJD*tr-Q78 in the Eye

Depending on the severity of the effect caused by the chromosomal positional effects on the level of transgene expression[14], the transgenic flies were grouped as weak (*MJD*tr-Q78w), moderate (*MJD*tr-Q78m) or strong (*MJD*tr-Q78s).

It was previously reported[9] that when expressed in the eye, using glass gene promoter *gmr-GAL4*, which drives the expression of all cells in the developing eye, *MJD*tr-Q27 didn't affect the eye at either the macroscopic or structural level (Fig. 1A). However, *MJD*tr-Q78 protein disrupted both eye morphology and pigmentation, with a phenotype severity that was dependent on the strength of the transgene insertion. *MJD*tr-Q78w transgenic flies had a normal eye structure and pigmentation on the first day after eclosion, but showed a progressive loss of eye pigmentation (data not shown). For *MJD*tr-Q78m or *MJD*tr-Q78s, the effect of the expanded polyglutamine was easily seen when the flies were eclosed. The red pigment of the

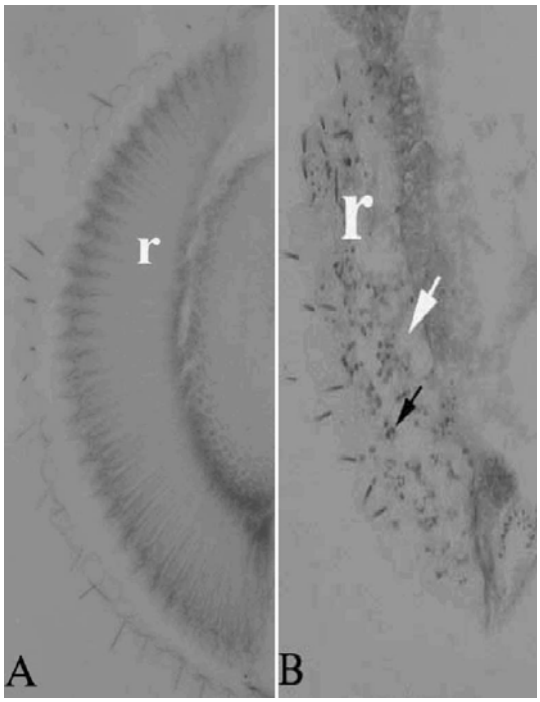

Fig. 1. Expanded polyglutamine protein causes adult eye degeneration. The structures of the eyes expressing *MJD*tr-Q27 **(A)** or *MJD*tr-Q78s **(B)** in the flies at 1 day after eclosion. While *MJD*tr-Q27 flies had compact retina structure, *MJD*tr-Q78s transgenic flies showed large holes in the retina, indicating retinal cell death (B; white arrow). Nuclear inclusions were visualized by anti-HA immunohistochemistry (B; black arrow). r : retina.

eye was lost and the retinal structure damaged (Fig. 1B).

2. *MJD*tr-Q78 causes behavioral dysfunction when expressed in the nervous system

When expression was targeted to the nervous system using *elav-GAL4* drivers, a similar degree of lethality could be observed in *MJD*tr-Q78s transgenic flies, which died during the late pupal stages. We refined these kinetics to reveal that, although the elav gene is expressed from the embryonic stages, no difference in larval CNS structures could be identified in *MJD*tr-Q27 controls and *MJD*tr-Q78 transgenic flies. Also, locomotor behavior at the larval stage showed no difference. This suggests that *MJD*tr-Q78 expression causes late-onset degeneration when expressed in the nervous system, and that this starts during the pupal stages. Moreover, we also examined functional aspects of the nervous system, i.e., climbing activity and olfactory response, to evaluate the behavioral manifestations of this late-onset neurodegeneration.

The climbing ability of *Drosophila* is known to decline with age, old flies tend to make short abortive climbs and

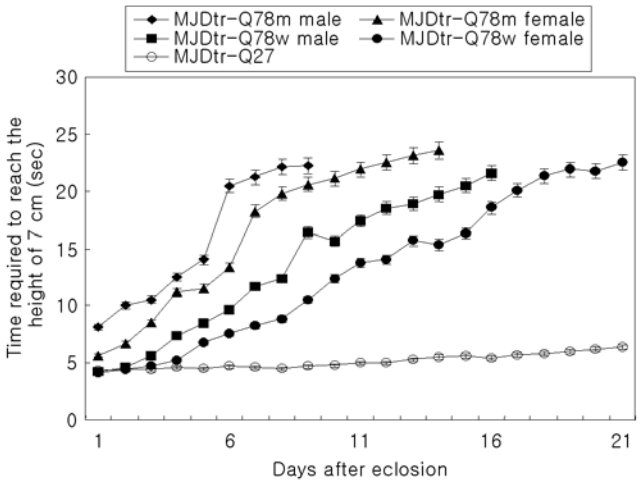

Fig. 2. Loss of climbing ability in *MJD*tr transgenic flies. *MJD*tr-Q78 transgenic flies in trans to *elav-GAL4*drivers were tested for climbing response. Curves represent means (±SEM) of the time required to reach the height of 7 cm after 10 sec of mechanical shock. At the first day of eclosion, *MJD*tr-Q78m flies readily showed a decrease in climbing ability, while *MJD*tr-Q78w flies showed similar climbing response when compared with control *MJD*tr-Q27 flies. However, this climbing ability seen in MJDtr-Q78w flies is decreased as they age. Both transgenic flies showed a progressive decline in climbing ability, with severity depending on the strength of the transgene expression. Difference in climbing ability between male and female is the due the dosage compensation of *elav-GAL4*, located on the X chromosome. In each genotype and sex, more than 100 flies were tested.

then fall back to the bottom of the tube[15]. This loss of climbing response is commonly used to monitor age-related changes in *Drosophila*.

Flies transgenic for *MJD*tr-Q78w, initially climbed normally, as did the *MJD*tr-Q27controls, but *MJD*tr-Q78m flies showed a reduced climbing response even on the first day of eclosion. Over time, transgenic *MJD*tr-Q78 flies showed a much more rapid performance deterioration than the controls, and this difference in severity was attributed to the strength of *MJD*tr-Q78 expression (Fig. 2). In addition, males and females showed different rates of decline. This difference is probably due to a *elav-GAL4* dosage effect, as it is located on the X chromosome, though dosage compensation in mammals occurs by the inactivation of one of the two female X chromosomes, in Drosophila it is accomplished by over-expressing the X chromosome of the male[16]. In summary, this accelerated, progressive decline in climbing response demonstrated that a functional deficit had been produced by *MJD*tr-Q78 expression in the nervous system.

3. Effects of *bcl-2* on *MJD*tr-Q78s

Much evidence indicates that polyglutamine induced degeneration is associated with apoptotic cell death[17, 18], but when p35 was coexpressed in polyglutamine disease *Drosophila* models, little evidence indicated a reduction or rescue of polyglutamine induced toxicity[9, 19]. To examine if this neural cell death in *MJD*tr-Q78s transgenic flies is inhibited by another caspase inhibitor, the anti-apoptotic gene, *bcl-2*, was coexpressed in *MJD*tr-Q78s flies.

However, the coexpression of *bcl-2* did not show any evidence of reduced cell death. When targeted to the eye, both transgenic flies showed similar degrees of pigment loss and rough eye structure (Fig. 3A, 3B). Also, the underlying retina structures showed little, if any, difference in damage, refer to Fig. 3C and 3D, which show large holes where retinal neurons have undergone cell death, throughout the retina. When targeted to the nervous system, no flies succeeded to eclose, both transgenic flies died during the late pupal stages.

4. Effects of chemical chaperones on *MJD*tr-Q78

Molecular chaperones such as HSP family proteins are thought to reduce the severity or inhibit cytotoxicity by affecting the misfolding caused of the expanded polyglutamine tract. Tatzelt et al.[12] showed that organic solvents glycerol and dimethylsulfoxide (DMSO), referred to as chemical chaperones, interfere with the aggregation of Prion proteins which causes Prion disease, another disease that is caused by abnormal protein folding. By feeding *MJD*tr-Q78m and *MJD*tr-Q78s transgenic flies with chemical chaperones, glycerol and DMSO, at concentrations from 0.01 M to 1 M, the effect of chemical chaperones was examined (more than 30 larvae of each genotypes were tested). Transgenic flies mated to *elav-GAL4* did not show any change when fed with chemical chaperones. No *MJD*tr-Q78s transgenic flies eclosed, and the activity of *MJD*tr-Q78m flies showed little difference form *MJD*tr-Q78m transgenic flies fed with normal fly medium.

The flies mated to *gmr-GAL4* were observed on their first day after eclosion to document the differences by pseudopupil analysis. *MJD*tr-Q78m flies fed 0.5 M or 1 M glycerol seemed to show more red eye pigment than control flies (Fig. 4). However, no clear difference between both flies could be observed.

5. Ataxin-2 expressing SCA2 transgenic *Drosophila* lines

Spinocerebellar ataxia 2 (SCA2) is a polyglutamine disease which shows difference in that the expanded protein does not seem to form NIs. Because SCA2 seems to be more frequent than SCA3 in Korean population[8], we gen-

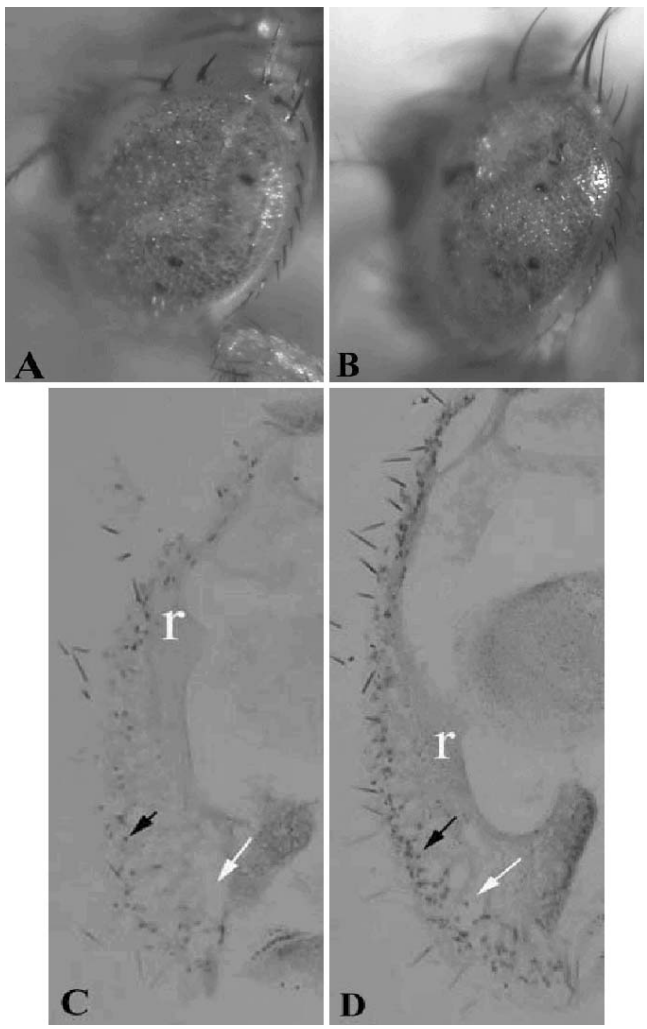

Fig. 3. Expression of *bcl-2* in *MJD*tr-Q78s transgenic flies. A and C : Flies expressing *MJD*tr-Q78s alone; B and D : Flies coexpressing *MJD*tr-Q78s and *bcl-2*. Genotypes UAS : *MJD*tr-Q78s or UAS : *MJD*tr-Q78s; UAS : *bcl-2* in trans to gmr-GAL4. Dissection micrographs **(A-B)** and frontal section **(C-D)** images of eyes of 1-day-old flies. Flies coexpressing bcl-2 **(B)** showed similar degree of eye structure damage as that of MJDtr-Q78s expressing flies **(A)**. The structure of the eye showed no apparent differences between the two transgenic flies **(C-D)**. Both lines showed severe damage to the retinal cells, seen by large holes formed in the retina tissue (C and D : white arrows). The brown spots seen in the retina are Nuclear inclusions (NIs) visualized by anti-HA immunohistochemistry (C and D : black arrows). The bright spots seen in A and B are reflections from photographs and thus, are not part of the phenotype. r : retina.

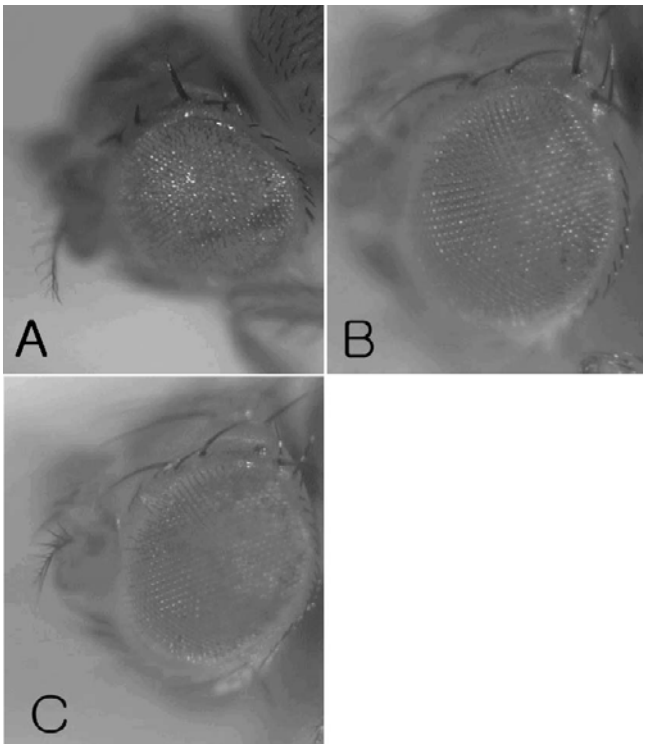

Fig. 4. Effects of glycerol on *MJD*tr-Q78m transgenic flies. Micrographs of fly eyes expressing *MJD*tr-Q78m at the first day after eclosion. Flies were fed normal cormeal/molass medium **(A)**, 1 M glycerol **(B)** or 0.5 M glycerol **(C)** from the 2nd instar larval stage. The structure of the eye showed little difference. The red pigment of the eye is lost in flies fed with normal medium **(A)**. However, flies fed with 1 M **(B)** and 0.5 M **(C)** glycerol showed much red pigment. The bright spots seen in the pictures are reflections from photographs and thus, are not part of the phenotype.

erated transgenic SCA2 flies bearing 22 and 58 polyglutamine repeats. 22 repeats are within the range of normal ataxin-2 CAG repeats whereas 58 repeats are well above the disease threshold. As can be seen from Fig. 5, SAC2-CAG58 fly lines showed no signs of degenerating retina structure when targeted to the eye. No significant change could be observed even when it was targeted to the nervous system.

Discussion

The coding regions of the genes associated with polyglutamine disorders do not share sequence homology, except for the highly polymorphic CAG tract. These polyglutamine diseases form a class of human neurodegenerative disease characterized by late-onset, progressive neural degeneration in specific brain regions. Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease

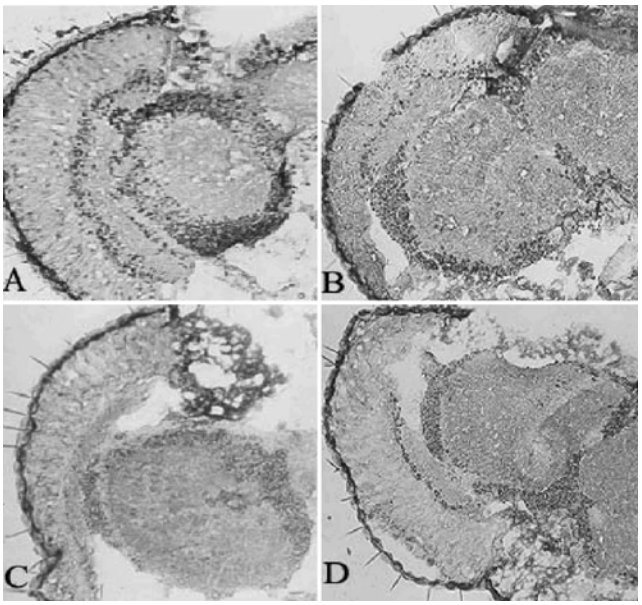

Fig. 5. Ataxin-2 expression in the eye of SCA2 transgenic flies. Frontal section images of eyes of 7-day-old flies. The structures of the eyes expressing SCA2-CAG22 **(A)** and SCA2-CAG58 **(B, C, D)** in the flies at 7 days after eclosion. The red pigmentation and retina structure of expanded polyglutamine protein expressing flies **(B, C, D)** show no retardation or degeneration compared to control flies **(A)**. A : SCA2-CAG22; B : SCA2-CAG58 line1; C : SCA2-CAG58 line2; D : SCA2-CAG58 line3.

(MJD), is the most common ataxia associated with the polyglutamine disease family.

In order to apply the power of genetics to the problems posed by these human hereditary neurodegenerative diseases, many *Drosophila* models of polyglutamine disease have been devised. The *GAL4/UAS* expression system[20] in flies allowed the tissue-specific expression of expanded polyglutamine protein. Such studies show that *Drosophila* displays the fundamental characteristics of neuronal toxicity and loss, which are conserved between humans and flies.

However, previous studies have mainly focused on toxic effects in the eye (Fig. 1) and the lethality caused by expressions in the nervous system[9, 19, 21, 22], and although eye degeneration has a late-onset and is progressive, no data is available regarding progressive neuronal dysfunction. Given the neuronal toxicity shown by *MJD*tr transgenic flies, we searched for behavioral manifestations of nervous system dysfunction.

Wild type *Drosophila* displays strong negative geotaxis. This climbing activity is grossly preserved in young flies, but as they age their ability to climb decreases. However, in transgenic flies showing a pan-neural expression of

*MJD*tr-Q78, a progressive decrease in climbing activity can be observed even at the young adult stage (Fig. 2). This progressive, accelerated decline in climbing ability in *MJD*tr transgenic flies reflects a functional deficit produced by *MJD*tr expression in the nervous system of *Drosophila*.

Proteins with expanded polyglutamine tracts seem to kill affected neurons through apoptotic pathways[23], and in SCA3/MJD disease, caspase-1, -3, and -8 seem to be involved[24].

Moreover, the BCL-2 family, which contains both pro- and anti-apoptotic members, constitutes a decisional checkpoint within the common portion of the apoptotic pathway[25]. Two models have been proposed to explain the manner in which BCL-2 family members act on the apoptotic pathway. Although there are some differences, both models agree on the point that BCL-2 family members are associated with mitochondrial structures and that the anti-apoptotic BCL-2 family members, BCL-2 and BCL-xL, prevent mitochondrial rupture and thus inhibit apoptosis.

In this study, we mated *MJD*tr-Q78s transgenic flies in trans to *bcl-2* bearing flies, and expressed both genes in the eye and in the nervous system by using different GAL4 drivers (Fig. 6). The results obtained show that *bcl-2* doesn't seem to affect *MJD*tr-Q78s induced polyglutamine toxicity in *Drosophila*. The eyes of *bcl-2* co-expressing MJDtr-Q78s transgenic flies were severely damaged as were the eyes of *MJD*tr-Q78s flies, and no progeny resulted from pan-neural expressing crosses.

Increasing evidence indicates that expanded polyglutamine confers a novel toxic property upon the otherwise unrelated polyglutamine disease proteins. Polyglutamine expansion leads to an altered, presumably misfolded, domain within the protein. One clear manifestation of this misfolding is the formation of intracellular aggregates by the disease protein, in particular intranuclear aggregates or NIs. The fact that NIs are ubiquitinated supports the view that they contain misfolded and aggregated protein. The presence of ubiquitinated aggregates implies that alterations in the major intracellular system for degrading proteins, the ubiquitin-proteasome pathway may contribute to the pathogenesis of polyglutamine diseases. Also results show that HSP family of molecular chaperones reduce the toxicity of expanded polyglutamine supports that polyglutamine tract leads to abnormal conformation[22].

Evidence suggesting that small molecules and chemical chaperones reduce the toxicityof abnormal conformation is growing[12, 26, 27]. Heiser et al.[26] shows that small molecules such as Congo red and thioflavine S interfere with the formation of mutant protein aggregates, thereby reducing cell death. Glycerol and dimethylsulfoxide (DMSO) are low molecular weight compounds that have been shown to protect proteins from thermal denaturation and aggregation *in vitro*. In addition, glycerol and DMSO were found to interfere with the formation of an insoluble form of mutant prion protein[12].

In our present study, both glycerol and DMSO didn't seem to show any effects on the normal development of wild type or *MJD*tr-Q27 transgenic flies. Both reagents had no effect on flies expressing MJDtr-Q78 in the nervous system. DMSO applied at concentrations from 0.01 M to 1 M had no significant effect in slowing or reducing the degeneration of adult eye in either transgenic flies. Glycerol seemed to reduce, or at least slow down, the degeneration of *MJD*tr-Q78m flies at concentrations above 0.5 M (Fig. 4), although we could not confirm if there appeared to be a dosage dependent reduction in pigment loss.

However these studies were primarily macroscopic results of the outer structure of the eye. Following studies focused on microscopic structures of the retina and on the formation of insoluble NIs in flies fed these chemical chaperones should give us evidence of the role of chemical chaperones. Also, *Drosophila* neuronal cell culture should allow us to study the effect of these chemicals on polyglutamine toxicity in neuron cells.

Spinocerebellar ataxia 2 (SCA2) is an autosomal dominant disorder leading to neuronal degeneration, affecting partly the cerebellum, pontine neuclei, inferior olive and substantia digra, while the striatum appears spared[28]. The CAG repeat in the SCA2 gene is located in the 5′-coding region of exon 1[29]. Disease brain tissues show a more intense ataxin-2 immunoreactivity than normal brain tissues and do not reveal the presence of intranuclear ubiquitinated inclusions[30]. The physiological function of ataxin-2 is proposed to be implicated in RNA splicing and protein interaction.

When we expressed expanded SCA2 protein (SCA2-CAG58) in the eye or the nervous system of our transgenic lines, there was no degenerative effect compared to the control SAC2-CAG22 control line. All the transgenic SCA2-CAG58 lines expressed the expanded ataxin-2 protein in both RNA and protein levels. However when we compared the expression level to SCA3 transgenic lines we

found that our SCA2 transgenic Drosophila model showed weaker transgene expression level than SCA3 transgenic flies (Data not shown). This may be the reason that we could observe little or no difference between SCA2-CAG22 controls and SCA2-CAG58 disease lines. We are currently developing more SCA2-CAG58 lines. Also we have expanded the CAG repeats to 100 and more repeats to see if a more expanded polyglutamine repeat is needed for flies to show the deleterious effect of SCA2.

Our study shows that expression of an expanded polyglutamine protein in *Drosophila* causes behavioral deficits that are similar to those observed in human patients. Moreover, although these cell deaths were not reduced by bcl-2 co-expression, further studies using other anti-apoptotic genes are felt warranted to allow the characterization of the role of apoptosis in polyglutamine disease. The role of chaperones, molecular and chemical, in curing or ameliorating the deleterious effect of polyglutamine disease needs to be further analyzed.

국문 요약

증가된 글루타민에 의해 초래되는 뇌신경질환의 초파리 모델에 대한 연구

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목 적 : 폴리글루타민 질환은 해당 발현단백질의 연속되는 글루타민 아미노산 서열이 신장되기 때문에 일어나는 질환군이다. 현 연구는 폴리글루타민 질환 형질전환 초파리 모델들이 환자들에서와 유사한 장애를 나타내는지 확인하기 위해 수행되었다.

방 법 : 폴리글루타민 질환 (SCA3) 형질전환 초파리를 대상으로 벽을 기어오르는 운동 능력을 검사하였다. 또한 유전학적인 방법을 통해서 아폽토시스를 억제하는 *bcl-2* 유전자와 화학적 샤페론이 뇌신경의 퇴행에 어떤 영향을 미치는지 확인하였으며 향후의 연구를 위해 척수소뇌 운동실조증 타입 2 (SCA2) 질환을 발현하는 형질전환 초파리를 생산하였다.

결 과 : SCA3 형질전환 초파리에서 신장된 폴리글루타민 배열을 지니는 질환성 초파리의 경우 신경계에서 해당 단백질을 발현하였을 경우 전형적인 운동 능력 상실을 나타냈다. 아폽토시스를 억제하는 유전자인 *bcl-2*를 함께 발현했을 경우, 신장된 단백질의 유독한 영향을 약화시키지 못했지만, 화학적 샤페론인 글리세롤의 경우 적어도 눈에서의 유독한 영향은 억제하는 것으로 보인다. 본 연구진에 의해 개발된 SCA2 형질전환 초파리의 경우 유해 단백질의 발현 정도가 낮아서 정확한 분석이 어려웠다.

결 론 : SCA3 형질전환 초파리는 환자들에서 발견되는 운동실조증을 보였다. 글리세롤과 같은 화학적 샤페론이 현재 치료가 전무한 이 종류의 질환군의 치료에 효과적일 것으로 사료된다.

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